The same rat Δ6-desaturase not only acts on 18- but also on 24-carbon fatty acids in very-long-chain polyunsaturated fatty acid biosynthesis

Sabine D'ANDREA*, Hervé GUILLOU*, Sophie JAN*†, Daniel CATHELINE*, Jean-Noël THIBAULT‡, Monique BOURIEL*, Vincent RIOUX* and Philippe LEGRAND*[1]

*Laboratoire de Biochimie, INRA-ENSA, 65 rue de Saint-Brieuc, CS84215, 35042 Rennes cedex, France, †Université du Maine, Avenue Olivier Messiaen, 72085 Le Mans Cedex 9, France, and ‡UMR-VP INRA-ENSA, 35590 Saint-Gilles, France

The recently cloned Δ6-desaturase is known to catalyse the first step in very-long-chain polyunsaturated fatty acid biosynthesis, i.e. the desaturation of linoleic and α-linolenic acids. The hypothesis that this enzyme could also catalyse the terminal desaturation step, i.e. the desaturation of 24-carbon highly unsaturated fatty acids, has never been elucidated. To test this hypothesis, the activity of rat Δ6-desaturase expressed in COS-7 cells was investigated. Recombinant Δ6-desaturase expression was analysed by Western blot, revealing a single band at 45 kDa. The putative involvement of this enzyme in the Δ6-desaturation of $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$ was measured by incubating transfected cells with $C_{22:5}$ $n-3$. Whereas both transfected and non-transfected COS-7 cells were able to synthesize $C_{24:5}$ $n-3$ by elongation of $C_{22:5}$ $n-3$, only cells expressing Δ6-desaturase were also able to produce $C_{24:6}$ $n-3$. In addition, Δ6-desaturation of [1-^{14}C]$C_{24:5}$ $n-3$ was assayed *in vitro* in homogenates from COS-7 cells expressing Δ6-desaturase or not, showing that Δ6-desaturase catalyses the conversion of $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$. Evidence is therefore presented that the same rat Δ6-desaturase catalyses not only the conversion of $C_{18:3}$ $n-3$ to $C_{18:4}$ $n-3$, but also the conversion of $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$. A similar mechanism in the $n-6$ series is strongly suggested.

Key words: docosahexaenoic acid, $n-3$ fatty acid, α-linolenic acid, tetracosahexaenoic acid.

INTRODUCTION

In animal cells, the availability of $n-6$ and $n-3$ very-long-chain polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA; $C_{22:6}$ $n-3$), depends both on the diet providing the precursors and on the activity of enzymes involved in very-long-chain PUFA biosynthesis [1]. The described pathway for DHA synthesis from α-linolenic acid involves alternating steps of desaturation and elongation [2]. The first part of the biosynthesis (Δ6-desaturation of α-linolenic acid, elongation, Δ5-desaturation and elongation) leading to $C_{22:5}$ $n-3$ has been well-documented [3,4]. The mammalian genes encoding Δ6- and Δ5-desaturases involved in the two initial desaturation steps have been identified [5–8]. However, the final conversion of $C_{22:5}$ $n-3$ to $C_{22:6}$ $n-3$ remains controversial. For years, the presence of a Δ4-desaturase has been assumed [9]. In 1991, a modified pathway independent of a Δ4-desaturase was proposed [10]. The pathway includes, successively, an elongation, a Δ6-desaturation and a final peroxisomal β-oxidation [11,12], as follows: $C_{22:5}$ $n-3 \rightarrow C_{24:5}$ $n-3 \rightarrow C_{24:6}$ $n-3 \rightarrow C_{22:6}$ $n-3$.

The hypothesis that the same Δ6-desaturase, without chain-length specificity, could act on both 18-carbon essential fatty acids and 24-carbon highly unsaturated fatty acids has never been tested. Competitive studies with rat liver microsomes indicated that a single Δ6-desaturase may act on both 18- and 24-carbon PUFA substrates [13]. Recent work based on the identification of the first Δ6-desaturation-step deficiency in humans [14,15] has also suggested that a single Δ6-desaturase may be involved in the two different desaturations. However, metabolic studies based on different conversion rates have suggested that there may also be two different Δ6-desaturase isoforms [16,17]. These studies support the existence of distinct enzymes catalysing Δ6-desaturation of 18- and 24-carbon fatty acids.

In the present work, the hypothesis that the protein encoded by the rat liver Δ6-desaturase cDNA [6] could act on both 18- and 24-carbon essential fatty acids was investigated. Evidence is presented that recombinant rat Δ6-desaturase expressed in mammalian cells catalyses not only the conversion of $C_{18:3}$ $n-3$ to $C_{18:4}$ $n-3$, but also the conversion of $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$.

MATERIALS AND METHODS

Chemicals

cis-7,10,13,16,19-Docosapentaenoic acid ($C_{22:5}$ $n-3$) and *cis*-9,12,15,17,21-tetracosapentaenoic acid ($C_{24:5}$ $n-3$) were purchased from Matreya (Pleasant Gap, PA, U.S.A.) and American Radiolabelled Chemicals (St. Louis, MO, U.S.A.), respectively. The characterized fatty acid methyl ester of $C_{24:6}$ $n-3$ [18] was a gift from Dr K. Ishihara (National Research Institute of Fisheries Science, Yokohama, Japan). Other unlabelled fatty acids were from Sigma (St Quentin Fallavier, France). Radiolabelled [1-^{14}C]$C_{18:3}$ $n-3$ (52 mCi/mmol) and [1-^{14}C]$C_{24:5}$ $n-3$ (55 mCi/mmol) were purchased from NEN Life Science (Paris, France) and American Radiolabelled Chemicals respectively. Foetal calf serum (FCS) was purchased from Perbio (Bezons, France). Solvents (HPLC grade) were purchased from Fischer

Abbreviations used: DHA, docosahexaenoic acid; FCS, foetal calf serum; GST, glutathione S-transferase; PUFA, polyunsaturated fatty acids; DMEM, Dulbecco's modified Eagle's medium.

[1] To whom correspondence should be addressed (e-mail Philippe.Legrand@agrorennes.educagri.fr).

Scientific (Elancourt, France). Nonidet P-40 was obtained from Boehringer Mannheim (Mannheim, Germany). Other reagents were from Sigma.

Plasmid construction

A plasmid coding for rat Δ6-desaturase was constructed using pCMV for expression in mammalian cells and is referred to as pCMV/Δ6. From the published rat Δ6-desaturase sequence [6] (GenBank accession number AB02 1980), oligonucleotide primers were designed to amplify, by PCR, the entire coding sequence with its stop codon using the high-fidelity *Pfu* polymerase from Promega (Lyon, France).

The forward primer (5′-CAGTGGATCC*ATG*GGGAAGGG-AGGTA-3′) included the translation start codon (in italics) and an *Nco*I restriction site (underlined). The reverse primer (5′-TGTGCGGCCGC*TCA*TTTGTGGAGGTAGGCATCC-3′) contained the translation stop codon (in italics) and a *Not*I site (underlined). The PCR product amplified from rat liver cDNA was treated with *Nco*I and *Not*I before cloning into pCMV/*myc*/cyto (Invitrogen, San Diego, CA, U.S.A.). The integrity of the construct was confirmed by DNA sequencing.

Two plasmids, coding for fusion proteins consisting of glutathione S-transferase (GST) attached to the 131 N-terminal or the 108 C-terminal amino acids of rat Δ6-desaturase, were constructed and are referred to as pGEX/Δ6N and pGEX/Δ6C, respectively. The sequence corresponding to the N-terminal section (amino acids 1–131) was amplified by PCR with the forward primer 5′-CAGTGGATCCATGGGGAAGGGAGG-TA-3′, introducing a *Bam*HI site (underlined), and the reverse primer 5′-CTGCTCGAG*TCA*CAGGTGGTTGGTTTTGAA-AAGG-3′, introducing a *Xho*I site (underlined) and a stop codon (in italics).

The sequence corresponding to the C-terminal section (amino acids 337–444) was amplified with the forward primer 5′-ATGGGATCCGTCACACAGATGAACCAC-3′, introducing a *Bam*HI site (underlined), and the reverse primer 5′-CTAG-GTCGACGAGGTCTGCTGCTTC-3′, introducing a *Sal*I site (underlined). The PCR fragments were digested with *Bam*HI and *Xho*I or *Sal*I and ligated into the bacterial expression vector pGEX-4T-1 (Amersham Bioscience, Les Ulis, France) restricted with the same enzymes.

Anti-rat Δ6-desaturase sera

Two anti-rat Δ6-desaturase sera were produced by immunizing rabbits with bacterially expressed fusion proteins encoded in pGEX/Δ6N and pGEX/Δ6C. Serum 1 (S1) and serum 2 (S2) were raised using inclusion bodies containing the 108 C-terminal amino acids of rat Δ6-desaturase (Δ6C-GST) and affinity-purified GST fusion protein containing the 131 N-terminal amino acids of the desaturase (Δ6N-GST), respectively, as immunogens. Recombinant protein expression was induced with 0.1 mM isopropyl β-D-thiogalactoside for 3 h at 37 °C (Δ6C-GST) or 0.05 mM isopropyl β-D-thiogalactoside for 3 h at room temperature (Δ6N-GST). The bacterial pellet was resuspended in resuspension buffer (50 mM Tris/HCl, pH 8, 50 mM EDTA, 250 mM sucrose and 1 mg/ml lysozyme), lysed by addition of 1 vol. of lysis buffer (1 % Triton X-100, 1 % Tween-20 and 10 mM dithiothreitol, in PBS), sonicated and centrifuged (12000 ***g***, 15 min, 4 °C). Inclusion bodies containing Δ6C-GST were prepared by washing the pellet with 2 M urea. Soluble Δ6N-GST antigen was affinity-purified on glutathione–Sepharose 4B (Amersham Bioscience) according to the manufacturer's protocol. Rabbits were immunized by three successive subcutaneous injections at weekly intervals. At monthly intervals, five boosters were administered subcutaneously and blood was collected a week later. Antigen (100–600 μg) was emulsified in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the following ones.

Immunoprecipitation of rat Δ6-desaturase

Brain and liver microsomal fractions were prepared from Sprague–Dawley adult rat tissues. Tissues were homogenized at 4 °C with a Potter–Elvehjem homogenizer in 0.25 M sucrose dissolved in 50 mM phosphate buffer solution (pH 7.4). Mitochondria were removed by centrifugation (10000 ***g***, 10 min, 4 °C) and microsomes were pelleted at 100000 ***g*** for 30 min at 4 °C. Microsomes were resuspended in PBS (150 mM NaCl/5 mM sodium phosphate, pH 7.4) and sonicated. The protein content was estimated by a modified Lowry procedure [19]. Microsomal proteins were made soluble in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 10 % glycerol, 1 % Nonidet P-40, 1 mM PMSF and 10 μg/ml aprotinin). Immunoprecipitation of rat Δ6-desaturase was carried out using a mixture of S1 and S2 sera (1 μl each for 500 μg of microsomal protein). Preimmune sera were used as a control for this experiment. The immunoprecipitates were collected using Protein G–Sepharose, washed eight times with lysis buffer and then used for Western blotting.

Anti-rat Δ6-desaturase Western blot

Reduced protein samples were analysed by SDS/PAGE and blotted on to nitrocellulose (Schleicher and Schuell, Dassel, Germany). The membrane was probed with a mixture of S1 and S2 sera, each at 1:2000 dilution. Rabbit antibodies were revealed with horseradish peroxidase-conjugated Protein G (Sigma). Saturation and incubation with antibodies were carried out for 90 min in Tris-buffered saline (20 mM Tris/HCl, 150 mM NaCl, pH 7.4) containing 0.05 % Tween-20 and 10 % non-fat dried milk. Washes were performed in Tris-buffered saline containing 0.05 % Tween-20. Peroxidase activity was revealed by following the procedure provided for the ECL Plus detection system kit (Amersham Bioscience).

Expression of rat Δ6-desaturase

Rat Δ6-desaturase was expressed by transiently transforming COS-7 cells with pCMV/Δ6. COS-7 cells were routinely maintained at about 50 % confluence and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FCS, 50 i.u./ml penicillin and 50 μg/ml streptomycin. The cells were split 1 day before transfection to 30 % confluence, and transfected the following day by using the Easyject Plus electroporator (Equibio, Ashford, Middx, U.K.) according to the manufacturer's instructions. Briefly, 10^6 COS-7 cells in 0.8 ml of DMEM were mixed with 30 μg of purified plasmid, electroporated at 250 V and 1500 μF with unlimited resistance and seeded on to a 10 cm dish containing culture medium.

Incubation of transfected COS-7 cells with fatty acid albuminic complex

The functionality of the expressed protein was investigated by incubating the transfected COS-7 cells with different fatty acid albuminic complexes. Fatty acid albuminic complex was prepared

as described previously [20]. Briefly, each fatty acid was saponified by incubation for 30 min at 70 °C with 2 M KOH in ethanol. The resulting fatty acid salt was dissolved at pH 10 in DMEM containing 1 % BSA. After 15 min of sonication followed by 5 h of shaking, the pH was adjusted to 7.3. FCS was added to 10 % and the final fatty acid concentration of the incubation medium was 0.2 mM unless stated otherwise. At 3 h post-transfection, the incubation of COS-7 cells was initiated by replacing the culture medium with 20 ml of the fatty acid-containing medium per 10 cm dish. Incubation was carried out for 24 h at 37 °C in a 5 % CO_2 atmosphere.

Fatty acid analysis

COS-7 cells were washed twice with ice-cold PBS and scraped into PBS. After centrifugation, the cell pellet was resuspended in PBS and sonicated at 20 W for 5 s. The protein content of the cell homogenate was determined by a modified Lowry procedure [19]. Cellular lipids were extracted with hexane/isopropanol (3:2, v/v) as described previously [20]. After saponification, fatty acids were methylated with BF_3 (14 % in methanol) at 70 °C for 30 min. Fatty acid methyl esters were extracted with pentane and analysed by GC using a GIRA 1600 chromatograph (GIRA, Morlaas, France) with a split injector (1:10) at 240 °C and a bonded silica capillary column (30 m × 0.25 mm inner diameter, BPX 70; SGE, Villeneuve-St-Georges, France) with a stationary phase of 70 % cyanopropylpolysilphenylene-siloxane (0.25 μm film thickness). Helium was used as gas vector (10^5 Pa). The column temperature program started at 150 °C, ramping at 2 °C/min to 220 °C and holding at 220 °C for 10 min. The flame ionization detector temperature was 260 °C. Identification of fatty acid methyl ester peaks was based upon retention times obtained for methyl esters prepared from fatty acid standards.

Enzyme assay

Cell homogenates were prepared as described above. Desaturase activity was assayed in a 1 ml mixture containing 100 μl of cell homogenate (7–7.8 mg of protein/ml), 150 mM phosphate buffer (pH 7.2), 6 mM $MgCl_2$, 7.2 mM ATP, 0.54 mM CoA and 0.8 mM NADH. The reaction was started by adding 60 nmol of [1-^{14}C]$C_{24:5}$ $n-3$ (55 mCi/mmol) or [1-^{14}C]$C_{18:3}$ $n-3$ (52 mCi/mmol) and stopped with 1 ml of 2 M KOH in ethanol after 1 h of incubation at 37 °C. To assess substrate quality, a control assay was also run by stopping the reaction before addition of the substrate. Fatty acid saponification was carried out at 70 °C for 30 min. After acidification, the fatty acids were extracted with diethylether, converted to fatty acid naphthacyl esters and separated on HPLC as described previously [21]. Collected fractions were subjected to liquid scintillation counting (Tri-Carb 1600 TR; Packard, Meriden, CT, U.S.A.). From the amount of radioactivity found in the product fraction versus the total radioactivity (i.e. substrate plus product), the percentage of substrate converted to its desaturated product was calculated. The percentage of conversion measured in the control assay was subtracted from that measured in transfected or non-transfected cells.

RESULTS

Western-blot analysis of Δ6-desaturase expressed in COS-7 cells

The 1335 bp open reading frame of the rat Δ6-desaturase cloned by Aki et al. [6] was amplified and inserted into a mammalian expression vector. The resulting plasmid, named pCMV/Δ6, was used for transient transfection in COS-7 cells. Recombinant rat Δ6-desaturase expressed in COS-7 cells was analysed by Western blotting with sera S1 and S2, raised against the two more hydrophilic fragments of rat Δ6-desaturase, i.e. the 108 C-terminal fragment (Δ6C-GST) and the 131 N-terminal fragment (Δ6N-GST) respectively. Western blotting revealed that both sera were able to recognize rat Δ6-desaturase expressed in COS-7 cells (results not shown), but in order to enhance the sensitivity of detection a 1:1 (v/v) mixture of S1 and S2 sera was used to probe immunoblots. Figure 1 shows the result of Western-blot analysis of total homogenate obtained from COS-7 cells transfected with pCMV/Δ6 compared with non-transfected cells. A single band (45 kDa) was detected in COS-7 cells expressing rat Δ6-desaturase (Figure 1, lane 3). This band was not revealed in non-transfected COS-7 cells (Figure 1, lane 4). The control with preimmune sera was negative (results not shown).

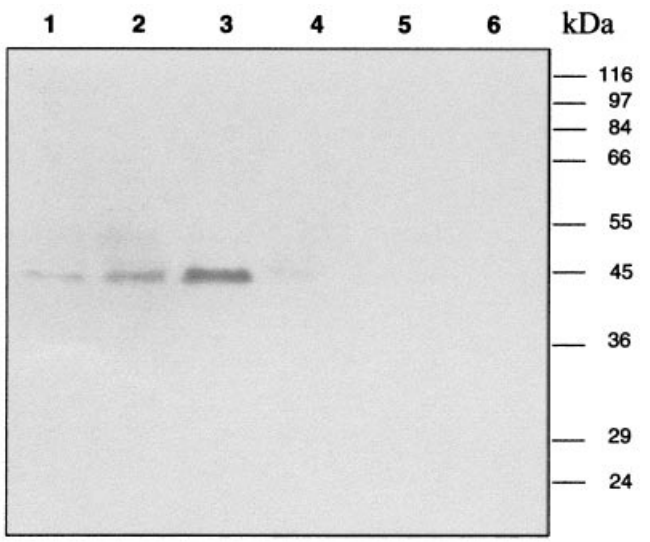

Figure 1 Detection of rat Δ6-desaturase in tissues and in transfected COS-7 cells by Western-blot analysis

Rat brain (lanes 1 and 6) and liver (lanes 2 and 5) microsomes were immunoprecipitated with anti-rat Δ6-desaturase S1 and S2 sera (lanes 1 and 2) or preimmune sera (lanes 5 and 6). COS-7 cells were transfected with pCMV/Δ6 (lane 3) or not (lane 4). Immunoprecipitates (from 500 μg of microsomal proteins) and COS-7 total cell homogenates (2 μg of protein) were resolved by SDS/PAGE and blotted. The blot was immunoprobed with anti-rat Δ6-desaturase sera S1 and S2, both at a 1:2000 dilution. Detection was performed using chemifluorescence (ECL Plus detection system). Molecular masses are indicated in kDa.

Recombinant Δ6-desaturase was compared by Western blotting with the Δ6-desaturase expressed in rat liver and brain. Because Western-blot analysis was not sensitive enough to specifically detect Δ6-desaturase from these tissues, the protein was immunoprecipitated with S1 and S2 sera before Western blotting. Immunoprecipitates from brain and liver microsomes are shown in lanes 1 and 2 of Figure 1, respectively. In both tissues, one band was detected at 45 kDa after immunoprecipitation with immune sera, whereas no band was revealed in immunoprecipitates with preimmune sera (Figure 1, lanes 5 and 6). This result shows that recombinant rat Δ6-desaturase expressed in COS-7 cells has the same apparent molecular mass as the native protein. This comparison also indicates that the expression level in transfected cells is higher than in liver and brain.

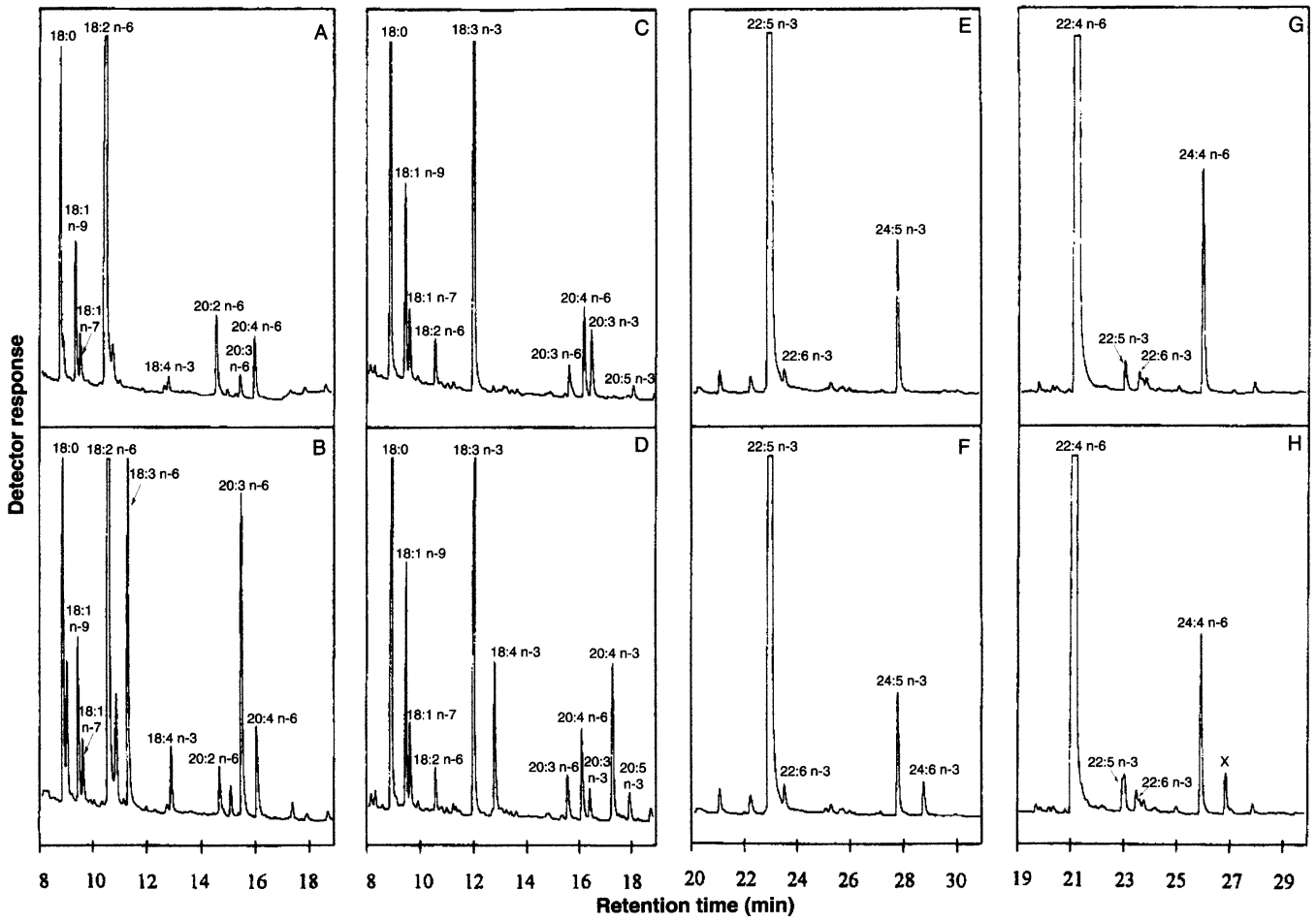

Figure 2 GC analysis of cellular fatty acid methyl esters from COS-7 cells containing rat Δ6-desaturase and control COS-7 cells

COS-7 cells transfected with pCMV/Δ6 (**B**, **D**, **F** and **H**) or non-transfected (**A**, **C**, **E** and **G**) were cultivated for 24 h with different albumin-bound fatty acids: 200 μM $C_{18:2}$ $n-6$ (**A** and **B**), 80 μM $C_{18:3}$ $n-3$ (**C** and **D**), 200 μM $C_{22:5}$ $n-3$ (**E** and **F**) and 200 μM $C_{22:4}$ $n-6$ (**G** and **H**). The identity of each fatty acid is indicated above its respective peak. The peak identified as $C_{24:6}$ $n-3$ (**F**) was confirmed using the methyl ester of $C_{24:6}$ $n-3$. Peak X (**H**), thought to correspond to $C_{24:5}$ $n-6$, could not be identified positively due to the lack of a standard. Identities of other peaks were determined by comparing their retention times with those of authentic standards. The same results were obtained three times with $C_{22:4}$ $n-6$ and $C_{22:5}$ $n-3$ treatments and once with $C_{18:2}$ $n-6$ and $C_{18:3}$ $n-3$ treatments.

Fatty acid analysis of COS-7 cells expressing Δ6-desaturase

For functional analysis of rat Δ6-desaturase, COS-7 cells transfected with pCMV/Δ6 were grown for 24 h in culture medium supplemented with various putative fatty acid substrates. First of all, because rat Δ6-desaturase was characterized previously as the enzyme able to catalyse the conversion of linoleic acid ($C_{18:2}$ $n-6$) to γ-linolenic acid ($C_{18:3}$ $n-6$), and α-linolenic acid ($C_{18:3}$ $n-3$) to stearidonic acid ($C_{18:4}$ $n-3$) [6], linoleic acid or α-linolenic acid were added to the culture medium. When the medium was supplemented with linoleic acid, GC analysis of fatty acids in COS-7 cells expressing Δ6-desaturase (Figure 2B) revealed a higher $C_{18:3}$ $n-6$ content, produced by Δ6-desaturation of $C_{18:2}$ $n-6$, and a higher $C_{20:3}$ $n-6$ content, produced by elongation of $C_{18:3}$ $n-6$, than non-transfected cells (Figure 2A). Similarly, COS-7 cells transfected with pCMV/Δ6 converted α-linolenic acid to its Δ6-desaturase product, $C_{18:4}$ $n-3$ (Figure 2D), whereas non-transfected COS-7 cells were unable to produce detectable level of $C_{18:4}$ $n-3$ (Figure 2C).

To investigate the putative involvement of this enzyme in the Δ6-desaturation of $C_{24:4}$ $n-6$ to $C_{24:5}$ $n-6$ and $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$, COS-7 cells transfected or not with pCMV/Δ6 were cultivated in medium supplemented with $C_{22:5}$ $n-3$ or $C_{22:4}$ $n-6$. Concerning the $n-3$ series, both transfected and non-transfected cells were able to elongate $C_{22:5}$ $n-3$ to $C_{24:5}$ $n-3$ (Figures 2E and 2F). The difference in their fatty acid contents was the presence of an additional peak in COS-7 cells expressing Δ6-desaturase (Figure 2F). This peak was identified as $C_{24:6}$ $n-3$ by comparing its retention time with standard $C_{24:6}$ $n-3$ methyl ester [18]. Cells expressing Δ6-desaturase were able to produce $C_{24:6}$ $n-3$ whereas no detectable $C_{24:6}$ $n-3$ was present in non-transfected cells. This result indicates that Δ6-desaturase is able to catalyse the synthesis of $C_{24:6}$ $n-3$.

Regarding the $n-6$ series, a similar result was obtained. Both transfected and non-transfected cells were able to elongate $C_{22:4}$ $n-6$ to $C_{24:4}$ $n-6$ (Figures 2G and 2H). One additional peak was present in transfected versus non-transfected cells cultivated in medium supplemented with $C_{22:4}$ $n-6$ (Figures 2G and 2H).

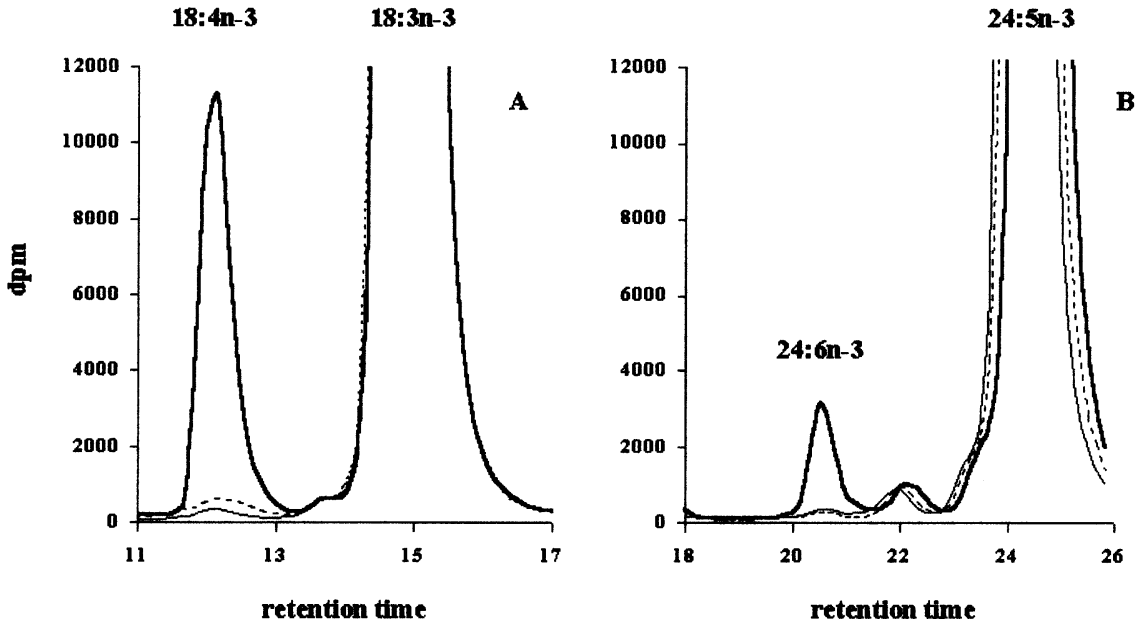

Figure 3 *In vitro* desaturation of [1-^{14}C]$C_{18:3}$ $n-3$ (A) and [1-^{14}C]$C_{24:5}$ $n-3$ (B) measured in COS-7 cell homogenates containing rat Δ6-desaturase or not

Cell homogenates prepared from COS-7 cells transfected with pCMV/Δ6 (thick line) or non-transfected (broken line) were incubated with 60 μM [1-^{14}C]$C_{18:3}$ $n-3$ (**A**) or [1-^{14}C]$C_{24:5}$ $n-3$ (**B**), as described in the Materials and methods section. The reaction was stopped after 1 h of incubation at 37 °C (thick and broken lines). A control assay (thin line) was also run by stopping the reaction before addition of the labelled substrate. After extraction and derivatization as naphthacyl esters, fatty acids were separated on HPLC and collected fractions were subjected to counting. Identification of fatty acids was based upon retention times obtained for naphthacyl esters prepared from fatty acid standards.

The identity of this peak (labelled X in Figure 2H) was thought to be $C_{24:5}$ $n-6$ but could not be positively established because no standard was available.

In vitro Δ6-desaturase assay using radiolabelled fatty acid

In order to confirm the Δ6-desaturase-catalysed conversion of $C_{18:3}$ $n-3$ to $C_{18:4}$ $n-3$ and $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$, [1-^{14}C]$C_{18:3}$ $n-3$ and [1-^{14}C]$C_{24:5}$ $n-3$ were incubated with COS-7 cell homogenates expressing or not rat Δ6-desaturase. Figure 3 shows the HPLC separation of the radiolabelled fatty acids produced after 1 h incubation of cell homogenate with labelled $C_{18:3}$ $n-3$ (Figure 3A) and $C_{24:5}$ $n-3$ (Figure 3B). As shown by the control assay, the substrates [1-^{14}C]$C_{18:3}$ $n-3$ (Figure 3A) and [1-^{14}C]$C_{24:5}$ $n-3$ (Figure 3B) correspond to the highest peaks. For each substrate, another radioactive peak with a shorter retention time was detected in the assay run with homogenate prepared from COS-7 cells expressing Δ6-desaturase. These radiolabelled products were identified as $C_{18:4}$ $n-3$ (Figure 3A) and $C_{24:6}$ $n-3$ (Figure 3B) from the standard naphthacyl esters. Neither was detected when the incubations were carried out with homogenate prepared from non-transfected cells. This *in vitro* desaturase assay confirms that rat Δ6-desaturase catalyses the conversion of both $C_{18:3}$ $n-3$ to $C_{18:4}$ $n-3$ and $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$.

The relative Δ6-desaturase activity between $C_{18:3}$ $n-3$ and $C_{24:5}$ $n-3$ was estimated from the percentage of substrate converted to its product. In COS-7 cells expressing Δ6-desaturase, 5.2 % of $C_{18:3}$ $n-3$ was converted to $C_{18:4}$ $n-3$ (compared with 0.3 % in non-transfected cells) and only 1.2 % of $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$ (compared with 0.1 % in non-transfected cells). These results suggest that Δ6-desaturase is more active on $C_{18:3}$ $n-3$ than on $C_{24:5}$ $n-3$.

DISCUSSION

The biosynthesis of long-chain PUFA from $C_{18:2}$ $n-6$ and $C_{18:3}$ $n-3$ involves successive desaturation and elongation steps. Various animal desaturases of this pathway have recently been characterized [5–8]. Several elongases necessary for the biosynthesis of long-chain PUFA have also been identified [22,23]. Thus only the controversial step from $C_{22:4}$ $n-6$ to $C_{22:5}$ $n-6$ and from $C_{22:5}$ $n-3$ to $C_{22:6}$ $n-3$ remains to be determined. One opinion is that this step depends on a microsomal Δ4-desaturation. Although a functional Δ4-desaturase has recently been cloned from *Thraustochytrium* sp. [24], the presence of a microsomal Δ4-desaturase in animals has never been demonstrated. Another theory proposed by Sprecher and co-workers [10] is that of a Δ4-desaturase-independent pathway. According to their proposal, synthesis of DHA ($C_{22:6}$ $n-3$) from eicosapentaenoic acid ($C_{20:5}$ $n-3$) and docosapentaenoic acid ($C_{22:5}$ $n-6$) from arachidonic acid ($C_{20:4}$ $n-6$) occurs through two successive elongations producing 24-carbon fatty acid intermediates, and Δ6-desaturation of these 24-carbon fatty acids in the microsomes followed by a two-carbon shortening in the peroxisomes [2]. Therefore, the aim of this study was to address the possible role of the Δ6-desaturase, known to act on $C_{18:2}$ $n-6$ and $C_{18:3}$ $n-3$, in the conversion of $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$. Transient transfection of the 1335 bp open reading frame of the rat Δ6-desaturase [6] in COS-7 cells was used to investigate this hypothesis.

By Western-blot analysis of transfected COS-7 cells (Figure 1), anti-Δ6-desaturase antibodies yielded a single band showing that

transient transfection effectively resulted in a high level of Δ6-desaturase expression as compared with the control. These antibodies were also able to recognize and to immunoprecipitate Δ6-desaturase from tissues. Both native and recombinant rat Δ6-desaturase displayed an apparent molecular mass of 45 kDa, lower than the 52.4 kDa predicted from its amino acid sequence [6]. Therefore, the protein may undergo post-translational modifications, especially proteolytic cleavage. This apparent molecular mass is also much lower than the 66 kDa estimation by Okayasu et al. [25], who reported purification of a linoleyl-CoA desaturase from rat liver microsomes.

In the present work, the high level of Δ6-desaturase expression in transfected COS-7 cells was correlated with a significant Δ6-desaturation of linoleic and α-linolenic acids. When the medium was supplemented with $C_{18:2}$ $n-6$ or $C_{18:3}$ $n-3$, the recombinant protein was able to produce $C_{18:3}$ $n-6$ (Figure 2B) or $C_{18:4}$ $n-3$ (Figure 2D) respectively. These data are consistent with previous reports [5,6].

Since the COS-7 cell line displayed very low endogenous Δ6-desaturase activity (Figures 2A, 2C and 3A) but was capable of expressing a high level of recombinant rat Δ6-desaturase after transfection, we used this model to investigate the putative involvement of this enzyme in the Δ6-desaturation of $C_{24:4}$ $n-6$ to $C_{24:5}$ $n-6$ and $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$. When incubated with $C_{22:5}$ $n-3$, both cell types were able to synthesize $C_{24:5}$ $n-3$ by elongation (Figures 2E and 2F). Then only cells expressing Δ6-desaturase produced significant amounts of $C_{24:6}$ $n-3$ (Figure 2F). Furthermore, an *in vitro* Δ6-desaturase assay of radiolabelled $C_{24:5}$ $n-3$ (Figure 3B) showed without doubt that the recombinant protein catalysed the conversion of the substrate to radiolabelled $C_{24:6}$ $n-3$. Therefore we report for the first time that the same rat Δ6-desaturase is able to catalyse both the desaturation of $C_{18:3}$ $n-3$ to $C_{18:4}$ $n-3$ and of $C_{24:5}$ $n-3$ to $C_{24:6}$ $n-3$. Moreover, estimation of the relative Δ6-desaturase activities suggests that the enzyme is more active on $C_{18:3}$ $n-3$ than on $C_{24:5}$ $n-3$. This result is consistent with a previous report [13] demonstrating by competitive studies that there was preferential Δ6-desaturation of $C_{18:3}$ $n-3$ rather than $C_{24:5}$ $n-3$ in rat liver microsomes. Finally, results obtained with the $n-3$ series showed that COS-7 cells exhibited functional endogenous fatty acid elongation activities but seemed to be devoid of functional endogenous peroxisomal activity. Indeed, no significant change in the content of $C_{22:6}$ $n-3$ between transfected and non-transfected cells was observed (Figures 2E and 2F).

With regard to the $n-6$ series, similar mechanisms are strongly suggested. After cell incubation with $C_{22:4}$ $n-6$, a peak X was detected only in cells expressing Δ6-desaturase (Figure 2H). Although the identity of this peak was not established definitely, from the retention times observed we concluded that X is likely to be $C_{24:5}$ $n-6$ produced by Δ6-desaturation of $C_{24:4}$ $n-6$.

Our results, demonstrating that one Δ6-desaturase is able to desaturate both 18- and 24-carbon PUFA, are consistent with the pathway described by Sprecher and co-workers [10,11]. Several recent studies also support this hypothesis. First, in human skin fibroblasts, Δ6-desaturation deficiency was shown to equally affect 18- and 24-carbon fatty acid Δ6-desaturation [14]. Moreover, in rat brain astrocytes, DHA production from $n-3$ precursors was shown to follow the proposed pathway [26]. Finally, in human skin fibroblasts, Su et al. [27] have identified peroxisomal enzymic activities necessary for the final retroconversion step involved in DHA synthesis. However, our results do not reject the potential existence of a Δ4-desaturase [28] or another chain-length-specific Δ6-desaturase in animals. An uncharacterized gene of the desaturase family (termed FADS3) has been identified in the same region of human chromosome 11 as the Δ6- and Δ5-desaturase genes [29]. FADS3 could be either a Δ4-desaturase or another chain-length-specific Δ6-desaturase but, to our knowledge, its function has not yet been established.

Desaturase enzymes are known to introduce double bonds between defined carbons of the fatty acyl chains. Our results clearly establish for the first time that the protein encoded by an animal Δ6-desaturase gene acts on both 18- and 24-carbon fatty acids from $n-3$ and $n-6$ series. Together with recent studies, our data further support the involvement of a unique Δ6-desaturase in the DHA synthetic pathway initially proposed by Sprecher and co-workers.

We thank Dr K. Ishihara (National Research Institute of Fisheries Science, Yokohama, Japan) for supplying the $C_{24:6}$ $n-3$ methyl ester used in this study. We thank A. Leborgne and K. L. Cung for able technical assistance.

REFERENCES

1 Spector, A. A. (1999) Essentiality of fatty acids. Lipids. **34** suppl, S1–S3
2 Sprecher, H. (2000) Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim. Biophys. Acta **1486**, 219–231
3 Los, D. A. and Murata, N. (1998) Structure and expression of fatty acid desaturases. Biochim. Biophys. Acta **1394**, 3–15
4 Horrobin, D. F. (1993) Fatty acid metabolism in health and disease: the role of Δ-6-desaturase. Am. J. Clin. Nutr. **57**, 732S–736S
5 Cho, H. P., Nakamura, M. T. and Clarke, S. D. (1999) Cloning, expression, and nutritional regulation of the mammalian Δ-6 desaturase. J. Biol. Chem. **274**, 471–477
6 Aki, T., Shimada, Y., Inagaki, K., Higashimoto, H., Kawamoto, S., Shigeta, S., Ono, K. and Suzuki, O. (1999) Molecular cloning and functional characterization of rat Δ-6 fatty acid desaturase. Biochem. Biophys. Res. Commun. **255**, 575–579
7 Cho, H. P., Nakamura, M. and Clarke, S. D. (1999) Cloning, expression, and fatty acid regulation of the human delta-5 desaturase. J. Biol. Chem. **274**, 37335–37339
8 Zolfaghari, R., Cifelli, C. J., Banta, M. D. and Ross, A. C. (2001) Fatty acid Δ(5)-desaturase mRNA is regulated by dietary vitamin A and exogenous retinoic acid in liver of adult rats. Arch. Biochem. Biophys. **391**, 8–15
9 Rosenthal, M. D. (1987) Fatty acid metabolism of isolated mammalian cells. Progr. Lipid Res. **26**, 87–124
10 Voss, A., Reinhart, M., Sankarappa, S. and Sprecher, H. (1991) The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. J. Biol. Chem. **266**, 19995–20000
11 Luthria, D. L., Mohammed, B. S. and Sprecher, H. (1996) Regulation of the biosynthesis of 4,7,10,13,16,19-docosahexaenoic acid. J. Biol. Chem. **271**, 16020–16025
12 Sprecher, H. (1999) An update on the pathways of polyunsaturated fatty acid metabolism. Curr. Opin. Clin. Nutr. Metab. Care **2**, 135–138
13 Geiger, M., Mohammed, B. S., Sankarappa, S. and Sprecher, H. (1993) Studies to determine if rat liver contains chain-length-specific acyl-CoA 6-desaturases. Biochim. Biophys. Acta **1170**, 137–142
14 Williard, D. E., Nwankwo, J. O., Kaduce, T. L., Harmon, S. D., Irons, M., Moser, H. W., Raymond, G. V. and Spector, A. A. (2001) Identification of a fatty acid Δ(6)-desaturase deficiency in human skin fibroblasts. J. Lipid Res. **42**, 501–508
15 de Antueno, R. J., Allen, S. J., Ponton, A. and Winther, M. D. (2001) Activity and mRNA abundance of Δ-5 and Δ-6 fatty acid desaturases in two human cell lines. FEBS Lett. **491**, 247–251
16 Marzo, I., Alava, M. A., Pineiro, A. and Naval, J. (1996) Biosynthesis of docosahexaenoic acid in human cells: evidence that two different Δ6-desaturase activities may exist. Biochim. Biophys. Acta **1301**, 263–272
17 Chen, Q. and Nilsson, A. (1993) Desaturation and chain elongation of n-3 and n-6 polyunsaturated fatty acids in the human CaCo-2 cell line. Biochim. Biophys. Acta **1166**, 193–201
18 Ishihara, K., Murata, M., Kaneniwa, M., Saito, H., Shinohara, K., Maeda-Yamamoto, M., Kawasaki, K. and Ooizumi, T. (1998) Effect of tetracosahexaenoic acid on the content and release of histamine, and eicosanoid production in MC/9 mouse mast cell. Lipids **33**, 1107–1114
19 Bensadoun, A. and Weinstein, D. (1976) Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**, 241–250
20 Rioux, V., Lemarchal, P. and Legrand, P. (2000) Myristic acid, unlike palmitic acid, is rapidly metabolized in cultured rat hepatocytes. J. Nutr. Biochem. **11**, 198–207
21 Rioux, V., Catheline, D., Bouriel, M. and Legrand, P. (2000) High performance liquid chromatography of fatty acids as naphtacyl esters. Analysis **27**, 186–193

22 Moon, Y. A., Shah, N. A., Mohapatra, S., Warrington, J. A. and Horton, J. D. (2001) Identification of a mammalian long chain fatty acyl elongase regulated by sterol regulatory element-binding proteins. J. Biol. Chem. **276**, 45358–45366
23 Leonard, A. E., Bobik, E. G., Dorado, J., Kroeger, P. E., Chuang, L. T., Thurmond, J. M., Parker-Barnes, J. M., Das, T., Huang, Y. S. and Mukerji, P. (2000) Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. Biochem. J. **350**, 765–770
24 Qiu, X., Hong, H. and Mackenzie, S. L. (2001) Identification of a Δ4 fatty acid desaturase from *Thraustochytrium* sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. J. Biol. Chem. **276**, 31561–31566
25 Okayasu, T., Nagao, M., Ishibashi, T. and Imai, Y. (1981) Purification and partial characterization of linoleoyl-CoA desaturase from rat liver microsomes. Arch. Biochem. Biophys. **206**, 21–28
26 Williard, D. E., Harmon, S. D., Kaduce, T. L., Preuss, M., Moore, S. A., Robbins, M. E. and Spector, A. A. (2001) Docosahexaenoic acid synthesis from n-3 polyunsaturated fatty acids in differentiated rat brain astrocytes. J. Lipid Res. **42**, 1368–1376
27 Su, H. M., Moser, A. B., Moser, H. W. and Watkins, P. A. (2001) Peroxisomal straight-chain acyl-CoA oxidase and d-bifunctional protein are essential for the retroconversion step in docosahexaenoic acid synthesis. J. Biol. Chem. **276**, 38115–38120
28 Infante, J. P. and Huszagh, V. A. (1998) Analysis of the putative role of 24-carbon polyunsaturated fatty acids in the biosynthesis of docosapentaenoic (22:5n-6) and docosahexaenoic (22:6n-3) acids. FEBS Lett. **431**, 1–6
29 Marquardt, A., Stohr, H., White, K. and Weber, B. H. (2000) cDNA cloning, genomic structure, and chromosomal localization of three members of the human fatty acid desaturase family. Genomics **66**, 175–183

Received 16 November 2001/25 January 2002; accepted 6 March 2002