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DELTA-9 DESATURASE ACTIVITY IN DEVELOPING COTYLEDONS OF SUNFLOWER (*HELIANTHUS ANNUUS*. L)

GARETH GRIFFITHS,* W. TREVOR GRIFFITHS† and A. KEITH STOBART‡

Department of Plant Genetics and Biotechnology, Horticulture Research International, Wellesbourne, Warwickshire, CV35 9EF, U.K.; † Department of Biochemistry, School of Medicine, University of Bristol, Bristol BS8 1TD, U.K.; ‡ School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, U.K.

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Key Word Index—*Helianthus annuus*; Compositae; seed development; oil synthesis; Δ9 desaturase; ferredoxin.

Abstract—Delta-9-desaturase (Δ9d) activity has been studied in the 100,000 *g* soluble protein fraction derived from the developing seed cotyledons of sunflower at a stage of active oil synthesis. In the presence of NADPH, ferredoxin (fd) and ferredoxin oxidoreductase (FdR) stimulated Δ9d activity. Fractionation of the Δ9d from endogenous fd by gel filtration resulted in the complete loss of activity and this could be restored by the addition of exogenously supplied fd together with its reductase. A soluble cytochrome-b_5 could not substitute for fd, indicating specificity in the binding site for the electron carrier, and this specificity was further supported by inhibitor studies using bis(salicylidene)-1,3-propanediamine (DSPD) and trifluoperazine (TFP). Δ9d was confirmed as a non-haem (no inhibition with CO) iron-containing, KCN-sensitive, 35 kDa protein on SDS-PAGE. Physiological levels of hydrogen peroxide (H_2O_2) up to 10 μM did not inhibit desaturation although catalase stimulated activity 2–3 fold. The biochemical evidence presented here supports the mechanistic model of Δ9d activity recently proposed from crystallographic studies of the castor enzyme.

INTRODUCTION

In unsaturated fatty acid synthesis, the introduction of a double bond into a saturated acyl chain at the C-9 position is catalysed by the enzyme Δ9 desaturase (Δ9d). This enzyme can utilise both palmitoyl- and stearoyl-esters yielding palmitoleic (16:1, Δ9) and oleic (18:1 Δ9) acids [1–3]. In mammals and yeast the desaturase is bound to the endoplasmic reticulum membranes and the substrate is the CoA ester (18:0–CoA) [4, 5]. Cytochrome-b_5 and cytochrome-b_5 reductase have been identified as the electron carriers from NADH to water during oxygen dependent desaturation [6, 7]. In plants, on the other hand, the Δ9d is a soluble plastidic protein with a marked preference for stearoyl-acyl carrier protein (18:0–ACP) as substrate [8, 9]. The putative electron carriers in this reaction are ferredoxin (fd) and ferredoxin oxidoreductase (FdR) [10, 11]. Here we report further studies on the biochemical characterisation and properties of Δ9d from the developing seed cotyledons of sunflower.

* Author to whom correspondence should be addressed.

RESULTS

Δ9 *desaturase activity*

Previous reports indicate that the higher plant Δ9d is a soluble protein [8, 9]. We have therefore characterised the activity of the 100,000 *g* soluble protein fraction prepared from cotyledons with active oil deposition, 14–18 days post anthesis [12]. Enzyme activity was measured by the release of tritium from [$^{9,10\text{-}3}$H] 18:0–CoA to water in the presence of putative cofactors [11]. The CoA ester was used in preference to the ACP substrate (V_{max} is the same for both) to avoid any potential denaturation of the protein moiety of the ACP by inhibitors used in later expts in the present study. Optimal rates of desaturation were achieved, in the presence of NADPH and catalase (see later), by the addition of fd and FdR and without the addition of BSA (Table 1). The desaturation rate was linear for up to 20 min at 25° (Fig. 1) and directly related to protein concn up to 0.4 mg/ml (data not given). Although some desaturation occurred with NADH as reductant (0.1 nmol/min/mg), this was much lower than the activity achieved with NADPH (0.8 nmol/min/mg).

Table 1. Effect of putative cofactor additions on Δ9 desaturation

NADPH	Fd	FdR	BSA	% Activity
—	—	—	—	<1
✓	—	—	—	11±2
✓	✓	—	—	41±5
✓	—	✓	—	18±3
✓	✓	✓	—	100
✓	✓	✓	✓	53±7

Soluble (100,000 *g*) supernatant protein preparations of developing sunflower seeds (30 μg) were incubated with [$^{9,10\text{-}3}$H] 18:0–CoA (10 nmol) in the presence of various putative cofactors as indicated in the Table in a final vol. of 140 μl adjusted with K*P*$_i$ buffer (50 mM, pH 7.1); NADPH (200 nmol), Fd (20 μg), FdR (0.01 unit), BSA (100 μg) for 20 min at 25°. 100% activity corresponds to 3.8±0.1 × 10^6 dpm/mg protein.

Values are mean±S.D. (n = 4).

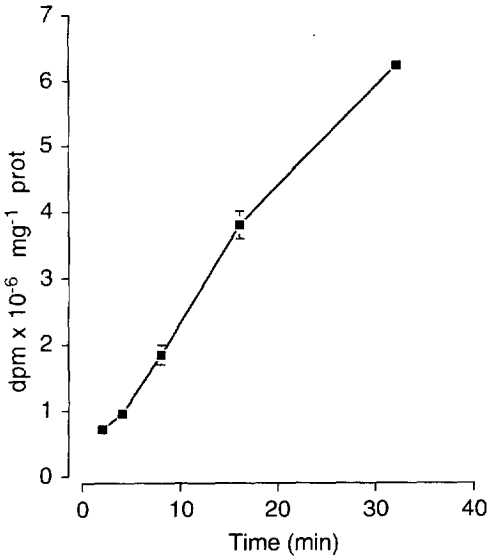

Fig. 1. Time course of 18:0–CoA desaturation. Soluble 100,000 *g* supernatant fractions (20 μg protein) were incubated with fd (20 μg), FdR (0.01 unit), NADPH (200 nmol), catalase (50 μg) and [$^{9,10\text{-}3}$H] 18:0–CoA (10 nmol) in *tris* pH 7.7 at 25°. Radioactivity was determined in aq EtOH phase by liquid scintillation after extraction (see Experimental).

Fractionation of the Δ9d system

The putative role of fd in Δ9d was studied by removal of the endogenous fd from the soluble enzyme extract by gel filtration. The fd in the column fractions was detected on Western blots using IgG fractions raised against spinach fd. Fd eluted after the peak protein fraction containing the Δ9d and slightly later than its predicted Ve, assuming an M_r of 12.5 kDa for fd isolated from non-photosynthetic tissue [13] (Fig. 2). Δ9d activity was only detectable in the peak protein fraction (peak 1) when exogenous fd and FdR were provided in the assay mixture (Table 2).

Purification of Δ9d

For further studies, attempts were made to purify the Δ9d. The enzyme was purified from a 100,000 *g* soluble supernatant fraction on a DEAE column (Q Fast flow) followed by affinity chromatography on ACP-sepharose. In the ion exchange step the enzyme eluted in an NaCl gradient at a concn of 0.3 M (Fig. 3). This resulted in a 5-fold purification and an almost 90% recovery of total activity applied to the column. Peak activity fractions were pooled, diluted 2-fold with H_2O and applied to an ACP-sepharose column. Over 95% of the protein eluted in the void (no activity detected) and a small trailing peak eluted with 0.5–0.75 M NaCl. When assayed in the reconstitution assay (i.e. containing fd, FdR, NADPH and 18:0–CoA), little activity was recovered (1–3% of total activity applied). Attempts to regain the activity by desalting were unsuccessful. SDS-PAGE of the affinity purified fraction showed the presence of a single major protein of 35 kDa (Fig 4). This was confirmed as Δ9d by Western blot analysis using two rabbit IgG antibodies raised to conserved region of the protein (data not shown). Because of the low yield of highly purified desaturase coupled with its low activity, all further expts were conducted using the Δ9d which had undergone ion exchange only.

Effect of catalase and H_2O_2 on Δ9d activity

DEAE-purified preps were active in 18:0–CoA desaturation on addition of fd and FdR in the presence of NADPH. Catalase stimulated this rate about twofold (Table 3). This appeared to be a specific property of active catalase since no stimulation was observed using the denatured protein or by using another haem protein, myoglobin. Stimulation of desaturation could be due to the breakdown of inhibitory H_2O_2 [10]. However, H_2O_2 (up to 10 μM) in the reaction mixture had no inhibitory effect on Δ9d in the absence of catalase (Table 3). In addition to its role in H_2O_2 degradation, however, catalase also exhibits peroxidase activity [14] and this might have a beneficial role in catalysis. In this regard, CO difference spectra of the soluble unfractionated extracts clearly revealed peroxidase protein in this fraction (Fig. 5) and this was largely eliminated in the DEAE-step (data not presented).

Effect of inhibitors

DEAE-preps were preincubated with inhibitor (typically 1 mM or 0.1 mM) for 15 min and the reaction initiated by the addition of cofactors and substrates giving final inhibitor concs of 450 μM or 45 μM in the reaction mixture (Table 4). No inhibition

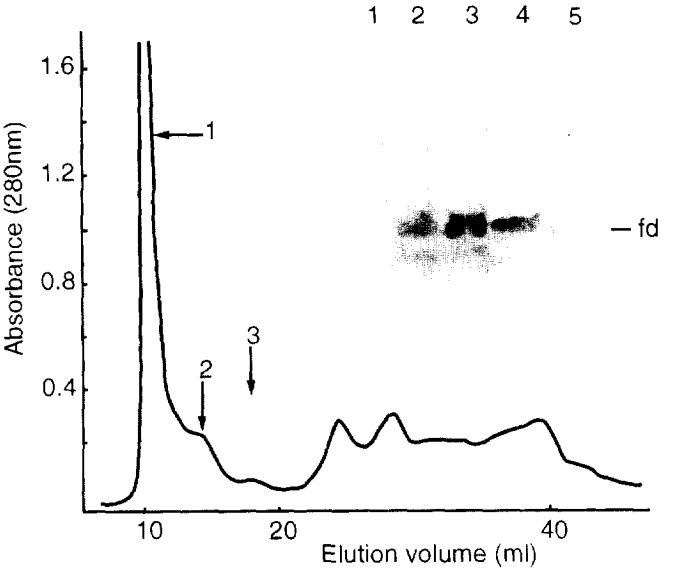

Fig. 2. Gel filtration of 100,000 *g* supernatant on Sephacryl HR-100. Proteins were fractionated by gel filtration and analysed by SDS-PAGE electrophoresis (insert) Lane 1 = peak protein; Lanes 2+3 = elution of fd; Lane 4 = marker fd. Lane 5 = unfractionated 100,000 *g* prep.

Table 2. Reconstitution of Δ9d activity

NADPH	Fd	FdR	Activity dpm × 10^{-6}/mg
✓	—	—	N.D.
✓	✓	—	0.45 ± 0.02
✓	—	✓	N.D.
✓	✓	✓	2.55 ± 0.07

Partially purified Δ9d preps (peak 1, Fig. 2) were incubated with [$^{9,10\text{-}3}$H] 18:0–CoA (10 nmol) plus NADPH (200 nmol); fd (20 μg) and FdR (0.01 unit) as indicated. Results are mean ± S.D. (n = 3).

of Δ9d was observed with four commonly used iron chelating agents under these conditions. However, by increasing the concn (5 mM) and incubation time (20 h) with *o*-bathophenanthroline disulfonate, a substantial reduction in activity was observed (80 ± 4%). Of the thiol inhibitors examined, *p*-CMBS and DTNB were the most potent. Almost complete inhibition was achieved with the flavoprotein inhibitors FAD, FMN and DPI at concs below 100 μM. Both superoxide dismutase and the superoxide anion scavenger 5′-5′ dimethyl-pyrroline oxide were ineffective inhibitors as were thiourea (a copper chelator) and EDTA (a magnesium chelator). Methyl viologen (a very low potential electron acceptor) was a potent inhibitor

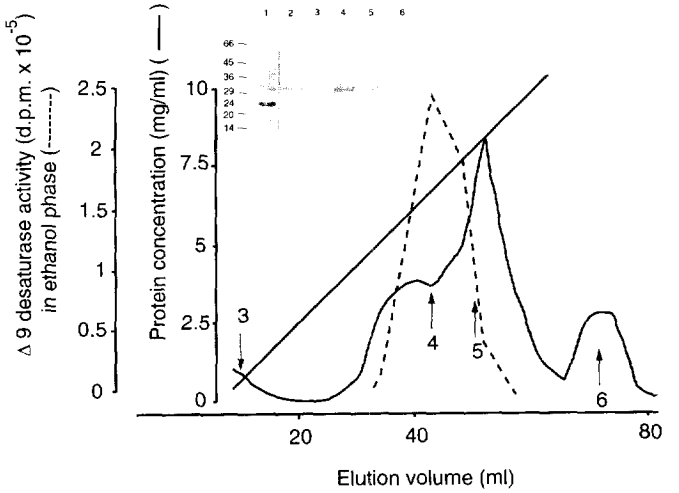

Fig. 3. Anion exchange chromatography of 100,000 *g* supernatant on DEAE (Q Fast Flow). Lane 1 = M_r markers; Lane 2 = unfractionated 100,000 *g* supernatant; Lane 3 = unbound protein; Lane 4 = peak Δ9d activity; Lane 5 = peak protein; peak 6 = tightly bound protein.

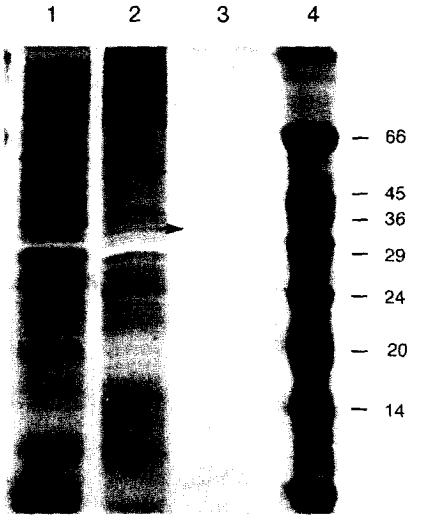

Fig. 4. SDS-PAGE of affinity chromatography (ACP-sepharose) purified fraction. Ion exchanged purified active fractions were pooled, diluted 2-fold and 1 ml (1.2 mg protein) applied at a flow rate of 0.4 ml/min to an ACP-Sepharose column (5 cm × 1 cm). Δ9d indicated by arrow at 35 kDa. Lane 1 = unfractionated 100,000 *g* soluble proteins; Lane 2 = ion exchanged (DEAE) proteins containing peak Δ9des activity; Lane 3 = ACP-sepharose purified protein; Lane 4 = M_r markers.

Table 3. Effect of haem proteins and hydrogen peroxide on desaturation

Additions	Activity dpm × 10^{-6}/mg
Control	2.35 ± 0.03
Catalase	
(5 μg)	4.55 ± 0.05
(50 μg)	5.20 ± 0.05
Boiled catalase (50 μg)	2.07 ± 0.02
Myoglobin	
(5 μg)	2.19 ± 0.02
(50 μg)	1.99 ± 0.02
Boiled myoglobin (50 μg)	1.99 ± 0.02
H_2O_2 (10 μM)	2.34 ± 0.02
H_2O_2 (10 μM) + catalase (50 μg)	5.31 ± 0.03

DEAE-purified enzyme was incubated with [^{9,10,3}H] 18:0–CoA, fd and FdR in the presence of NADPH as given in Table 1. Samples were preincubated with H_2O_2 for 15 min prior to assay. Results are mean ± S.D. (n = 3).

while some inhibition was noted for trifluoperazine (TFP), an anticalmodulin drug. Bis(salicylidene)-1,3-propanediamine(DSPD) which is considered an inhibitor of fd-mediated reactions, also caused a marked inhibition of Δ9d. The enzyme was sensitive to KCN but insensitive to CO.

DISCUSSION

Δ9d activity has been studied in soluble 100,000 *g* supernatant preparations from the developing cotyledons of sunflower. The presence of NADPH, fd and FdR stimulated Δ9d. Fractionation of the Δ9d from endogenous fd by gel filtration resulted in the complete loss of activity which could be restored by the addition of exogenously supplied fd together with its reductase. Attempts to replace fd with another one electron carrier, cytochrome-b_5 (a soluble form lacking the hydrophobic anchoring domain expressed in transgenic tobacco [15]) were unsuccessful, indicating specificity in the binding site for fd. Such a potential fd binding site has recently been identified in the crystal structure of Δ9d [16]. This was further supported in studies with the fd inhibitor DSPD which also markedly inhibited desaturation. Furthermore, the inhibition by TFP (a calmodulin antagonist) could be explained on the basis that fd is a Ca^{2+}-binding protein [17] which displays multiple carboxyl groups on its surface [18] which interact with the FdR in complex formation to facilitate electron transfer.

The chromatographic behaviour of Δ9d on DEAE was notably different from that reported previously in safflower [10], even though the apparent M_rs (35 kDa) on SDS-PAGE were very similar. In the present study, protein samples were prepared without acetone precipitation and, unlike the safflower enzyme, bound to a DEAE column. It is possible that acetone removes negatively charged components non-covalently bound to Δ9d (such as acidic phospholipids) resulting in less binding to the positively charged support. Alternatively, acetone could have altered the quaternary (dimeric) structure of the protein again resulting in changes in the net surface charge to give a product interactive with DEAE.

Stimulation of Δ9d by catalase has been observed previously [10, 19] and Mckeon and Stumpf reported an almost complete inhibition with 1 mM H_2O_2 [10]. However, using more physiologically relevant concentrations [20] we observed that Δ9d was unaffected by H_2O_2 up to 10 μM (a concn which inhibits photosynthetic carbon fixation by 50% [21]). This lack of inhibition, coupled with the stimulation of Δ9d activity by catalase, indicates that catalase may act as another component of the desaturase machinery in an analogous role to the coupling protein of the methane mono-oxygenase system [22] by facilitating the transfer of electrons and regulating the rate and regioselectivity of the Δ9d.

Δ9d is KCN sensitive but insensitive to CO indicating the unlikely involvement of cytochrome p450 type pigment. This has been supported by the inability of iodobenzene (an O-atom donor) to replace electrons and dioxygen in a peroxide shunt type mechanism [23] exhibited by p450 based systems (data not given). Initial iron chelator treatments were ineffective at inhibiting desaturation. Prolonged exposure at elevated concn, however, resulted in substantial inhibition consistent with the report in which iron was identified in Δ9d expressed in *E. coli* transformed with the *Ricinus* gene [24]. The deeply buried location of the iron atoms leading to restricted access to added inhibitors

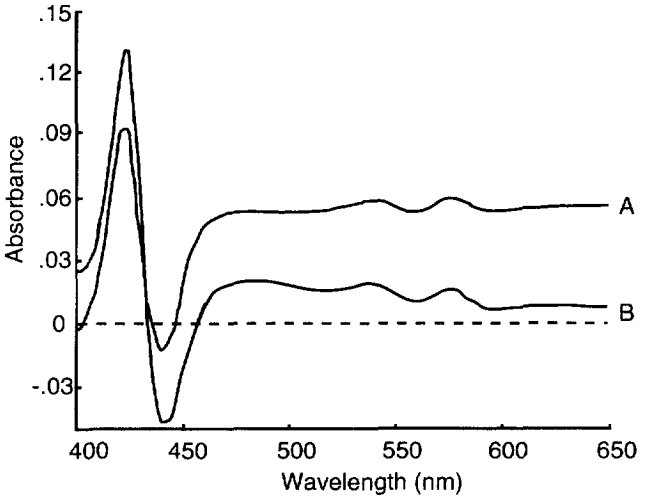

Fig. 5. Reduced plus CO-minus reduced difference spectra. 100,000 *g* soluble supernatants (0.6 ml) in KP_i buffer (pH 7.1) were dithionite reduced. Absorbance spectra were recorded before and after bubbling CO gas through the suspension. A = horseradish peroxidase; B = unfractionated 100,000 *g* supernatant.

supports this conclusion [16]. The KCN sensitivity, CO insensitivity and iron chelator data suggest that sunflower Δ9d is a non-haem iron protein. The inhibition of desaturation with flavoprotein inhibitors is likely to act through an inhibition of FdR, an essential component of the reconstituted assay system. Inhibition with water-soluble thiol inhibitors further suggests the importance of cysteine (-SH) groups in the desaturation process. In this respect, three cys residues are found in cDNA deduced amino acid sequences of castor and cucumber Δ9d [25]. A redox role for the SH group has been proposed in the catalytic mechanism of methane mono-oxygenase which, like Δ9d, is also a di-iron oxo protein [22]. In addition, indirect inhibition of Δ9d activity is possible through binding to SH groups in the electron transport component, FdR [26]. Lack of inhibition with the radical scavenger 5′-5′-dimethyl pyrroline oxide and superoxide dismutase suggests that radicals and/or superoxide are not generated in the catalytic cycle in contrast to the proposed role of tyrosine radicals in ribonucleotide reductase [16]. The characterisation of sunflower Δ9d as an iron-containing 35 kDa protein is consistent with structural studies of *Ricinus* Δ9d expressed in *E. coli* (actually a 70 kDa dimer). However, the plant Δ9d obtained from transgenic *E. coli* has a lower enzymic activity (16 nmol/min/mg protein) compared with that obtained for the partially purified enzyme in the present study (2 μmol/min/mg-calculated on 100% purity basis). This may reflect incorrect protein folding in the transgenic system and/or less than optimum conditions of assay, i.e. a requirement for other co-factors. A mechanistic model for Δ9d has recently been proposed from crystallographic evidence of the heterologously expressed castor enzyme [16]. The biochemical data for the sunflower enzyme presented in this paper can readily be interpreted in terms of this model.

EXPERIMENTAL

Plant material and chemicals

Developing sunflower seeds (*Helianthus annuus* L cv Sunbread 236) were harvested 14–18 days post anthesis (d.p.a) from plants grown under greenhouse conditions in a 16 h photoperiod at 25° and an 8 h night at 18°. All chemicals were of Analar grade or better. [^{9,10,3}H]stearic acid was custom synthesised by the chemical hydrogenation of oleic acid by DuPont (U.S.A.) and the CoA ester was synthesised from the mixed anhydrides as described [27]. The radioactive 18:0–CoA had a purity greater than 96% as determined by TLC and GC with a sp. act. of 122,000 dpm/nmol.

Preparation of 100,000 g *soluble extracts*

Cotyledons were removed from seeds 14–18 d.p.a. and stored on ice. All further procedures were carried out at 1–4°. The cotyledons were ground in a mortar with two parts (w/v) 0.1 M-KP_i buffer, pH 7.2, containing 0.33 M sucrose and 1 mM each of EDTA, DTT, Mg Cl_2 and PVP (insoluble form, 3%). The homogenate was filtered through two layers of Miracloth, diluted 2-fold with fresh grinding medium and centrifuged for 20 min at 20,000 *g*. The supernatant was filtered through Miracloth to remove the fat pad and re-centrifuged at 105,000 *g* for 90 min. The supernatant was finally split into 5 ml aliquots and stored at −80° at protein concs in the range of 5–8 mg/ml.

Delta-9d assays

Δ9d was routinely assayed by the release of ^{3}H from [^{9,10,3}H] 18:0–CoA to H_2O in the presence of exogenously supplied fd, FdR, catalase and NADPH.

Table 4. Effect of various chelating agents and enzyme inhibitors on Δ9 desaturation

Compound		% Inhibition
Iron chelators		
a-a bipyridyl	450 μM	0
Desferrioxamine	—	0
o-phenanthroline	—	0
**o*-phenanthroline disulfonate	5 mM	80
Thiol inhibitors		
p-CMB	450 μM	0
p-CMBS	—	100
	45 μM	49
NEM	450 μM	0
Phenylmaleimide	—	0
DTNB	450 μM	85
	45 μM	20
Flavoprotein inhibitors		
5-Deaza-FAD	450 μM	0
Quinacrine	—	0
FAD	100 μM	95
FMN	100 μM	100
DPI	45 μM	100
Others		
Thiourea	450 μM	0
TFP	100 μM	26
Methyl viologen	45 μM	100
Superoxide dismutase	5–100 μg	0
5′-5′ Dimethyl-pyrroline oxide	100 μM	0
EDTA	450 μM	0
DSPD	1 mM	63
KCN	1 mM	58
CO	saturated	0

Anion exchange purified Δ9des active fractions were preincubated with inhibitors for 15 min or overnight (indicated *) and then assayed using cofactors and [9,10 ^{3}H] 18:0-CoA as described in Experimental.

Incubations were performed in 70–150 μl vol reaction mixtures adjusted to pH 7.1 with 50 m MKP_i buffer in Eppendorf tubes at 25°. Reactions were terminated by the addition of EtOH (1.4 ml) and the proteins removed by centrifugation. Excess [^{9,10,3}H] 18:0–CoA substrate was removed by addition of Norit A followed by centrifugation. An aliquot of the EtOH phase was then assayed for radioactivity by liquid scintillation counting in PCS/xylene (2/1, v/v).

Preparation of IgG to Δ9d and fd

IgG to Δ9d were raised in rabbits challenged with synthetic peptides raised to two highly conserved regions of the protein from consensus sequence data given in [25] (from amino acid 260–278 and 385–397, Molecular Recognition Centre, Bristol University). IgGs were purified from serum by Na_2SO_4 pption and anion exchange [28]. During Western blotting of soluble extracts of sunflower seeds, both antibody preparations highlighted a single band of ca 35 kDa but did not inhibit enzyme activity. IgGs to spinach fd (Sigma) were also raised and purified from rabbit serum as given above.

Purification of Δ9d

Soluble 100,000 *g* supernatants in 0.1 M KP_i buffer, pH 7.2 were diluted 5-fold and the pH was adjusted to 6.8 with KH_2PO_4. 45 ml (250 mg protein) was applied to a Q Fast Flow column (25 ml bed vol., Pharmacia) equilibrated in the same buffer at a flow rate of 1 ml/min. Activity eluted in an NaCl gradient over 0.3–0.5 M. Peak activity fractions were diluted with H_2O 2-fold, and 1 ml (1.2 mg protein) was applied to an ACP-Sepharose 4-B column prepared essentially as described in [10].

Removal of endogenous fd

Soluble 100,000 *g* supernatants (10 mg protein) prep. as above, were fractionated on a Sephacryl HR-100 column (25 cm × 1 cm) at a flow rate of 0.3 ml/min. Fd was detected in the fractions by Western blot analysis.

SDS-PAGE and Western blotting

Protein samples were resolved on SDS-PAGE in a tris/glycine system [29] using a 12% separating gel and 4.5% stacking gel on a Biorad mini protean 11 cell. Proteins were transferred to HighBond membranes using a semi dry blotter (Biorad). After treatment with primary antibody, proteins were detected with goat anti rabbit peroxidase and H_2O_2 using 3,3′-diaminobenzidine.

Effect of inhibitors on Δ9d

DEAE-purified Δ9d was used to test the effects of inhibitors on enzyme activity. The prepn (50 μg total protein) was preincubated with the inhibitors (typically 1 mM or 0.1 mM) for 15 min and the reactions were initiated by the addition of substrates consisting of FdR (0.01 unit), fd (20 μg), catalase (50 μg), NADPH (200 nmol) and [^{3}H]stearoyl-CoA (10 nmol) adjusted to pH 7.1 with 50 mM KP_i buffer (150 μl final vol.). The final concn of inhibitors in the assay was then 450 μM or 45 μM. In cases where the inhibitor was soluble in EtOH or DMSO (e.g. diphenylene iodonium) corresponding control samples were used. No differences were observed between H_2O, EtOH (5% final concn) and DMSO (1.6% final concn) controls. Activity was measured by the production of tritiated H_2O as given above.

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