A Dual Function α-Dioxygenase-Peroxidase and NAD+ Oxidoreductase Active Enzyme from Germinating Pea Rationalizing α-Oxidation of Fatty Acids in Plants[1,2]

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An enzyme with fatty acid α-oxidation activity (49 nkat mg^{-1}; substrate: lauric acid) was purified from germinating pea (*Pisum sativum*) by a five-step procedure to apparent homogeneity. The purified protein was found to be a 230-kD oligomer with two dominant subunits, i.e. a 50-kD subunit with NAD+ oxidoreductase activity and a 70-kD subunit, homolog to a pathogen-induced oxygenase, which in turn shows significant homology to animal cyclooxygenase. On-line liquid chromatography-electrospray ionization-tandem mass spectrometry revealed rapid α-oxidation of palmitic acid incubated at 0°C with the purified α-oxidation enzyme, leading to (*R*)-2-hydroperoxypalmitic acid as the major product together with (*R*)-2-hydroxypalmitic acid, 1-pentadecanal, and pentadecanoic acid. Inherent peroxidase activity of the 70-kD fraction decreased the amount of the (*R*)-2-hydroperoxy product rapidly and increased the level of (*R*)-2-hydroxypalmitic acid. Incubations at room temperature accelerated the decline toward the chain-shortened aldehyde. With the identification of the dual function α-dioxygenase-peroxidase (70-kD unit) and the related NAD+ oxidoreductase (50-kD unit) we provided novel data to rationalize all steps of the classical scheme of α-oxidation in plants.

Fatty acid hydroperoxides are reactive intermediates in the oxylipin pathways of fatty acid oxygenation in plants and fungi (Hamberg and Gardner, 1992; Mueller, 1997; Blée, 1998; Grechkin, 1998). Their metabolites include fatty acid epoxides and epoxy alcohols (Blée and Schuber, 1990; Hamberg and Hamberg, 1990), dihydroxy acids (Hamberg and Gerwick, 1993), divinyl ethers (Galliard and Phillips, 1972; Grechkin et al., 1995), and aldehydes (Gardner, 1991; Hatanaka, 1993), as well as a number of derivatives originating from the jasmonic acid pathway (Boland et al., 1998).

A hydroperoxide has also been proposed as key intermediate (Shine and Stumpf, 1974) (Fig. 1) for the α-oxidation of fatty acids in higher plants, such as pea (*Pisum sativum*) leaf (Hitchcock and James, 1966), germinating peanut (*Arachis hypogaea*) (Shine and Stumpf, 1974), cucumber (*Cucumis sativus*) (Galliard and Matthew, 1976), and potato (*Solanum tuberosum*) (Laties and Hoelle, 1967), as well as in marine green algae (*Ulva pertusa*) (Kajiwara et al., 1988). This hypothesis was supported by our findings that incubations of fatty acids with a preparation from germinating peas in the presence of stannous chloride afforded enantiomerically pure (*R*)-2-hydroxy acids at the expense of aldehydes (Adam et al., 1998);

it was also supported by the recent identification of (*R*)-2-hydroperoxypalmitic acid and (*R*)-2-hydroperoxylinolenic acid in the α-oxidation system from marine green algae (Akakabe et al., 1999) and by the identification of a pathogen-induced oxygenase (PIOX) from tobacco leaf (Sanz et al., 1998) and its homologous enzyme from Arabidopsis, respectively (Hamberg et al., 1999).

To date, structural and mechanistic information about the initial step of the α-oxidation of fatty acids in plants has been provided. However, experimental data about the complete pathway, in which both reduction of the 2-hydroperoxy fatty acid and its decomposition to the corresponding chain-shortened aldehyde and CO_2 are involved (Fig. 1), are still rather scarce. In this paper we describe the isolation and characterization of an enzyme from germinating pea whose subunits exhibit α-dioxygenase and peroxidase as well as NAD+ oxidoreductase activities, rationalizing all steps of the previously proposed α-oxidation mechanism (Fig. 1).

RESULTS

Purification of α-Oxidation Enzyme

We achieved purification of an enzyme with α-oxidation activity to apparent homogeneity by using a five-step protocol that included $(NH_4)_2SO_4$ and pH precipitations as well as three successive chromatographic steps (Table I). The separations are outlined in Figure 2. After the last chromatographic step, a 230-fold overall purification was obtained. The

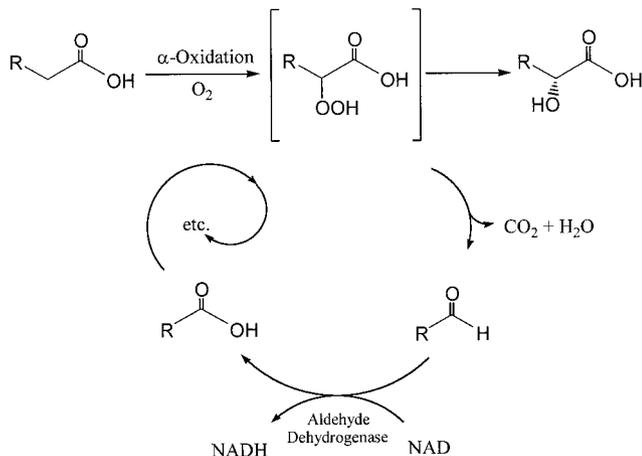**Figure 1.** α-Oxidation of fatty acids in higher plants (adapted from Shine and Stumpf, 1974).

pure α-oxidation enzyme had a specific activity of 49 nkat mg^{-1} using lauric acid as substrate.

Properties of α-Oxidation Enzyme

The calculated K_m and V_{max} values of the purified enzyme were 70 μM and 31.7 nkat mg^{-1} for oxygen, and 55 μM and 29.2 nkat mg^{-1} for lauric acid. The apparent molecular mass of the native α-oxidation enzyme determined by a calibrated Sephacryl S-300HR column was approximately 230 kD. When the purified enzyme was denatured with 1% (w/v) SDS and 10 mM 2-mercaptoethanol and then subjected to SDS-PAGE, two bands were consistently observed (Fig. 3, lane 2). Their relative molecular masses were estimated to be 50 and 70 kD, respectively. The pH optimum was determined post Sephacryl S-300HR in citric acid/sodium phosphate buffer over a range of pH 5 to 9 under saturating substrate conditions. Enzymatic activity was optimal between pH 6.8 and 8, with a maximum at pH 7.4. The pI of the α-oxidation enzyme was found to be 4.7 (data not shown).

The peroxidase-activity of the purified enzyme measured with the guaiacol/2-hydroperoxypalmitic acid system was found to be 0.5 nkat mg^{-1}, whereas oxidoreductase NAD$^+$ activity was found to be 2 nkat mg^{-1}.
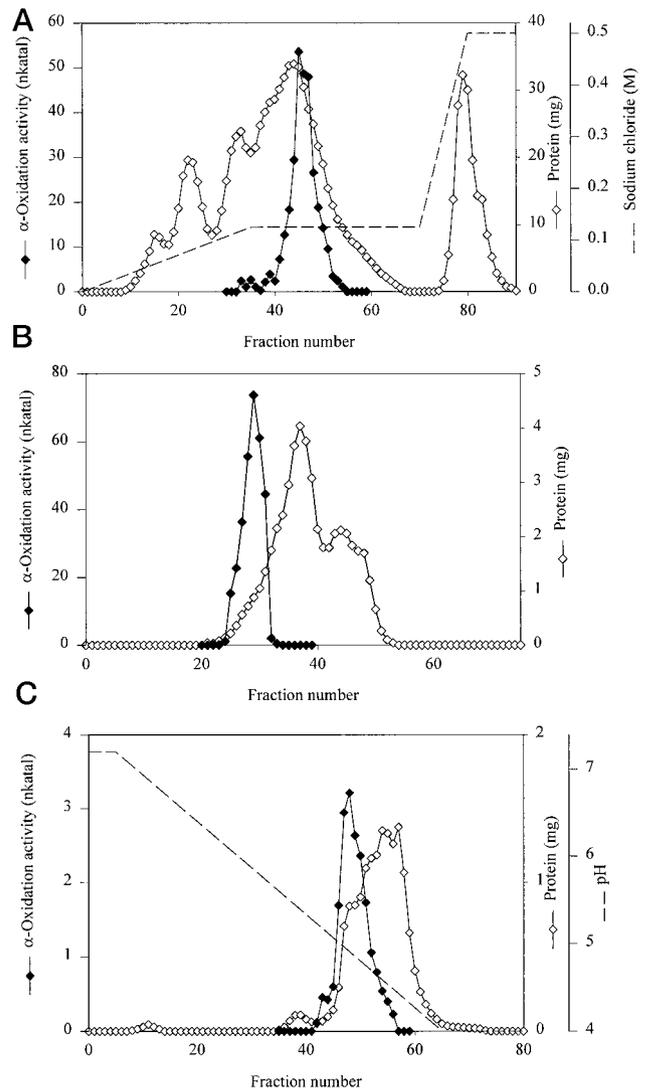

Figure 2. Chromatographic purification of α-oxidation enzyme from 3-d-old pea seedlings. The amount of protein (55) and α-oxidation activity (54) in each fraction of the DEAE-Sepharose (A), Sephacryl S-300 (B), and Mono-P (C) columns is given. The shape of the gradient in A and C is indicated by a dashed line.

Table I. *Purification of an enzyme with α-oxidation activity from whole germinating pea seedlings*

The results presented are for a typical purification starting from 100 g fresh wt.

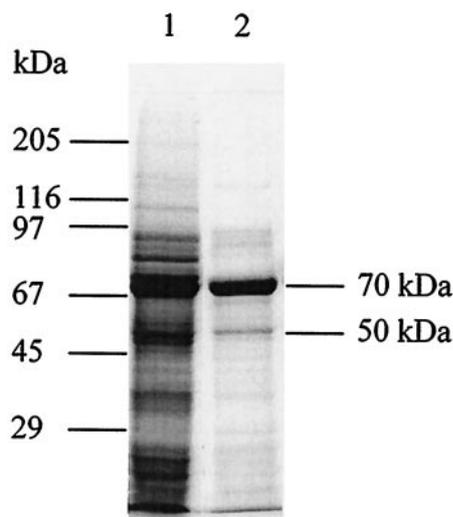| Purification Step	Total Protein	Total Activity	Specific Activity	Purification Factor	Yield
	mg	*nkat*	*nkat/mg*		*%*
Crude extract	4227	874.7	0.21	–	100
(NH$_4$)$_2$SO$_4$	2634	595.7	0.23	1.1	68.1
pH 5.6	1540	528.5	0.34	1.6	60.4
DEAE-Sepharose	323	363.9	1.13	5.4	41.6
Sephacryl S-300	22.2	270.5	12.2	58	30.9
Mono-P	0.7	34.2	49	233	3.9

Figure 3. Coomassie Blue-stained SDS-PAGE documenting the progress of purification of α-oxidation enzyme after gel filtration. Lane 1, Sephacryl S-300 chromatography; lane 2, Mono-P chromatography. The positions of molecular mass markers are indicated on the left in kD.

N-Terminal and Internal Peptide Amino Acid Sequence

The amino acid sequence of the 10 N-terminal amino acid residues was obtained from the purified 50-kD subunit. When this sequence was compared with published protein sequences, 100% amino acid identity with a turgor-responsive NAD$^+$ oxidoreductase cDNA from pea (Guerrero et al., 1990) was found (Fig. 4A). In contrast, the 20 N-terminal amino acid residues of the 70-kD subunit revealed no significant similarity to any protein in the database (data not shown). However, two internal peptides obtained by cyanogen bromide fragmentation showed complete identity to PIOX from tobacco (Fig. 4B). This 643-amino acid protein with a predicted molecular mass of approximately 73 kD in turn shows a high degree of identity to an Arabidopsis cDNA and, in addition, has significant similarity to the amino acid sequence of ovine and murine cyclooxygenase (COX)-1 and -2, respectively (Sanz et al., 1998).

α-Oxygenation of Palmitic Acid

Purified enzyme (post Sephacryl S-300HR) was stirred for 30 min at 0°C with 1 mM palmitic acid. The products were analyzed by reversed phase HPLC-evaporating light scattering detection (ELSD) and identified by on-line liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using authentic reference compounds. After incubation four peaks (1–4) appeared on reversed-phase HPLC together with the unconverted substrate (Fig. 5). The major product (product 1) cochromatographed with (R,S)-2-hydroperoxypalmitic acid, and their subsequent LC-ESI-MS/MS analyses revealed identical prominent adduct ions $[M + NH_4]^+$ with

A

Oxidoreductase NAD$^+$

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1    MGSDSNNLGF LKEIGLGATN IGSFINGQWK ANGPTVHSVN PSTNQVIASV
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B

'Pathogen-induced oxygenase'

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1    MSLVMDSLKN LLLSPLRGFI HKDLHDIFER MTLLSKLLFL IVHLVDKLNL
51   WHRLPVLLGL LYLGARRHLH QEYNLINVGK TPIGVRSNPA DHPYRTADGK
101  YNDPFNEGAG SELSFFGRNM LPVDQHNQLK KPDPMVVATK LLARRNFVDT
151  GKQFNMIAAS WIQFMIHDWI DHLEDTKQIE LKAAEEVASQ CPLKSFRFFK
201  TKEIPTGFYE IKTGHLNTRT PWWDGSAIYG SNAEVLKKVR TFKDGKLKLS
251  ADGLLEIDKN GKIISGDVRN TWAGLSALQA LFVQEHNSVC DALKKEYPEL
301  EEEDLYRHAR LVTSAVIAKV HTIDWTVELL KTDTLLAGMR ANWYGLLGKK
351  FKDTFGHVGG SILGGFVGMK KPENYGVPYS LTEEFTSVYR MHQLLPDKLQ
401  LRNIDATPGP NKSLPLTNEI PLEDLIGGKG EKNLSKIGFT KQMVSMGHQA
451  CGALELWNYP VWMRDLIPQD VDGTDRPDHI DLAALEIYRD RERSVARYNE
501  FRRGMLQIPI SKWEDLTDDE EVINTLGEVY GDDVEELDLM VGMAAEKKIK
551  GFAISETAFF IFLVMASRRL EADRFFTSNY NEETYTKKGL EWVNTTESLK
601  DVLDRHYPEI TEKWMNSSSA FSVWDSTPQP HNPIPLYFRV PPQ*
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Figure 4. Comparison of partial amino acid sequences of the purified α-oxidation enzyme to the deduced amino acid sequences of oxidoreductase NAD$^+$ and PIOX of tobacco. A, The N-terminal 50 amino acids of pea turgor-responsive oxidoreductase NAD$^+$ (accession no. X54359) are shown. The identity to the N-terminal sequence of the 50-kD protein is indicated by white letters on a black background. B, Amino acid identity between two cyanogen bromide-derived fragments of the 70-kD protein from pea and the PIOX from tobacco (accession no. AJ007630) is indicated in the same manner.

m/z 306 for the reference and the analytical product. Production spectra obtained by low-energy collision-induced dissociation (12 eV) of $[M+NH_4]^+$ precursor ions revealed authentic characteristic fragment patterns (Fig. 6). The most abundant product ions resulted from the consecutive loss of water $[M-H_2O+NH_4]^+$ m/z
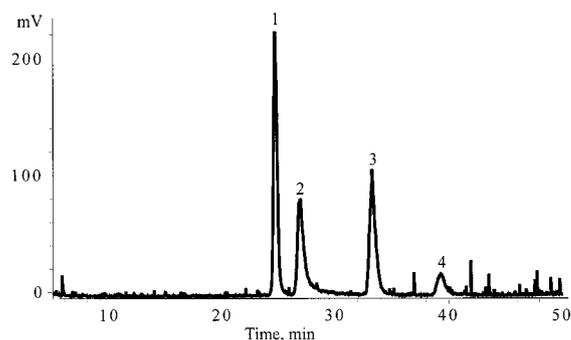

Figure 5. HPLC-ELSD analysis of products formed after a 30-min incubation at 0°C with 1 mM palmitic acid and 250 µg of purified α-oxidation enzyme from pea. The peaks are: 1, 2-hydroperoxypalmitic acid; 2, 2-hydroxypalmitic acid; 3, 1-pentadecanal and pentadecanoic acid; and 4, unconverted palmitic acid.
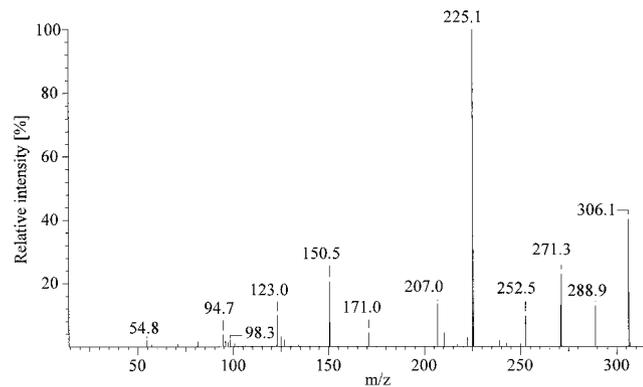
Figure 6. Product ion spectrum of 2-hydroperoxypalmitic acid (positive mode, −12 eV, 1.8 mTorr of argon as collision gas), precursor ion $[M+NH_4]^+$ m/z 306, $[M-H_2O+NH_4]^+$ m/z 288.9, $[M-H_2O+H]^+$ m/z 271.3, $[M-2xH_2O+H]^+$ m/z 252.5, and $[C_{15}H_{29}O]^+$ m/z 225.1.

289, $[M-H_2O+H]^+$ m/z 271, $[M-2xH_2O+H]^+$ m/z 253, and subsequent loss of formic acid $[C_{15}H_{29}O]^+$, yielding m/z 225 (Schneider et al., 1997).

Incubations at room temperature or for more than 30 min at 4°C led to a decrease of product 1 and an increase of product 2, the latter cochromatographed with (R,S)-2-hydroxypalmitic acid on reversed-phase HPLC. This indicates a dual activity of the α-oxidation enzyme as both an α-dioxygenase and peroxidase. For products 3 and 4, identical retention times on reversed-phase HPLC were found with authentic references of pentadecanoic acid and pentadecanal, respectively. The identities of products 2 to 4 were further confirmed by on-line high resolution gas chromatography (GC)-MS analysis. In agreement with our recent findings (Adam et al., 1998), high resolution GC of the methylated product 2 esterified with (−)-menthyl chloroformate revealed the presence of pure (R)-enantiomer (data not shown).

DISCUSSION

With the findings of Akakabe et al. (1999) and Hamberg et al. (1999), together with the data represented here, there is sufficient experimental proof for the existence of the previously postulated 2-hydroperoxide as an intermediate in the α-oxidation of fatty acids in plants. An alternative pathway via 2-hydroxy and 2-oxo acid, recently proposed to be operative in cucumber (Andersen Borge et al., 1999), seems unlikely. There is also good evidence that the (R)-selective α-dioxygenation of fatty acids is catalyzed by a new type of dioxygenase as shown by Hamberg et al. (1999) on the basis of the earlier identification of PIOX in tobacco leaves and a homologous 75-kD enzyme from Arabidopsis (Sanz et al., 1998). As shown in Figure 4, the 70-kD subunit of the α-oxidation enzyme from germinating pea possesses two stretches of amino acid sequence identity to this α-dioxygenase.

Sequence and functional analysis of the tobacco PIOX cDNA-encoded protein showed striking similarity with COX-1 and -2, key enzymes in the synthesis of lipid signal molecules such as prostaglandin and thromboxane in vertebrates. COXs are known to be dual function enzymes possessing both COX and peroxidase activities (Smith et al., 1996). To investigate the possibility that plant dioxygenases are similar dual function enzymes, we have thus analyzed our purified 70-kD protein and, in fact, the α-dioxygenase showed also peroxidase activity. Despite the fact that the natural electron donor for the peroxidase activity is still unknown, similar to the situation with the peroxidase activity of COXs (Smith et al., 1996), this finding rationalizes the formation of (R)-2-hydroxy fatty acid from its precursor, the (R)-2-hydroperoxy derivative.

All hitherto characterized COXs are heme proteins: In the PIOX protein from tobacco and Arabidopsis (Sanz et al., 1998), the proximal and distal heme-binding histidines of COX-1 (His-388 and His-207, respectively) as well as the distal Gln (Gln-203) are conserved (Smith et al., 1996; Landino et al., 1997), indicating that these heme proteins are capable of further transformation of fatty acid hydroperoxides. From the available information it can thus be concluded that the dual function α-dioxygenase-peroxidase from germinating pea is a heme protein. However, in contrast to the α-dioxygenase in germinating pea, no hydroperoxide converting activity has been described for the tobacco PIOX protein.

For the third enzymatic activity of the α-oxidation system in germinating pea, i.e. the NAD$^+$ oxidoreductase associated with the purified 50-kD subunit, additional experimental evidence was provided for the operation of the enzymatic cascade outlined in Figure 1. The oxidoreductase is the link for the oxidation of the formed aldehyde to the next chain-shortened homologous fatty acid, which in turn functions as a substrate in the α-oxidation cycle (Shine and Stumpf, 1974).

The α-oxidation pathway in mammals was elucidated by degradation of naturally occurring phytanic acid and other β-methyl-branched fatty acids (Verhoeven et al., 1998). In contrast the function of the corresponding pathway in plants is not fully understood yet. PIOX activity suggests the importance of a similar α-oxidation pathway in plants as defense reaction against pathogens (Hamberg et al., 1999), with 2-hydroperoxides functioning as signal compounds for induction of defense mechanisms (Reymond and Farmer, 1998). Possibly, germination should be important for induction, since α-dioxygenase activity in pea seeds or leaves was found to be less distinct in our study (data not shown).

In conclusion, isolation of the α-oxidation enzyme of germinating pea confirms the classical scheme of α-oxidation in plants and provides further evidence

that a multifunctional enzyme catalyzes the α-oxidative mechanism.

MATERIALS AND METHODS

Chemicals

Pentadecanal was synthesized by oxidation of 1-pentadecanol using pyridinium chlorochromate according to Harwood and Moody (1989) and (R,S)-2-hydroperoxy-palmitic acid was prepared as previously described (Konen et al., 1975). Unless otherwise specified, all other chemicals and enzymes were obtained from Sigma (St. Louis), Boehringer (Mannheim, Germany), and Pharmacia (Uppsala), respectively.

Plant Material

Dried green peas (*Pisum sativum*) were purchased from a local market. After 3 d of germination in water at room temperature the whole-plant material was collected.

Assay for α-Dioxygenase

For routine analysis and monitoring of activity in the purification steps, α-oxidation was assayed indirectly by aldehyde formation in the course of incubation of fatty acids (Shine and Stumpf, 1974) (compare with Fig. 1). Aldehyde analysis was performed at 30°C spectrophotometrically as 2,4-dinitrophenylhydrazine derivative at 430 nm as described by Yukawa et al. (1993) using lauric acid as substrate. The reaction mixture consisted of 1 mM lauric acid in 0.1 M Tris [tris(hydroxymethyl)aminomethane]-HCl buffer, pH 7.5, 200 μL of protein solution and water in a total volume of 500 μL. For HPLC analysis of the oxygenation products 1 mM palmitic acid in 0.1 M Tris-HCl buffer, pH 7.5, was incubated with 250 μg of purified enzyme in a total volume of 500 μL at 0°C for 30 min.

For the determination of kinetic constants, lauric acid was stirred with enzyme preparation at 30°C under the conditions described above, and the rate of oxygen uptake was monitored using a DW-1 Clark oxygen electrode (Bachofer, Reutlingen, Germany). The K_m and V_{max} values were determined from double-reciprocal plots of the maximum velocity of oxygen uptake and substrate concentration.

Purification of α-Oxidation Enzyme

All purification steps were carried out at 4°C. Germinating peas (100 g fresh weight) were homogenized in 500 mL of 0.1 M Tris-HCl buffer, pH 7.5. The homogenate was filtered through a nylon cloth and centrifuged at 14,000g for 30 min. The supernatant was subjected to $(NH_4)_2SO_4$ precipitation and the fraction precipitating between 30% and 70% saturation was resuspended in 60 mL of 20 mM Tris-HCl buffer, pH 7.5. After dialysis against 20 mM Piperazine-HCl buffer, pH 5.6, all material insoluble at this pH was removed by centrifugation at 14,000g for 10 min and discarded. The supernatant was adjusted to pH 7.5 with 0.1 N NaOH and loaded onto a DEAE-Sepharose FF

column (1.6 × 70 cm; Pharmacia) equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The enzyme was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in 20 mM Tris-HCl buffer, pH 7.5, at a flow rate of 1 mL min⁻¹. Fractions of 11 mL were collected and those exhibiting α-dioxygenase activity were combined, desalted, and concentrated to a volume of 1 mL by ultrafiltration using a Centricon-30 polycarbonate membrane (Amicon, Beverly, MA), and applied onto a Sephacryl S-300 HR FPLC column (1.6 × 60 cm; Pharmacia) equilibrated with 0.1 M Tris-HCl buffer, pH 7.5. Proteins were eluted with the same buffer at a flow rate of 0.2 mL min⁻¹ and 2-mL fractions were collected. α-Dioxygenase-active fractions were pooled, desalted, and concentrated to a volume of 500 μL as outlined above. The concentrate was loaded onto a Mono-P HR5/20 column (Pharmacia) equilibrated with 25 mM Bis-Tris-HCl buffer, pH 7.2, and 5% (w/v) betaine (Fluka, Buchs, Switzerland). The column was then rinsed with 3 mL of the same buffer and the α-dioxygenase activity was eluted with a reverse-stepwise gradient from pH 7 to 4 using polybuffer 74 (Pharmacia), pH 4, 5% (w/v) betaine (Fluka) at a flow rate of 0.5 mL min⁻¹. Purified α-dioxygenase fractions were stored at −22°C.

Assays for Peroxidase and Oxidoreductase NAD⁺ Activity

Inherent peroxidase activity of the purified enzyme (after chromatofocusing) was assayed using 2-hydroperoxy-palmitic acid by measuring the oxidation product of guaiacol spectrophotometrically at 470 nm (Maehly, 1955). Using pentadecanal the oxidoreductase NAD⁺ activity was assayed by measuring the increase of NADH spectophotometrically at 340 nm.

Determination of the Native Molecular Mass and pI

The partially purified enzymes were chromatographed on a Sephacryl S-300 HR column (1.6 × 60 cm; Pharmacia) equilibrated with 0.1 M Tris-HCl buffer, pH 7.5. Retention time was compared with that of gel filtration markers run simultaneously with the α-dioxygenase proteins. The markers used were thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), aldolase (158 kD), and bovine serum albumin (BSA; 67 kD). All proteins were loaded in a total volume of 1.2 mL, and elution was monitored at 280 nm. The estimation of pI was carried out using isoelectric focusing (Servalyt Precotes, Boehringer; Isoelectric Focusing System, Desaga, Heidelberg), pH 3 to 10. We loaded the proteins on two identical halves of one gel and focused them according to the manufacturer's instructions (Desaga). One-half of the gel was stained for protein using Coomassie Brilliant Blue R-250, the other one-half was sliced into 1-mm segments and assayed for enzyme activity using fuchsine sulfuric acid. We used standards with pIs of 3.5, 4.2, 4.5, 5.15/5.3, 6, 6.9/7.35, 7.75/8/8.3, 9.45, and 10.65 (Boehringer) for comparison, and estimated the pIs of the enzymes from the calibration curve and the distance of the active band from the anode.

Electrophoretic Analysis

Denaturating SDS-PAGE was carried out with a Mini-Protean II apparatus (Bio-Rad, Hercules, CA) using 1-mm-thick slab gels containing 10% (w/v) acrylamide according to the procedure of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 and destained in an ethanol:acetic acid:water solution (1:1:8, v/v). Molecular-mass standards (Boehringer) were myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (97.4 kD), BSA (67 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

N-Terminal Amino Acid Sequencing

Proteins purified as described above were resolved on an 8% (w/v) SDS-PAGE gel of 0.75-mm-thickness under reducing conditions. The proteins were electrotransferred on a polyvinylidene difluoride membrane and stained with Coomassie Brilliant Blue R-250. Bands were excised from the membrane, and sequence analysis was determined by automated Edman degradation with a 476A pulsed-liquid-phase protein sequencer (Applied Biosystems, Foster City, CA). The phenylthiohydantoin derivatives of amino acids were separated and identified by an on-line phenylthiohydantoin analyzer on a phenylhydantoin-C_{18} column. The peptide sequences obtained were subjected to homology searches using the public protein sequence databases (Swiss Prot, PIR, PRF, nr-aa, GenBank, and EMBL).

Internal Sequencing

Bands purified by 8% (w/v) SDS-PAGE gel were excised from the gel and treated with formic acid, 50% (w/v) cyanogen bromide (w/v) overnight at room temperature. The eluted peptides were resolved on a gel according to the method of Schägger and von Jagow (1987). After electrotransfer on a polyvinylidene difluoride membrane and staining with Coomassie Brilliant Blue R-250 the bands were excised and the amino acid sequence was determined as described above.

Determination of Protein Concentration

Protein concentration was measured by the bicinchoninic acid method of Smith et al. (1985) using the reagents obtained from Sigma, and BSA as the standard protein.

Chromatographic Product Analysis

(a) HPLC-evaporating light scattering detection (ELSD) analysis was carried out using a liquid chromatograph equipped with a Sedex 55 ELSD detector (Sedere, Alfortville, France). α-Oxygenation products of palmitic acid were separated on a Spherisorb ODS2 C_{18} column (250 × 4.6 mm, Knauer, Berlin). Elution was performed at a flow rate of 1 mL min^{-1} using an acetonitrile-water mixture containing 0.05% (v/v) formic acid. The injection volume was 20 μL and the ratio of acetonitrile to water increased with a linear gradient from 55% to 100% (w/v) acetonitrile over 45 min. ELSD detection was performed at 40°C and an air pressure of 2.4 bar.

(b) LC-ESI-MS/MS analysis was performed on an Eurospher 100 C_{18} column (100 × 2 mm; Knauer) with a binary gradient delivered by an Applied Biosystems 140 B pump using solvent A (THF:methanol:water:acetic acid, 25:30:44.9:0.1, v/v) and solvent B (methanol:water, 9:1, v/v). Both solvents A and B contained 5 mM NH_4OAc. After injection of the sample, a linear gradient from 20% to 100% solvent B was applied over 30 min with a flow rate of 0.2 mL min^{-1}. Injection volume was 1 μL both for reference compounds and biological samples. LC-ESI-MS/MS analyses were performed according to our previously described conditions (Schneider et al., 1997) using a triple-quadrupole TSQ 7000 apparatus with electrospray interface (Finnigan-MAT, Bremen, Germany). For pneumatically assisted electrospray ionization the spray capillary voltage was set to 4 kV and the temperature of the heated inlet capillary serving simultaneously as repeller electrode (8.3 V) was 170°C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 mL min^{-1}). Positive ions were detected by scanning from 100 to 400 amu with a total scan duration of 1.0 s for a single full spectrum. MS/MS experiments were carried out at a collision gas pressure of 1.8 mTorr of argon, scanning a mass range from 20 to 310 amu with a total scan duration of 1.0 s for a single full spectrum.

(c) The enantiomeric excess of α-oxygenation was determined by high resolution GC of the methyl 2-hydroxy fatty acid ester after derivatization with (−)-menthyl chloroformate as recently described (Adam et al., 1998).

(d) On-line high resolution GC-MS analysis was performed under electron impact conditions (70 eV) with an MD 800 system (Fisons, Bellvue, WA) equipped with a DB-5 fused silica capillary column (30 m × 2 mm i.d., film thickness = 0.25 μm, J&W Scientific, Folsom, CA). The temperature program was 60°C to 250°C at 5°C/min.

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