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3-Oxoacyl-(acyl-carrier protein) reductase from avocado (*Persea americana*) fruit mesocarp

Philip S. SHELDON,*‡ Roy G. O. KEKWICK,* Christopher SIDEBOTTOM,† Colin G. SMITH† and Antoni R. SLABAS†§

*Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, and †Biosciences Division, Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.

The NADPH-linked 3-oxoacyl-(acyl-carrier protein) (ACP) reductase (EC 1.1.1.100), also known as 'β-ketoacyl-ACP reductase', has been purified from the mesocarp of mature avocado pears (*Persea americana*). The enzyme is inactivated by low ionic strength and low temperature. On SDS/PAGE under reducing conditions, purified 3-oxoacyl-ACP reductase migrated as a single polypeptide giving a molecular mass of 28 kDa. Gel-filtration chromatography gave an apparent native molecular mass of 130 kDa, suggesting that the enzyme is tetrameric. The enzyme is inactivated by dilution, but some protection is afforded by the presence of NADPH. Kinetic constants have been determined using synthetic analogues as well as the natural ACP substrate. It exhibits a broad pH optimum around neutrality. Phenylglyoxal inactivates the enzyme, and partial protection is given by 1 mM-NADPH. Antibodies have been raised against the protein, which were used to localize it using immunogold electron microscopy. It is localized in plastids. *N*-Terminal amino-acid-sequence analysis was performed on the enzyme, and it shows close structural similarity with cytochrome *f*. Internal amino-acid-sequence data, derived from tryptic peptides, shows similarity with the putative gene products encoded by the *nodG* gene from the nitrogen-fixing bacterium *Rhizobium meliloti* and the *gra III* and *act III* genes from *Streptomyces spp.*

INTRODUCTION

Fatty acid synthetases have been classified into two types [1,2]. Animals, fungi and mycobacteria possess type 1 systems in which the acyl carrier and seven catalytic activities reside on one or two multifunctional polypeptide chains [3–7]. Type 2 systems, found in plants and prokaryotes such as *Escherichia coli*, and cyanobacteria, consist of a distinct acyl-carrier protein (ACP) and separable catalytic activities [8–15]. In comparison with animal and microbial systems, little is known concerning the enzymes of plant fatty acid synthetase.

In plants fatty acid synthesis *de novo* takes place in the plastid [16–20]. Following hydrolysis of the principal products, palmitoyl- and oleoyl-ACPs, fatty acids may be exported from the plastid and elongated and further desaturated in the endoplasmic reticulum [21].

3-Oxoacyl-ACP reductase catalyses the reduction of 3-oxoacyl-ACP to 3-hydroxy-ACP. Two forms have been reported. An NADPH-linked enzyme (EC 1.1.1.100) has been purified to homogeneity from spinach (*Spinacia oleracea*) leaf and partially purified from barley (*Hordeum vulgare*) leaf, safflower (*Carthamus tinctorius*) seed, *Euglena* and avocado (*Persea americana*) mesocarp [10–14,22]. In addition, an enzyme which catalyses the NADH-linked reduction of 3-oxoacyl thioesters, whose role in fatty acid synthesis is unclear, has been purified from avocado mesocarp plastids and *Euglena* [13,23].

Here we describe the purification and characterization of NADPH-linked 3-oxoacyl-ACP reductase from avocado mesocarp, amino acid sequences from its *N*-terminus and from two tryptic peptides, the production of a monospecific antiserum, and its subcellular localization by immunoelectron microscopy.

MATERIALS AND METHODS

Chemicals

Diketene, from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) was distilled under reduced pressure and stored at −20 °C before use. 1,3-Dibromopropanone was from Kodak Eastman Co (Liverpool, U.K.). Sephadex G-25 and Ultrogel AcA 34 were from Pharmacia LKB (Milton Keynes, Bucks., U.K.). Procion Red H-E3B–Sepharose 4B was made as described by Lowe & Pearson [24]. Assuming an absorption coefficient of 30000 $M^{-1} \cdot cm^{-1}$, the bound dye concentration was 0.79 μmol/g of moist gel. Hydroxyapatite, Bradford Reagent and 4-chloro-1-napthol were from Bio-Rad (Watford, Herts., U.K.). Acetoacetyl-*N*-acetylcysteamine was synthesized from diacetylcysteamine and diketene as described by Kass & Brock [25] and recrystallized from diethyl ether [26]. ^{125}I-labelled donkey anti-(rabbit Fab region) antiserum and iodo[^{14}C]acetamide were from Amersham International (Aylesbury, Bucks., U.K.). Goat anti-rabbit IgG–colloidal gold and Intense II were from Janssen (Wantage, Oxfordshire, U.K.). Other reagents were generally obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Plant material

Avocado pears (*Persea americana* var. Fuerte) were obtained from local wholesalers. Before use they were ripened at 25 °C and chilled overnight before homogenization.

Synthesis of acetoacetyl-ACP

ACP from *E. coli*, purified by the method of Vagelos *et al.* [27], was contained in Tris/HCl (pH 8.0)/approx. 0.4 M-NaCl and

Abbreviations used: ACP, acyl-carrier protein; PBS, phosphate-buffered saline.

‡ Present address and address for correspondence: Department of Biochemistry, University of Leeds, Leeds LS2 9JT, W. Yorks., U.K.

§ Present address: Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, U.K.

was concentrated by acid precipitation (HCl, pH 1.0). It was desalted on a Sephadex G-25 column (20 cm × 1.5 cm) equilibrated in 50 mM-Tris/HCl, pH 8.0, before use. Dithiothreitol was added to a final concentration of 10 mM to 10 mg (1 μmol) of ACP in 10 ml of buffer to reduce the pantetheine thiol groups. The solution was maintained under N_2 at 30 °C for 1 h. Diketene was added to a final concentration of 40 mM (a 2-fold excess over thiol groups), and the solution was maintained at 0 °C for 15 min with stirring. The mixture was then desalted using a Sephadex G-25 column (30 cm × 1.5 cm) equilibrated in 50 mM-acetate/NH_4^+, pH 5.0. The acetoacetyl-ACP was concentrated before use in kinetic studies by partial freeze-drying.

Enzyme assay

Activity was monitored by measuring the decrease in A_{340}. The standard assay mix (1 ml) contained potassium phosphate, pH 7.0 (final concn. 100 mM), NADPH (100 μM), enzyme solution (1–50 μl) and acetoacetyl-*N*-acetylcysteamine (final concn. 5 mM). With the exception of acetoacetyl-*N*-acetylcysteamine, the components of the assay were mixed in a 1 ml cell and preincubated for 5–10 min at 30 °C. The reaction was started by the addition of acetoacetyl-*N*-acetylcysteamine. An absorption coefficient of 6.22×10^3 M^{-1}·cm^{-1} for NADPH was used in calculation of enzyme activities [28]. All rates given have had the blank rate (no thioester present) subtracted from the enzymic rate (all components of assay present). An enzyme unit is defined as the amount of enzyme required to oxidize 1 nmol of NADPH/min in the above assays.

Determination of pseudo-Michaelis–Menten constants

The assay was essentially as described except that enzyme was added last. Enzyme stock solution was maintained at room temperature to prevent the possibility of cold-inactivation. Michaelis–Menten constants were calculated by linear regression from plots of [S] × [S]/v.

Purification of avocado 3-oxoacyl-ACP reductase

Preparation of a soluble extract and $(NH_4)_2SO_4$ fractionation. The initial operations were carried out at 4 °C. Routinely the mesocarp of ten ripe prechilled avocados was squeezed through one layer of muslin into 2 litres of homogenization buffer [0.33 M-sucrose/10 mM-KCl/20 mM-Tris/20 mM-KH_2PO_4/4 mM-*N*-acetylcysteine/0.5 mM-EDTA/1 mM-diethyldithiocarbamic acid/BSA (0.75 mg/ml), pH 7.4]. The mixture was stirred with a glass rod, and a further 2 litres of homogenization buffer were added. The slurry was squeezed through four layers of muslin, then centrifuged at 2000 *g* for 10 min. The upper two layers of lipid and supernatant were removed by suction and discarded, leaving a plastid-enriched layer. This was then frozen at −20 °C before subsequent use.

It was thawed by the addition of an approximately equal volume (about 1 litre) of 100 mM-sodium phosphate (pH 7.4)/1 mM-dithiothreitol/0.5 mM-EDTA at room temperature with stirring. It was subjected to six 30 s homogenizations in a Polytron instrument (Kinemetica, Lucerne, Switzerland) at maximum power and centrifuged at 14000 *g* for 60 min. The supernatant was filtered through glass wool. Solid $(NH_4)_2SO_4$ (208 g/litre) was added to 35% saturation, the pH being maintained by the addition of Tris base. After 20 min of gentle stirring the extract was centrifuged at 15000 *g*. $(NH_4)_2SO_4$ (62 g/litre) was added to the supernatant to 45% saturation, which was subsequently centrifuged at 15000 *g*. $(NH_4)_2SO_4$ (245 g/litre) was finally added to the '45%' supernatant to 80% saturation. After centrifugation the '45–80%' precipitate was dissolved in a minimum volume of 25 mM-sodium phosphate (pH 7.4)/0.5 mM-EDTA/10 mM-2-mercaptoethanol and dialysed against 3 × 5 litres of the same buffer at room temperature over a period of about 18 h. Finally it was centrifuged to remove the precipitated material. All the subsequent chromatographic steps were carried out at room temperature.

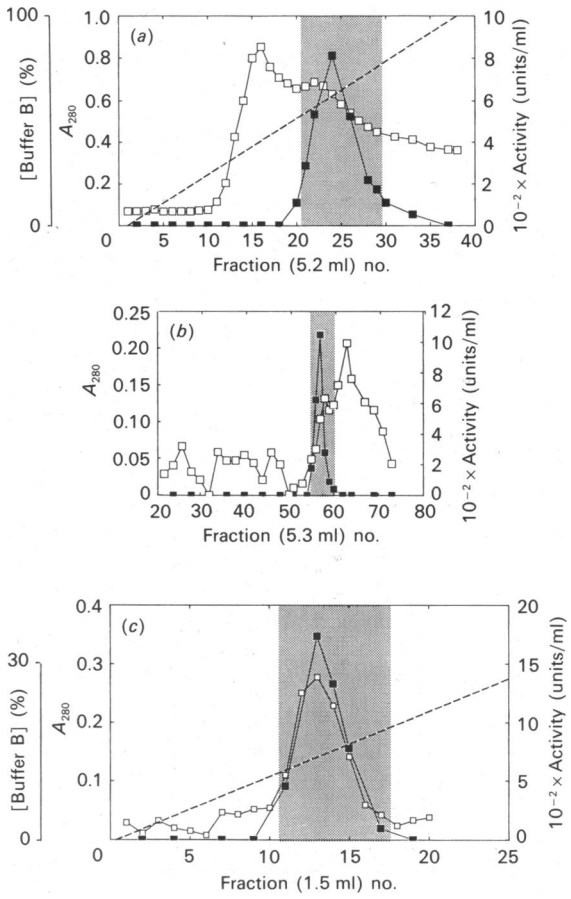

Fig. 1. Purification of 3-oxoacyl-ACP reductase

Chromatography conditions are given in the text. (*a*) Procion Red H-E3B–Sepharose chromatography of the 45–80%-satd.-$(NH_4)_2SO_4$ fraction. No activity was detected in the pass and wash (volume 1320 ml; A_{280} 1.9). (*b*) Gel filtration on Ultrogel AcA 34. (*c*) Hydroxyapatite chromatography. No activity or material absorbing at 280 nm was detected further in the salt gradient than is shown in the graph. The shaded areas represent the pooled fractions. □, A_{280}; ■, activity.

Procion Red H-E3B–Sepharose chromatography. The preparation was applied to a Procion Red H-E3B–Sepharose column (2.1 cm × 18 cm), previously equilibrated in 25 mM-sodium phosphate (pH 7.4)/0.5 mM-EDTA/1 mM-dithiothreitol. The column was washed at a flow rate of 1.2–1.5 ml/min for about 12 h. Activity was eluted with a linear gradient starting with the same buffer and leading to 100 mM-sodium phosphate/2 M-NaCl/1 mM-dithiothreitol, over a volume of 200 ml and at a flow rate of about 1 ml/min (Fig. 1*a*). The fractions of higher specific activity were pooled, then concentrated with an Amicon pressure cell with a PM10 membrane.

Gel-filtration chromatography. The next step was gel filtration, using Ultrogel AcA 34 (a 2.1 cm × 90 cm column). The column was equilibrated in 100 mM-sodium phosphate (pH 7.4)/0.5 mM-EDTA/1 mM-dithiothreitol. A flow rate of 0.5 ml·min^{-1} was used (Fig. 1*b*).

Hydroxyapatite chromatography. The final step was hydroxyapatite chromatography on a 0.8 cm × 10 cm column. The column was pre-equilibrated in 100 mM-sodium phosphate (pH 7.4)/1 mM-dithiothreitol. After sample application the enzyme was eluted with a gradient to 1 M-sodium phosphate (pH 7.4)/1 mM-dithiothreitol over a volume of 140 ml (Fig. 1*c*).

Analytical procedures

Protein estimation was carried out either by the dye-binding method [29] or by measurement of A_{280}. The final specific activity was determined from direct amino acid analysis.

SDS/PAGE was carried out using the discontinuous system of Laemmli [30]. Samples were prepared by dialysis against 20 mM-*N*-ethylmorpholine acetate, pH 8.0, followed by freeze-drying. Proteins were revealed by staining with Coomassie Blue G250 [31].

Amino acid analysis was carried out using an LKB 4400 analyser, the manufacturer's recommended procedure being followed. Cysteine residues were determined after performic acid oxidation and quantified by comparison with valine.

H.p.l.c./f.p.l.c. systems and sequential Edman degradation on an Applied Biosystems gas-phase sequencer, model 470, were as described in [32]. Before amino acid sequencing, cysteine residues were reductively alkylated with iodoacetamide [33].

Immunization of rabbits with 3-oxoacyl-ACP reductase

Cross-linking of antigen to keyhole-limpet haemocyanin. The method of cross-linking is from J. Knudsen (personal communication). Keyhole-limpet haemocyanin (7.5 mg) was suspended in 0.1 M-potassium phosphate, pH 6.8 (750 μl), by sonication in an Eppendorf tube. Glutaraldehyde [25 % (v/v), 225 μl] was added and the mixture was stirred at room temperature overnight. A Sephadex G-25 (Fine grade) column (in a Pasteur pipette) was equilibrated in 0.9 % NaCl and the void volume was determined. The keyhole-limpet haemocyanin/glutaraldehyde mixture was run through the column. In order to remove excess unbound glutaraldehyde and maintain a high protein concentration, only the first 100 μl to be eluted after the void volume was used, the remaining material being discarded. Avocado 3-oxoacyl-ACP reductase (100 μg), prepared by dialysis into 20 mM-*N*-ethylmorpholine acetate and freeze-drying, was dissolved in 36 μl of 1 M-Na_2CO_3 buffer, pH 9.5. Then 100 μl of Sephadex G-25 eluate (containing 1 mg of keyhole-limpet haemocyanin) was added and the mixture was stirred overnight at room temperature. Any remaining active groups were blocked by the addition of 1 M-glycine (36 μl). The mixture was stirred for another 60 min, and divided into three equal portions. They were each made up to 0.75 ml with sterile phosphate-buffered saline (PBS; 10 mM-sodium phosphate/150 mM-NaCl, pH 7.4) before being frozen at −20 °C before injection.

Immunization schedule. A rabbit was given four injections of antigen. The first three were of cross-linked keyhole-limpet haemocyanin–3-oxoacyl-ACP reductase; the final booster was with unlinked 3-oxoacyl-ACP reductase (100 μg). The procedure was devised such that, on the first three injections, the immunogenicity was raised by linkage of the protein to keyhole-limpet haemocyanin. After a fall in the titre, injection with unlinked antigen should result in a specific increase of the antibodies against the antigen as opposed to its carrier. Immunizations were carried out by subcutaneous injections in the back. In the first week the rabbit was immunized with 33 μg of cross-linked 3-oxoacyl-ACP reductase in complete adjuvant. At the beginning of weeks 7 and 9 respectively it was given boosters of 33 μg of cross-linked 3-oxoacyl-ACP reductase in incomplete adjuvant. At the beginning of week 12 it was given a final booster of 100 μg of unlinked 3-oxoacyl-ACP reductase in incomplete adjuvant. Serum was used from week 14.

Western blotting

After electrophoresis on 0.75 mm-thick gels, blotting was carried out as described by Towbin *et al.* [34]. During the following incubations, carried out successively at room temperature, a rotary shaker was used to agitate the liquid around the nitrocellulose paper: (*a*) 1 % haemoglobin in TBS [20 mM-Tris/HCl (pH 7.5)/0.5 M-NaCl] for at least 2 h or overnight; (*b*) antiserum (0.2 %) in TBSHb (1 % haemoglobin in TBS) for 2 h; (*c*) two 15 min washes with TBS; (*d*) ^{125}I-labelled donkey anti-(rabbit Fab region) antiserum (0.2 %) in TBSHb for 1 h; (*e*) two 30 min washes with TBS. After drying the blot was autoradiographed overnight.

Immunoelectron microscopy

Avocado mesocarp tissue was prepared for immunoelectron microscopy as previously described [35]. The rabbit antiserum raised against 3-oxoacyl-ACP reductase was used at 1:600 (v/v) dilution in PBS/0.1 % Tween 20, containing 1 % ovalbumin, for 60 min at 37 °C. After a thorough wash with PBS, the sections were incubated with goat anti-rabbit IgG–colloidal gold (5 nm-diameter particles), diluted 1:200 (v/v) in PBS/1 % ovalbumin for 30 min at 37 °C.

The preparations were silver-enhanced using Intense II for 3 min at room temperature. All sections were counterstained with aq. 2 % (v/v) uranyl acetate, or uranyl acetate together with lead citrate, before examination. Control experiments included (*a*) using pre-immune serum and (2) omitting the primary antibody.

Trypsin treatment

Reductively alkylated protein was freeze-dried and dissolved in 200 mM-NH_4HCO_3 saturated with $CaCl_2$. Tosylphenylalanylchloromethane ('TPCK')-treated trypsin was added at a proteinase/protein ratio of 1:100, and the mixture was incubated at 37 °C for 12 h. A further aliquot of proteinase (proteinase/protein ratio 1:100) was added and the mixture incubated again for 12 h at 37 °C. The digest was injected on to an OD-032 Spheri 5 C_{18} column (Anachem, Luton, Beds., U.K.), which was eluted with a linear gradient from 0.1 % (v/v) trifluoroacetic acid in water to 0.1 % (v/v) trifluoroacetic acid/75 % (v/v) acetonitrile in water over 7.5 ml at a flow rate of 100 μl/min.

RESULTS

Enzyme stability

A dialysed $(NH_4)_2SO_4$ fraction was diluted 1:5 into 10 mM-potassium phosphate (pH 7.2) ± 2 M-NaCl and incubated at either 4 or 27 °C for 24 h. The activity was then measured. It was found that incubation in 2 M-NaCl at 27 °C resulted in a 3-fold increase in preservation of activity compared with any of the other conditions.

Purification of the enzyme to electrophoretic homogeneity

The enzyme was purified by $(NH_4)_2SO_4$ fractionation, followed by chromatography on Procion Red H-E3B–Sepharose, Ultrogel AcA 34 and hydroxyapatite (Fig. 1). In order to maintain enzymic activity, the purification was carried out at room temperature using buffer solutions of as high an ionic strength as possible. Table 1 shows a typical purification of 3-oxoacyl-ACP reductase. The apparent increase in total activity after $(NH_4)_2SO_4$ fractionation may have been due to re-activation by high-ionic-strength buffer. The enzyme was purified approx. 280-fold at a yield of 20 %.

Table 1. Purification of 3-oxoacyl-ACP reductase from avocado mesocarp

Fraction	Volume (ml)	A_{280}	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
Supernatant	1540	–	1694*	56500	33.4*	1	100
45–80%-Satd. $(NH_4)_2SO_4$	81	–	1255*	63400	50.5*	1.5	112
Dialysis residue	91	–	865*	38000	43.0*	1.3	67
Procion Red H-E3B–Sepharose	47	0.579	27†	21500	790†	24	38
Ultrogel AcA 34	32	0.098	3.1†	20100	6400†	192	36
Hydroxyapatite	10.5	0.159	1.23‡	11600	9401‡	281	20.5

* Protein determined by Bradford [29] method.
† Protein determined by assuming a 1% solution has an A_{280} of 10.0.
‡ Protein determined by amino acid analysis.

On SDS/dithiothreitol/PAGE, the purified enzyme migrated as a single component of molecular mass 28 kDa (Fig. 2, lane A). In the absence of dithiothreitol, a number of additional components, presumably containing either inter- or intra-chain disulphide bonds, were also observed (lane B).

Native molecular mass and subunit structure

On f.p.l.c. gel filtration using Superose 12, the enzyme activity was eluted as a single peak at a position corresponding to a molecular mass of approx. 130 kDa.

Total amino acid analysis

The enzyme contains three cysteine residues/28 kDa subunit and apparently does not contain histidine (Table 2). A comparison with the composition of the spinach leaf enzyme [12] is also shown. There are quite notable differences in the amino acid composition. The avocado enzyme has half the quantity of proline and double the isoleucine content; in addition, it has considerably fewer aromatic residues than the spinach enzyme.

Kinetic characterization

When purified enzyme, dialysed against 50 mM-potassium phosphate/8 mM-Mops, pH 7.0, was diluted 1:10 in the same buffer containing 1 mg of BSA/ml and maintained at 0 °C, it lost 74% activity after 12 min. The undiluted enzyme was not significantly inactivated after this time. The inclusion of 500 μM-NADPH in the dilution buffer gave significant protection (27% loss of activity), suggesting that the binding of NADPH to the enzyme favours a more stable conformation.

When the concentration of acetoacetyl-*N*-acetylcysteamine was maintained at 5 mM and NADPH concentration was varied, it was found that, at low NADPH concentration, the initial rates did not appear to obey Michaelis–Menten kinetics (Fig. 3*a*). This may have been due to the protection against inactivation of the enzyme, on dilution into the assay mix, given by NADPH. This also may be an explanation for the variations previously reported for the K_m: 71 μM for the avocado enzyme [13], 25 μM and 15 μM for the spinach leaf enzyme [11,12] and 16 μM for the safflower seed enzyme [10]. No activity was detected when 100 μM-NADH was used as a cofactor instead of NADPH.

Pseudo single-substrate Michaelis–Menten constants at 100 μM-NADPH were determined for the acetoacetyl thioesters of *N*-acetylcysteamine, CoA and ACP from *E. coli* (Figs. 3*b*, 3*c* and 3*d*). In order to obtain measurable rates for the CoA and ACP substrates, the enzyme was diluted respectively 1:10 and 1:20 and incubated for 90 min at 25 °C before use. After this

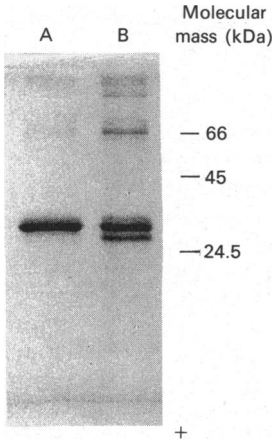

Fig. 2. Analysis of purified avocado 3-oxoacyl-(ACP) reductase by SDS/PAGE

The Figure shows a 12%-(w/v)-polyacrylamide gel stained with Coomassie Brilliant Blue. Lane A, purified enzyme solubilized in SDS/PAGE sample buffer containing 20 mM-dithiothreitol; lane B, purified enzyme solubilized in sample buffer in the absence of dithiothreitol.

Table 2. Total amino acid composition of 3-oxoacyl-ACP reductase from avocado mesocarp and comparison with that of the spinach leaf enzyme [12]

Amino acid	Composition (residues/subunit) Avocado (28 kDa)	Spinach leaf (24.5 kDa)
Asx	26	23
Thr	16	15
Ser	15	13
Glx	32	13
Pro	8	16
Gly	28	30
Ala	36	23
Val	28	23
Met	1	2
Ile	23	14
Leu	19	20
Tyr	2	6
Phe	4	19
His	0	6
Lys	20	15
Arg	9	3
Cys	3	2

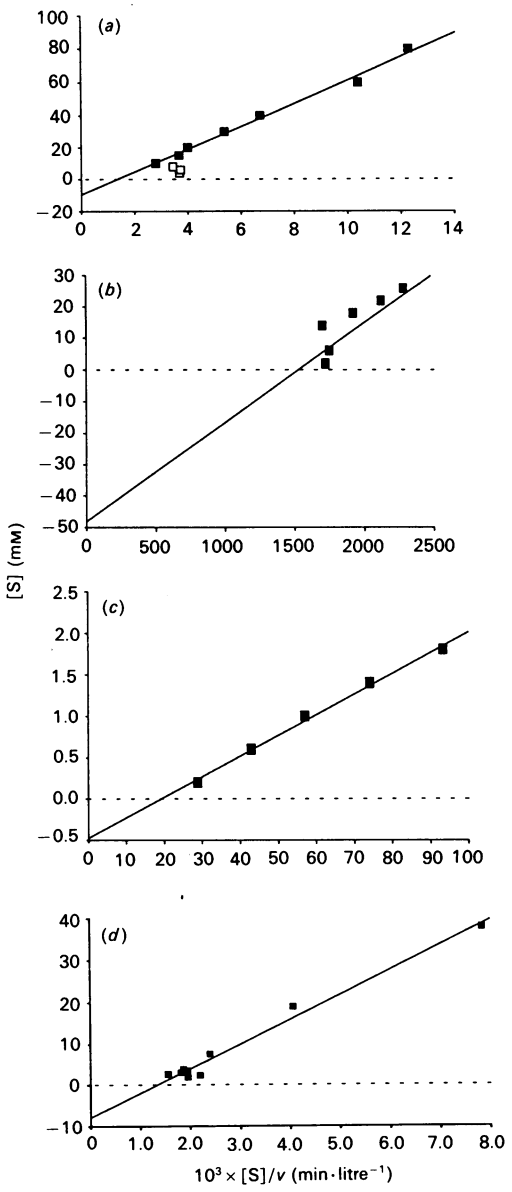

Fig. 3. Kinetics of 3-oxoacyl-ACP reductase: plots of [S] versus [S]/v

(*a*) NADPH (acetoacetyl *N*-acetylcysteamine concentration maintained at 5 mM). The points at low NADPH concentration (□), in which the enzyme was inactivated by dilution, were not used in the calculation of kinetic constants; (*b*) acetoacetyl-*N*-acetylcysteamine; (*c*) acetoacetyl-CoA; (*d*) acetoacetyl-ACP. For (*b*), (*c*) and (*d*) the NADPH concentration was maintained at 100 μM.

time the activity had stabilized, but since inactivation on dilution had occurred, the comparative $V_{max.}$ (apparent) values are likely to be underestimations. The kinetic data are summarized in Table 3. The K_m value of 7.9 μM for acetoacetyl-ACP is comparable with previously determined values, which range from 3.7 to 8.8 μM [11–13].

The order of specificity towards the three substrates is *N*-acetylcysteamine < CoA < ACP.

Dependence of activity on pH

The reductase appears to possess a pH optimum around neutrality, which is surprisingly broad for a reaction in which a proton participates (Fig. 4). The spinach leaf enzyme, by contrast, was reported to be more active at low pH [12]. At a pH below 5.0,

Table 3. Kinetic constants of 3-oxoacyl-ACP reductase

The constants for NADPH were determined with the acetoacetyl-*N*-acetylcysteamine concentration maintained at 5 mM; for the thioester substrates the NADPH concentration was maintained at 100 μM.

Substrate	K_m (μM)	$V_{max.}$ (μmol·min^{-1}·mg^{-1})
NADPH	9.3	13
Acetoacetyl-*N*-acetylcysteamine	48000	280
Acetoacetyl-CoA	470	450
Acetoacetyl-ACP	7.9	530

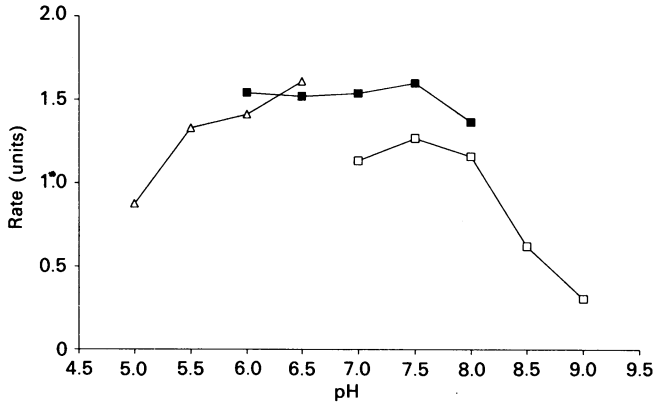

Fig. 4. Effect of pH on the activity of 3-oxoacyl-ACP reductase

The assay, using acetoacetyl-*N*-acetylcysteamine, was modified from that described in the Materials and methods section by substituting the indicated buffers for potassium phosphate. △, 0.2 M-Citrate/K$^+$; ■, 0.2 M-phosphate/K$^+$; □, 0.2 M-Tris/HCl.

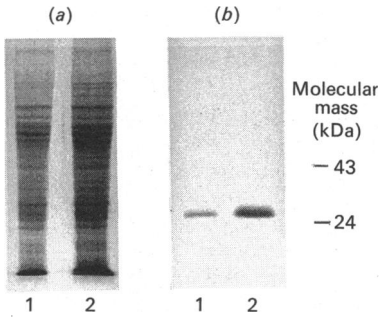

Fig. 5. SDS/PAGE and Western-blot analysis of avocado extracts

Plastids, prepared by differential centrifugation [19], were resuspended in Laemmli sample buffer. The sample was heated to 100 °C for 5 min. After removal of undissolved material by centrifugation, SDS/PAGE on 10% gels was carried out. (*a*) Coomassie Blue-stained gel; (*b*) autoradiograph of blot developed as described in the Materials and methods section, using antiserum raised against 3-oxoacyl-ACP reductase. Lane 1, 5 μl sample; lane 2, 10 μl sample.

NADPH becomes labile, and at high pH values acetoacetyl-*N*-acetylcysteamine forms an enolate ion (pK_a 8.5) [36].

Effects of phenylglyoxal, iodoacetamide and 1,3-dibromopropanone on 3-oxoacyl-ACP reductase activity

In 50 mM-$NaHCO_3$ buffer, pH 8.3, the addition of 10 mM-phenylglyoxal causes rapid loss of activity. When the reaction was carried out in the presence of 1 mM-NADPH, loss of activity

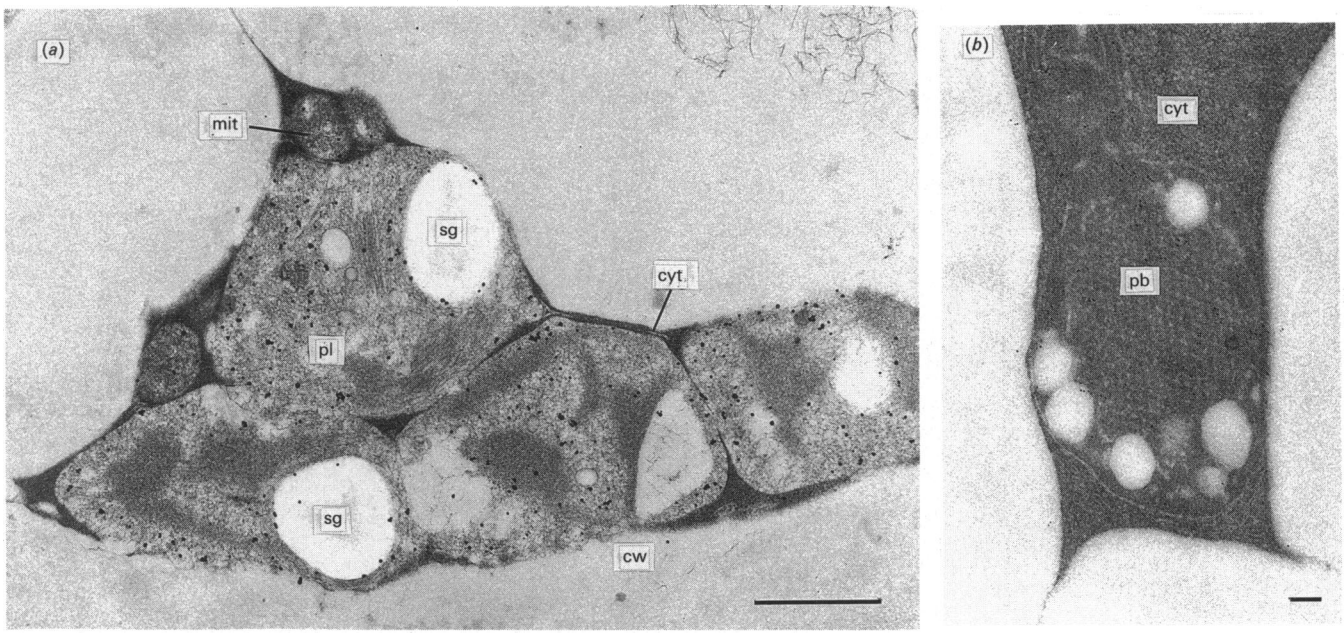

Fig. 6. Localization of 3-oxoacyl-ACP reductase in avocado mesocarp

Abbreviations: pl, plastids; pb, prolamella body; sg, starch grains; cw, cell wall; cyt, cytoplasm. In (*a*) the scale bar represents 1 μm; (*b*) shows an etioplast from the inner mesocarp and the scale bar represents 0.1 μm.

appears to take place in two phases. About 55% of enzyme activity is rapidly lost. The remaining activity is somewhat protected, compared with reaction in the absence of NADPH. In $NaHCO_3$ buffer, phenylglyoxal reacts specifically with arginine residues [37]. This confirms previously obtained data on the reaction of the enzyme with butane-2,3-dione, suggesting that the NADPH-binding site contains an arginine residue [13].

It has been reported that the 3-oxoacyl reductase activity of mammalian fatty acid synthetase is able to reduce phenylglyoxal itself [38]. Such an activity could not be detected at pH 7.0 in the avocado enzyme.

After removal of dithiothreitol from the purified enzyme by pressure dialysis, the activity of 3-oxoacyl-ACP reductase was unaffected by incubation in 1 mM-iodoacetamide or 1 mM-1,3-dibromopropanone over a period of 60 min. The enzyme therefore does not appear to possess an active-site thiol group.

Subcellular localization of 3-oxoacyl-ACP reductase by immunoelectron microscopy

A monospecific antiserum was raised against the purified enzyme which reacts against a single 28 kDa component after blotting against a total extract from a crude plastid fraction (Fig. 5). The subcellular distribution of the enzyme was determined by immunoelectron microscopy of avocado tissue. The enzyme is found in both chloroplasts and non-photosynthetic plastids (Fig. 6). As with ACP [35], the mitochondria are also occasionally labelled.

N-Terminal and internal amino acid sequencing

The sequence of the first 24 residues from the *N*-terminus is shown in Fig. 7. The initial yield was about 10%, suggesting that the *N*-terminus was partially blocked.

After digestion with trypsin and separation by reverse-phase h.p.l.c. the sequences of two tryptic peptides from avocado 3-oxoacyl-ACP reductase were also determined.

A search using the protein sequence database at Leeds University revealed similarities with cytochrome *f* from the liverwort *Marchantia polymorpha* [39], the putative *nodG*-gene product from *Rhizobium meliloti* [40], *gra III* open reading frames 5–6 from *Streptomyces violaceoruber* [41] and *act III* from *Streptomyces coelicolor* [42]. The relevant sequences are aligned in Fig. 7.

DISCUSSION

In the present paper we have described the purification, to electrophoretic homogeneity, of 3-oxoacyl-ACP reductase from avocado mesocarp. The purification method is designed to preserve activity by using buffers of as high an ionic strength as possible, and by being carried out at room temperature. The enzyme shows similarities to the 3-oxoacyl-ACP reductase from *E. coli* (also a type 11 fatty acid synthetase component), in that they are both inactivated by cold and low ionic strength [43]. Similarly the light-induced *Euglena* enzyme appears to lose activity after freeze–thawing [22]. More surprisingly the 3-oxoacyl reductase activity of multifunctional chicken liver fatty acid synthetase is also inactivated by low ionic strength and low temperature [44,45].

On SDS/PAGE analysis the enzyme appears as a single polypeptide of molecular mass 28 kDa. Since the native enzyme has an apparent molecular mass of 130 kDa on gel filtration, it would appear to be tetrameric.

The enzyme has a marked preference for use of its physiological substrate, acetoacetyl-ACP, over the model substrates acetoacetyl-*N*-acetylcysteamine and acetoacetyl-CoA, indicating that it is an authentic component of fatty acid synthetase. The enzyme is rapidly inactivated by phenylglyoxal, confirming earlier reports of the presence of an active-site arginine residue. In the presence of NADPH, about 55% activity is rapidly lost, but the remaining activity is somewhat protected. This appears to indicate 'half-the-sites' reactivity characteristic of many oxidoreductases. The avocado enzyme activity is not affected by thiol reagents, unlike the spinach leaf enzyme [12].

Immunoelectron microscopy indicates that the enzyme is

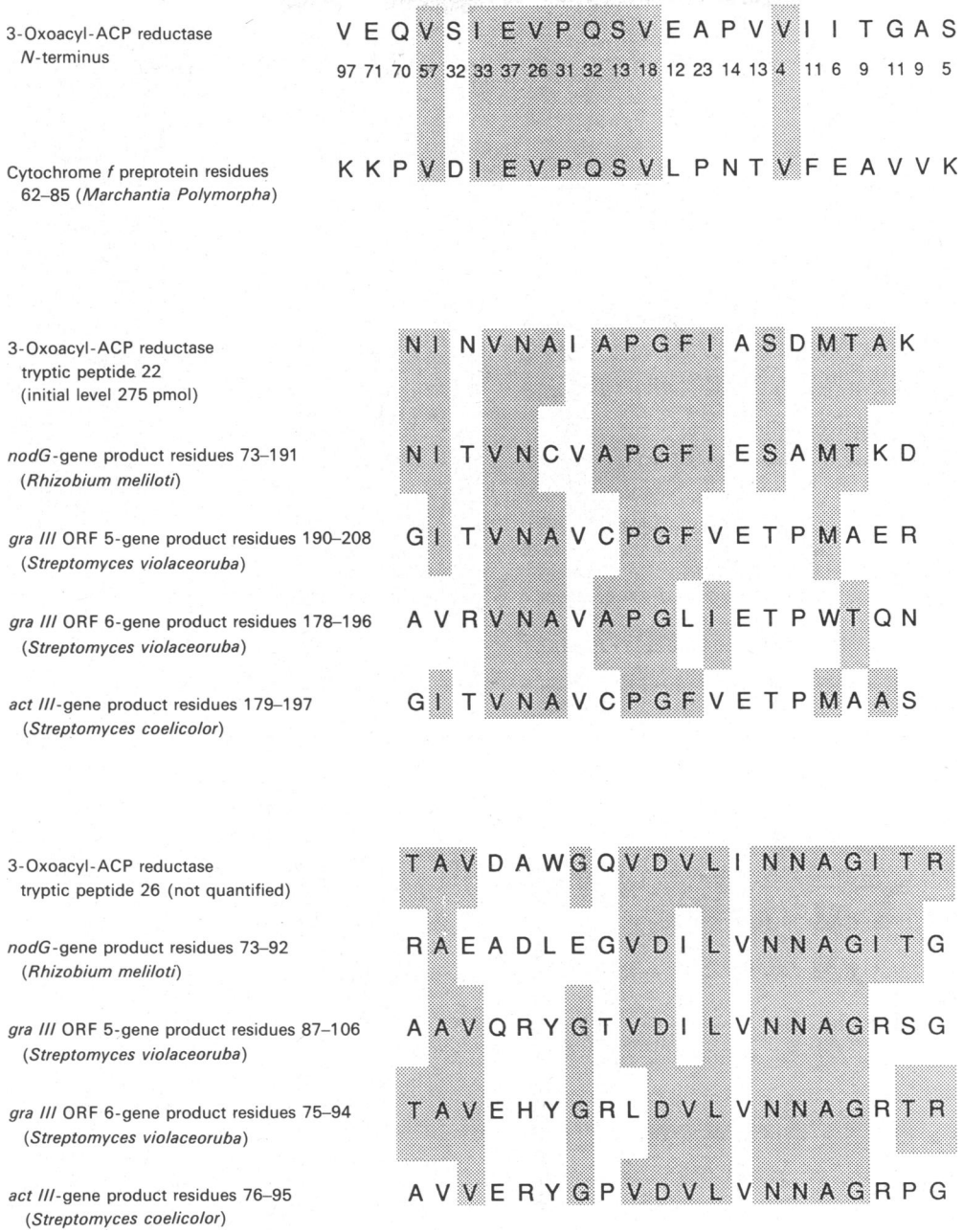

Fig. 7. Sequencing of the *N*-terminus and two tryptic peptides of 3-oxoacyl-ACP reductase: comparison with similar sequences

The numbers under the first sequence are amino acid yields in pmol. Abbreviation: ORF, open reading frame.

located predominantly inside the plastids of mesocarp tissue. This confirms the conclusion of Weaire & Kekwick [19], which was based on their results from the fractionation of homogenates of avocado mesocarp tissue. It has been observed in other plant tissues that fatty acid synthesis *de novo* and ACP are exclusively localized inside the plastid [16–20,33].

Part of the *N*-terminal sequence shows similarity to cytochrome *f* [39]. The similarity may reflect a functional domain common to both proteins.

Internal amino-acid-sequence information has been obtained by sequencing of two tryptic peptides. These both show strong similarity to sequences present in the putative *nodG*-gene product, a 26 kDa polypeptide from *Rhizobium meliloti* [40] and to the *gra III*- and *act III*-gene products from *Streptomyces* spp. [41,42]. It is noteworthy that the *nodE*- and *nodF*-gene products, which may be part of the same operon, show similarity to condensing enzyme and ACP [46,47], other components of fatty acid synthetase. It therefore appears that these genes, which may determine host specificity, encode enzymes catalysing a similar pathway to that of fatty acid synthesis. Recently the structure of a root-hair-deformation factor has been isolated and shown to be a sulphated *N*-acyl-tri-*N*-acetyl-β1,4-D-glucosamine tetrasaccharide. The acyl group was identified as hexadecadi-2,9-enoic acid [48].

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REFERENCES

1. Bloch, K. & Vance, D. (1977) Annu. Rev. Biochem. **46**, 263–298
2. Brindley, D. N., Matsumura, S. & Bloch, K. (1969) Nature (London) **244**, 666–669
3. Stoops, J. K., Arslanian, M. J., Oh, Y. H., Aune, K. C., Wakil, S. J. & Oliver, R. M. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 1940–1944
4. Guy, P. S., Law, S. & Hardie, D. G. (1978) FEBS Lett **94**, 33–37
5. McCarthy, A. D. & Hardie, D. G. (1983) Eur. J. Biochem. **130**, 185–193
6. Schweizer, E., Werkmeister, K. & Jain, M. K. (1978) Mol. Cell. Biochem. **21**, 95–107
7. Fulco, A. J. (1983) Prog. Lipid Res. **22**, 133–160
8. Volpe, J. J. & Vagelos, P. R. (1973) Annu. Rev. Biochem. **12**, 21–60
9. Vagelos, P. R. (1974) MTP Int. Rev. Sci. (Biochem. Lipids) **4**, 100–140
10. Shimakata, T. & Stumpf, P. K. (1982) Arch. Biochem. Biophys. **217**, 144–148
11. Shimakata, T. & Stumpf, P. K. (1982) Plant Physiol. **69**, 1257–1262
12. Shimakata, T. & Stumpf, P. K. (1982) Arch. Biochem. Biophys. **218**, 77–91
13. Caughey, I. & Kekwick, R. G. O. (1982) Eur. J. Biochem. **123**, 553–561
14. Høj, P. B. & Mikkesen, J. D. (1982) Carlsberg Res. Commun. **47**, 119–141
15. Slabas, A. R., Hellyer, A., Sidebottom, C., Bambridge, H., Cottingham, I. R., Kessell, R., Smith, C. G., Sheldon, P., Kekwick, R. G. O., de Silva, J., Lucas, C., Windhurst, J., James, C. M., Hughes, S. G. & Safford, R. (1988) NATO Advanced Study Institute, Series A, Vol. 140: Plant Molecular Biology (Wettstein, D. & Chua, N., eds.), pp. 265–277, Plenum Press, New York
16. Zilkey, B. F. & Canvin, D. T. (1972) Can. J. Bot. **50**, 323–326
17. Nakamura, Y. & Yamamada, M. (1979) Plant Sci. Lett. **14**, 291–295
18. Vick, B. & Beevers, H. (1978) Plant Physiol. **62**, 173–178
19. Weaire, P. J. & Kekwick, R. G. O. (1975) Biochem. J. **146**, 425–437
20. Ohlrogge, J. B., Kuhn, D. N. & Stumpf, P. K. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 1194–1198
21. Gurr, M. I. (1980) in The Biochemistry of Plants (Stumpf, P. K., ed.), vol. 4, pp. 205–248, Academic Press, New York
22. Hendren, R. W. & Bloch, K. (1980) J. Biol. Chem. **255**, 1504–1508
23. Ernst-Fonberg, M. L. (1986) Plant Physiol. **82**, 978–984
24. Lowe, C. R. & Pearson, J. C. (1984) Methods Enzymol. **104**, 97–113
25. Kass, L. R. & Brock, D. J. H. (1969) Methods. Enzymol. **14**, 696–700
26. Lynen, F. & Wieland, O. (1953) Methods Enzymol. **1**, 566–573
27. Vagelos, P. R., Alberts, A. W. & Majerus, P. W. (1969) Methods Enzymol. **14**, 60–63
28. Warburg, O. & Christian, W. (1941) Biochem. Z. **310**, 384–421
29. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
30. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
31. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) Biochemistry **10**, 2607–2617
32. Cottingham, I. R., Austin, A., Sidebottom, C. & Slabas, A. R. (1988) Biochim. Biophys. Acta **954**, 201–207
33. Johnson, D. M. A., Gagnon, J. & Reid, K. B. M. (1980) Biochem. J. **187**, 863–874
34. Towbin, H., Staehlin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
35. Slabas, A. R. & Smith, C. G. (1988) Planta **175**, 145–152
36. Lynen, F. & Ochoa, S. (1953) Biochim. Biophys. Acta **12**, 299–314
37. Cheung, S. T. & Fonda, M. L. (1979) Biochem. Biophys. Res. Commun. **90**, 940–947
38. Poulose, A. J. & Kollatukudy, P. E. (1986) Int. J. Biochem. **18**, 807
39. Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Shin-ichi, A., Inokuchi, H. & Ozeki, H. (1986) Nature (London) **322**, 572–574
40. Debellé, F. & Sharmi, S. B. (1986) Nucleic Acids Res. **14**, 7453–7472
41. Sherman, D. H., Malpartida, F., Bibb, M. J., Kieser, H. M., Bibb, M. J. & Hopwood, D. A. (1989) EMBO J. **8**, 2717–2725
42. Hallam, S. E., Malpartida, F. & Hopwood, D. A. (1988) Gene **74**, 305–320
43. Schulz, H. & Wakil, S. J. (1971) J. Biol. Chem. **246**, 1895–1901
44. Yuan, Z. & Hammes, G. G. (1986) J. Biol. Chem. **261**, 13643–13651
45. Kashem, M. A. & Hammes, G. G. (1988) Biochim. Biophys. Acta **956**, 39–48
46. Bibb, M. J., Biro, S., Motamedi, H., Collins, J. F. & Hutchinson, C. R. (1989) EMBO J. **8**, 2727–2736
47. Shearman, C. A., Rossen, L., Johnston, A. W. B. & Downie, J. A. (1986) EMBO J. **5**, 647–652
48. Lerouge, P., Roche, P., Faucher, P., Maillet, F., Truchet, G., Promé, J. C. & Dénarié, J. (1990) Nature (London) **344**, 781–784

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