Primary Structure, Regioselectivity, and Evolution of the Membrane-bound Fatty Acid Desaturases of Claviceps purpurea

Received for publication, March 13, 2007, and in revised form, May 7, 2007. Published, JBC Papers in Press, May 17, 2007, DOI 10.1074/jbc.M702196200

Dauenpen Meesapyodsuk1†, Darwin W. Reed‡, Patrick S. Covello§, and Xiao Qiu††

From the †Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Saskatchewan S7N5A8 and the ‡Bioriginal Food and Science Corporation and the §Plant Biotechnology Institute, Saskatoon, Saskatchewan S7N0W9, Canada

Two cDNAs with sequence similarity to fatty acid desaturase genes were isolated from the phytopathogenic fungus, Claviceps purpurea. The predicted amino acid sequences of the corresponding genes, named CpDes12 and CpDesX, share 87% identity. Phylogenetic analysis indicates that CpDes12 and CpDesX arose by gene duplication of an ancestral Δ12-desaturase gene after the divergence of Nectriaeae and Clavicipitaceae. Functional expression of CpDes12 and CpDesX in yeast (Saccharomyces cerevisiae) indicated that CpDes12 is primarily a “Δ12-ω3” desaturase, whereas CpDesX is a novel desaturase catalyzing “Δ12” and “Δ15” types of desaturation with ω3 activity predominating. CpDesX sequentially desaturates both 16:1–9c and 18:1–9c to give 16:3–9c,12c,15c and 18:3–9c,12c,15c, respectively. In addition, it could also act as an ω3-desaturase converting ω6-polyunsaturates 18:3–6c,9c,12c, 20:3–8c,11c,14c, and 20:4–5c,8c,11c,14c to their ω3-counterparts 18:4–6c,9c,12c,15c, 20:4–8c,11c,14c,17c, and 20:5–5c,8c,11c,14c,17c, respectively. By using reciprocal site-directed mutagenesis, we demonstrated that two residues (isoleucine at 152 and alanine at 206) are critical in defining the catalytic specificity of these enzymes and the C-terminal amino acid sequence (residues 302–477) was also found to be important. These data provide insights into the nature of regioselectivity in membrane-bound fatty acid desaturases and the relevant structural determinants. The authors suggest that the regioselectivity of such enzymes may be best understood by considering the relative importance of more than one regioselective preference. In this view, CpDesX is designated as a ν + 3 (ω3) desaturase, which primarily references an existing double bond (ν + 3 regioselectivity) and secondarily shows preference for ω3 desaturation.

Membrane-bound fatty acid desaturases are involved in the non-heme di-iron- and oxygen-dependent dehydrogenation of fatty acid chains. The membrane desaturases have a wide range of substrate specificity and regioselectivity (1–3). These enzymes are typically labeled according to their apparent regioselectivity. For instance, Δ12-desaturases introduce a double bond at position x referenced from the carboxyl end; ω3-desaturases introduce a double bond at position y referenced from the methyl end. Less commonly, desaturases are classified as ν + z, indicating the introduction of an additional double bond at carbon z as referenced from a pre-existing double bond (ν) (4, 5). An example of a Δ5-desaturase is the Saccharomyces cerevisiae acyl-CoA Δ9-desaturase introducing a Δ5 double bond into palmitoyl and stearoyl thioesters (6). The nematode Caenorhabditis elegans has an ω3-desaturase involved in producing long-chain polynsaturates (7). Although the plant extraplastidal oleate desaturase is often called Δ12-desaturase, strictly speaking it is a ν + 3 enzyme with a preference for introducing double bonds at or near the Δ12 position (8).

Membrane-bound desaturases are remarkable for their structural similarity and functional diversity. They all contain three conserved histidine motifs, which are believed to be responsible for di-iron binding at the catalytic center, and share similar hydrophobicity profiles that predict a common membrane topology (9, 10). This structural resemblance has provided the basis for the study of structure-function relationships in these enzymes. By using site-directed mutagenesis, Shanklin, Somerville, and colleagues showed that the eight histidine residues in three conserved histidine-rich boxes are essential for the functionality of a rat stearoyl-CoA Δ9-desaturase (11) and amino acid residues flanking the conserved boxes are critical for the catalytic properties of plant FAD2 desaturases and related enzymes (12, 13). Using domain swapping, Napier and colleagues showed that the regions of first two membrane-spanning helices and C terminus of borage front-end desaturases are important for the substrate specificity and/or regioselectivity (14). Despite the above work, our understanding of the structure, function, and evolution of membrane-bound fatty acid desaturases remains fragmentary. In the course of studying desaturases of the fungus Claviceps purpurea (15), we have uncovered two desaturases with very similar structure but differing function, which help to elucidate some of the structure-function-evolution relationships in this class of enzymes.

EXPERIMENTAL PROCEDURES

Organisms and Culture Conditions—C. purpurea kindly provided by Dr. Yu Chen, Dept. of Plant Science, University of Saskatchewan.

JULY 13, 2007 • VOLUME 282 • NUMBER 28
© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Manitoba was grown at 25 °C for 14 days in medium C (16). The S. cerevisiae strain INVSc1 (MATa his3Δ1 leu2 trpl–289 ura3–52 MATα his3Δ1 leu2 trpl–289 ura3–52, Invitrogen) was used as a heterologous host to study the expression of CpDes12 and CpDesX desaturases. The S. cerevisiae strain MKP-0 (MATa canl–100 ade2–1 lys2–1 trpl–52 leu2–3, 112 his3–2 A200 trpl–Δ901), kindly provided by Prof. Wei Xiao, University of Saskatchewan, Canada, was used as a host strain for a Δ9-desaturase gene (ole1) knock-out. The ole1Δ:TRPI1 disruption was made by cloning the EcoRI and Nhel fragments of YEpW (17) containing TRP1 marker into the Sall and KpnI sites of ole1Δ and then introducing the linear recombinant plasmid into the host. The knock-out mutant was selected on the selective medium. The mutant strain named ole1Δ-MKP-0 was used as a heterologous host to study substrate specificity of CpDesX desaturase. Yeast cells were grown at 28 °C either in complex medium (YPD) or synthetic minimal medium (SD).

Cloning of CpDes12 and CpDesX cDNAs from C. purpurea—The reverse transcription-polymerase chain reaction was used to clone CpDes12 and CpDesX. The single-stranded cDNA was synthesized by Superscript III reverse transcriptase (Invitrogen) using total RNA from mycelia of C. purpurea. The cDNA was then used as the template for the PCR reaction with two degenerate oligonucleotide primers, DM34 (forward primer) and DM36 (reverse primer) (see Table 1). These primers were designed based on the conserved amino acid regions of Δ12-desaturases and Δ9-desaturase-like enzymes from other fungal species such as Aspergillus nidulans and Neurospora crassa. The forward primer resides in the first conserved histidine box and reverse primer resides outside the histidine boxes corresponding to the amino acid sequences AHECGH(G/Q)AF (DM34) and WV(N/H)HWLVIALTY (DM36), respectively. The PCR reaction was carried out for 35 cycles with a program (95 °C for 30 s, 50 °C for 60 s, and 72 °C for 90 s) and final extension at 72 °C for 10 min using denatured first-stand cDNA from C. purpurea as templates. PCR products with the expected size (~600 bp) were separated by electrophoresis (1.2% agarose gel) and purified using a QIAGen gel extraction kit (Qiagen). The products were ligated into a pCR4-TOPO vector (Invitrogen) and sequenced.

To obtain the entire sequences of CpDes12 and CpDesX cDNAs, the 5′ and 3′ regions were amplified separately using the Marathon cDNA amplification kit (BD Biosciences, Clontech) according to the manufacturer’s instructions. The primers DM39 and DM40 were used to amplify the 5′ regions of CpDes12 and CpDesX, respectively. For amplification of the 5′ region, primers DM37 and DM41 were used for CpDes12 and DM38 and DM42 were used for CpDesX. The complete sequences, including untranslated and coding regions, were then amplified using specific primers DM43 and DM44 for CpDes12, and DM47 and DM48 for CpDesX, by Pfx50 DNA polymerase (Invitrogen). The resulting PCR products were gel-purified and ligated into a pCR4-TOPO-TA cloning vector to give plasmids pDM11 and pDM12 for CpDes12 and CpDesX, respectively.

Phylogenetic Analysis—Selected fungal fatty acid desaturase amino acid sequences were aligned with ClustalW as hosted at the European Bioinformatics Institute (18) using default parameters, including the Gonnet scoring matrix, a gap penalty of 10, and a gap extension penalty of 0.2. The resulting alignment was used to generate a distance-based unrooted phylogram using the neighbor-joining method performed using PROTDIST and NEIGHBOR in the PHYLIP software suite, version 3.6 (19) as hosted by the Institute Pasteur, Paris, France. Parameters for PROTDIST included the use of the Dayhoff PAM matrix and George/Hunt/Barker amino acid categories. The tree was visualized using TREEVIEW (20). The analysis was repeated with bootstrap analysis using 100 iterations and an extended majority rule tree was constructed using CONSENSE.

Functional Expression and Site-directed Mutagenesis of the CpDes12 and CpDesX Desaturases—The primers DM45 and DM46 for CpDes12 and DM49 and DM50 for CpDesX, respectively, were used to amplify coding regions using PfX50 DNA polymerase. Fragments were then ligated into the vector pYES2.1/V5-His-TOPO (Invitrogen) to yield plasmids pDM13 for CpDes12 and pDM14 for CpDesX. The sequence of the inserts was confirmed to be identical to the original cDNA and in the sense orientation relative to the GAL1 promoter.

For mutagenesis, oligonucleotide primers were used to introduce nucleotide substitutions into CpDes12 and CpDesX through the use of the overlap extension PCR technique (21). For the first step, two overlapping fragments were synthesized in separate PCR reactions with PfX50 DNA polymerase using mutagenic primer pairs as shown in Table 1. The pDM13 was used as a template to generate CpDes12 mutants (CpDes12[V152I] and CpDes12[V206A]), and the pDM14 was used to generate CpDesX mutants (CpDesX[I152V] and CpDesX[A206V]). The appropriate gel-purified products were combined and then used as templates for the second step PCR. The PCR products were then purified and ligated into the pCR4-TOPO-TA cloning vector to give plasmids pDM15 and pDM16 for CpDes12 and CpDesX, respectively.

**Table 1: Primers used in this study.**

| Primer name | Sequence (’5’ to ’3’) |
|-------------|-----------------------|
| DM34        | GCICAYGARTGVCYGAGGAC  |
| DM35        | TAATGTCAGTCCACCAATCG  |
| DM36        | CGGGGAAGGAAAACCATGTCG |
| DM37        | CGGACTTTGGATTATTGCCCACGA |
| DM38        | TCATGGGCAATAATCCAAAGTCCGG |
| DM39        | DAMAGCCAGCCGAAACGGTTGCCAAGTAAC |
| DM40        | DM78                  |
| DM41        | DM77                  |
| DM42        | DM76                  |
| DM43        | DM75                  |
| DM44        | DM74                  |
| DM45        | DM73                  |
| DM46        | DM72                  |
| DM47        | DM71                  |
| DM48        | DM70                  |
| DM49        | DM69                  |
| DM50        | DM68                  |
| DM51        | DM67                  |
| DM52        | DM66                  |
| DM53        | DM65                  |
| DM54        | DM64                  |
| DM55        | DM63                  |
| DM56        | DM62                  |
| DM57        | DM61                  |
| DM58        | DM60                  |
| DM59        | DM59                  |
| DM60        | DM58                  |
| DM61        | DM57                  |
| DM62        | DM56                  |
| DM63        | DM55                  |
| DM64        | DM54                  |
| DM65        | DM53                  |
| DM66        | DM52                  |
| DM67        | DM51                  |
| DM68        | DM50                  |
| DM69        | DM49                  |
| DM70        | DM48                  |
| DM71        | DM47                  |
| DM72        | DM46                  |
| DM73        | DM45                  |
| DM74        | DM44                  |
| DM75        | DM43                  |
| DM76        | DM42                  |
| DM77        | DM41                  |
| DM78        | DM40                  |
| DM79        | DM39                  |
| DM80        | DM38                  |
| DM81        | DM37                  |
| DM82        | DM36                  |

Nucleotides required for amino acid sequence changes are underlined.
acids at the C terminus domain of \textit{CpDes12} were substituted by 176 amino acids of equivalent position of \textit{CpDesX}. The double mutants \textit{CpDes12} [V152I, V206A] and \textit{CpDesX} [I152V, A206V] were generated using constructs \textit{CpDes12} [V206A] and \textit{CpDesX} [A206V] as templates, respectively. All variant fragments were gel-purified and ligated into pYES2.1/V5-His-TOP. The sequences of all mutant constructs were confirmed by sequencing.

\textit{Yeast Transformation and Growth Conditions—} \textit{S. cerevisiae} strain INVSc1 or ole1\&Delta;MKP-0 was transformed with each construct using the S. C. EasyComp transformation kit (Invitrogen) with selection on uracil-deficient medium and supplemented with 17:1–10c in the case of ole1\&Delta;MKP-0. For assessment of desaturase activity, recombinant yeast cells were grown to saturation in 10-ml cultures for 2 days at 28 °C on minimal medium (synthetic dropout) lacking uracil. Yeast cells were then washed and used to inoculate 10 ml of induction medium containing 2% galactose supplemented with or without 0.1 mM methyloxazoline (DMOX) and/or the fatty acyl diethylamide [(product(s))/(substrate + product(s)) \times 100], where the one or more products include those derived from further desaturation.

\textit{Fatty Acid Analysis—} For fatty acid analysis, yeast cells were pelleted by centrifugation, washed once with 1% Tergitol, and washed once with water, and FAMEs\(^2\) were prepared as previously described (8). The FAME samples were analyzed on an Agilent 6890N gas chromatograph equipped with a DB-23 column (30-m \times 0.25-mm) with 0.25-\(\mu\)m film thickness (J&W Scientific). The column temperature was maintained at 160 °C for 1 min, then raised to 240 °C at a rate of 4 °C/min.

The position of newly introduced double bonds in desaturated products was determined by the analysis of the 4,4-dimethylxazoline (DMOX) and/or the fatty acyl diethylamide derivatives as described previously (7, 22). GC-MS analysis was accomplished using an Agilent 5973 mass selective detector coupled to an Agilent 6890N gas chromatograph using the same column and conditions described above. The mass selective detector was run under standard electron impact conditions (70 eV), scanning an effective \(m/z\) range of 40–700 at 2.26 scans/s.

Purification of 16:2–9c,12c from isolated yeast FAMEs was accomplished by high-performance liquid chromatography fractionation using an Agilent 1100 Series high-performance liquid chromatography with the fraction collector connected to 2 \times 12.5 cm Whatman Partisphere C\(_{18}\) columns connected in series. A linear solvent elution gradient was used starting at 90% acetonitrile, 10% water with increasing acetone from 0 to 30% in 20 ml. Collected eluate fractions containing pure 16:2–9c,12c (\(\sim 98\%\) by GC) were pooled and saponified to the free fatty acid (8) for use in yeast medium supplementation experiments.

\textit{Topo\%y Prediction—} The topology of \textit{C. purpurea} desaturases were predicted using the combination software of TOPPRED (23), TMHMM (24), HMMTOP (25), and ConPred II (26).

\textbf{RESULTS}

\textit{Isolation of Two \textit{C. purpurea} cDNAs Encoding \(\Delta^{12}\) Desaturase-like Enzymes (\textit{CpDes12} and \textit{CpDesX})—} By using degenerate oligonucleotide primers targeted to conserved histidine motifs of known \(\Delta^{12}\)-desaturases, and total RNA isolated from mycelia of \textit{C. purpurea} as the template for reverse transcription-PCR, two cDNA fragments of \(\sim 600\) bp showing sequence similarity to fungal \(\Delta^{12}\)-desaturases were amplified. Subsequently, full-length cDNAs corresponding to the genes, given the names \textit{CpDesX} and \textit{CpDes12}, were obtained by 5’ and 3’ rapid amplification of cDNA ends. Sequence analysis indicated that \textit{CpDesX} encodes a polypeptide with 477 amino acids, whereas \textit{CpDes12} codes for a protein with one amino acid shorter than the \textit{CpDesX} polypeptide. \textit{CpDesX} and \textit{CpDes12} share 87% amino acid identity and 86% nucleotide identity. Both \textit{CpDes12} and \textit{CpDesX} possess three histidine motifs, which are believed to be involved in di-iron binding at the active site of membrane-bound fatty acid desaturases (11). BLAST searches showed that \textit{CpDesX} and \textit{CpDes12} have high amino acid sequence identity to \(\Delta^{12}\)-desaturases from \textit{Fusarium moniliforme} (69% for \textit{CpDesX} and 73% for \textit{CpDes12}), \textit{Aspergillus nidulans} (64% for \textit{CpDesX} and 65% for \textit{CpDes12}), \textit{Neurospora crassa} (64% for \textit{CpDesX} and 68% for \textit{CpDes12}), and \textit{Arabidopsis thaliana} (38% for \textit{CpDesX} and 39% for \textit{CpDes12}), as well as to recently identified “bifunctional \(\Delta^{12}\)−\(\Delta^{15}\)” desaturases from \textit{F. moniliforme} and \textit{Magnaporthe grisea} (45–46%).

As might be expected from inspection of the above sequence similarities, phylogenetic analysis supports the recent divergence of \textit{CpDes12} and \textit{CpDesX} (Fig. 1). Both sequences cluster together with fungal \(\Delta^{12}\)-desaturases from \textit{F. graminearum}, \textit{F. moniliforme}, \textit{M. grisea}, \textit{N. crassa}, and \textit{A. nidulans}, which form a group that has been classified as subfamily 2 by Damude and coworkers (27). A separate group includes the fungal bifunctional desaturases from \textit{F. graminearum}, \textit{F. moniliforme}, \textit{M. grisea}, and \textit{N. crassa} (27). Thus, both \textit{CpDes12} and \textit{CpDesX} appear to have evolved from an ancestral subfamily 2 type gene (probably a \(\Delta^{12}\)-desaturase) (27) after the divergence of the \textit{Clavicipitaceae} (including \textit{Claviceps}) and \textit{Nectriaceae} (including \textit{Fusarium}).

\textit{Functional Characterization of \textit{CpDes12} in \textit{S. cerevisiae)—} To determine the function of \textit{CpDes12} and \textit{CpDesX}, the coding regions of two cDNAs were cloned into the yeast expression vector pYES2.1 under control of GAL1 promoter, and the recombinant plasmids were then introduced into \textit{S. cerevisiae} INVSc1. The empty vector (pYES2.1) and the vector containing \textit{Arabidopsis AtFAD2} (28) were used as the negative and positive controls, respectively. The analysis of total FAMEs showed that, compared with the yeast negative control (pYES2.1/INVSc1), the yeast strain AtFAD2/INVSc1 expressing \textit{Arabidopsis FAD2} produced significant quantities of two additional fatty acids as previously described and identified as 16:2–9c,12c and 18:2–

\(^2\)The abbreviations used are: FAME, fatty acid methyl ester; 16:2–9c,12t, a fatty acid containing 16 carbons with 2 double bonds at position 9 and 12, counted from the C terminus with cis configuration at position 9 and trans configuration at position 12; 18c, fatty acid(s) containing 18 carbons; DMOX, 4,4-dimethylxazoline; GC, gas chromatography; MS, mass spectrometry.
The yeast strain CpDes12/INVSc1 expressing CpDes12 also produced two new fatty acids with the same retention time as 16:2–9c,12c and 18:2–9c,12c standards (Fig. 2A, chromatogram 1). Their identities were confirmed by GC/MS analysis of the FAMEs (see supplemental Fig. S1).

In addition, CpDes12 could also use 19:1–10c and 18:2–9c,12c as substrates, albeit to a much lesser extent, producing 19:2–10c,13c (see supplemental Fig. S2) and 18:3–9c,12c,15c (see below), respectively. When supplied with 19:1–10c substrate, the yeast strain CpDes12/INVSc1 expressing CpDes12 produced 19:2–10c,13c at a level of 0.4% of total fatty acids. These results indicated CpDes12 is primarily a Δ12-desaturase with ν + 3 regioselectivity. We propose the common name CpDes12 for this enzyme (see "Discussion").

Functional Characterization of CpDesX in S. cerevisiae—The yeast strain CpDesX/INVSc1 expressing CpDesX produced five new peaks compared with the negative control (pYES2.1/INVSc1) (Fig. 2A, chromatogram 2). The two FAMEs with longer retention times were identified as 18:2–9c,12c and 18:3–9c,12c,15c (Fig. 2A, chromatogram 2) based on their retention times and mass spectra being identical to that of authentic standards. The mass spectra of two FAMEs with lower retention times were consistent with 16:2 isomers (molecular ion \(m/z = 266\)), whereas the mass spectrum of a third peak with an intermediate retention time was consistent with 16:3 (molecular ion \(m/z = 264\)). The exact position of the double bonds of three novel 16C fatty acids was provided by GC-MS analysis of their DMOX (Fig. 3) and diethylamide derivatives (see supplemental Fig. S3). Fig. 3 (A and B) show the mass spectra of the DMOX derivatives of 16C dienoic acids. Comparison of the mass spectra of the derivatives indicates that they have identical molecular ion and fragmentation patterns. The diagnostic fragment pairs of DMOX derivatives at \(m/z\) 196 and 208, and 236 and 248, with gaps of 12 atomic mass units indicate that both fatty acids are 16C dienes with double bonds at the Δ12 and Δ15 positions. The mass spectra of diethylamide derivatives confirmed the result of DMOX derivatives (see supplemental Fig. S3). One of the 16C dienoic acids was eluted at the same retention time as authentic 16:2–9c,12c standard and was thus identified as 16:2–9c,12c. The earlier eluting 16C diene, as found in a similar study by Cahoon and colleagues (29), was identified as 16:2–9c,12t. Chemically speaking, the data are also consistent with 16:2–9t,12c and 16:2–9t,12t isomers, but, given the likelihood that the isomer is derived from 16:2–9c (see below), we consider those possible structures improbable. Under the growth conditions used, 16:2–9c,12t isomer was not detected in C. purpurea.
Fig. 3C shows the mass spectrum of the DMOX derivative of the 16C trienoic acid. The derivative has a molecular ion at m/z 303 and diagnostic fragments at m/z 182, 196, 208, 222, 236, 248, 274, and 288, identical to the pattern of 16:3–9c,12c,15c, a product of a recently identified "bifunctional" desaturase from Acanthamoeba castellanii (30).

To verify the substrates of CpDesX for the biosynthesis of the 16C and 18C polyunsaturates, a Δ⁹-desaturase knock-out mutant (ole1Δ) of the yeast strain MKP-0 was generated using the one-step gene disruption approach (31). When CpDesX was expressed in the mutant strain (ole1Δ-MKP-0) in the presence of 16:1–9t, no new fatty acid was detected. On the other hand, supply of 16:1–9c to CpDesX/ole1Δ-MKP-0 yielded two new fatty acids, 16:2–9c,12t and 16:2–9c,12c. Supply of 16:2–9c,12t to the strain produced 16:3–9c,12c,15c (see supplementary Fig. S4). These results provided further evidence that the 16C diene with the lower retention time described above is 16:2–9c,12t and that CpDesX catalyzes desaturation of 16C fatty acids at both Δ¹² and Δ¹⁵ positions. The Δ¹² desaturation of 16:1–9c produced two 16C dienes, 16:2–9c,12c and 16:2–9c,12t. The ratio of the two isomers was ~2.6:1, with 16:2–9c,12c predominating. In addition, CpDesX also has Δ¹²- and Δ¹⁵-desaturase activities on 18:1–9c for the sequential synthesis of Δ¹²- and Δ¹⁵-polyunsaturates (18:2–9c,12c and 18:3–9c,12c,15c) in yeast. It was noted, however, that the Δ¹⁵ activity of CpDesX on 18C monoene was much lower compared with its Δ¹⁵ activity on the 18C diene (see Table 2).

To define the substrate specificity of the CpDesX, a range of possible substrates from 16C to 22C fatty acids were exogenously supplied to CpDesX/ole1Δ-MKP-0 or CpDesX/INVSc1. Among 14 fatty acids tested, 16:1–9c, 16:2–9c,12c, 18:1–9c, 18:2–9c,12c, 18:3–6c,9c,12c, 20:2–11c,14c, 20:3–8c,11c,14c, and 20:4–5c,8c,11c,14c could be used by CpDesX as substrates, whereas desaturation products of 16:1–9t, 18:1–6c, 18:1–9t, 18:1–11c, 20:1–11c, and 22:5–4c,7c,10c,13c,16c were not detected. These results suggest that CpDesX is a novel desaturase with a very wide range of substrates. In addition to its apparent ν + 3 desaturase activity, referencing both Δ⁹ and Δ¹² double bonds on 16 and 18 carbon fatty acids, it could also function as a typical ω⁶-desaturase, introducing a ω⁶ double bond into 18C and 20C ω⁶-polyunsaturates, such as 18:2–9c,12c, 18:3–6c,9c,12c, 20:3–8c,11c,14c, and 20:4–5c,8c,11c,14c (Fig. 2B).

To obtain quantitative data for the substrate preferences of CpDesX for ω⁶-polyunsaturated fatty acids, cultures of CpDesX/INVSc1 were grown in minimal medium supplemented separately with 18:2–9c,12c, 18:3–6c,9c,12c, 20:2–11c,14c, 20:3–8c,11c,14c, and 20:4–5c,8c,11c,14c and analyzed by GC. As shown in Table 3, CpDesX shows a preference for 18C fatty acids, linoleic acid in particular. The highest conversion efficiency (products as a percentage of the sum of substrates) was obtained with 18:2–9c,12c.

**TABLE 2**

Effects of the mutagenesis on Δ¹² and Δ¹⁵ desaturation of 18C fatty acids by CpDes12 and CpDesX

| Enzyme | Accumulation | Conversion | Δ¹² | Δ¹⁵/Δ¹² ratio relative to wild type |
|--------|--------------|------------|-----|-----------------------------------|
| CpDes12 wild type | 10.4 ± 0.55 | 16.4 ± 0.97 | 0.06 ± 0.0 | 61.3 ± 0.4 | 0.37 ± 0.02 | 0.006 ± 0.00 | 1.0 |
| CpDes12[152V,122V] | 10.8 ± 0.18 | 15.6 ± 0.25 | 0.40 ± 0.02 | 59.7 ± 0.7 | 2.52 ± 0.11 | 0.042 ± 0.002 | 7.0 |
| CpDes12[152V,206A] | 9.5 ± 0.23 | 15.0 ± 0.15 | 1.97 ± 0.13 | 64.1 ± 0.9 | 11.57 ± 0.59 | 0.180 ± 0.007 | 29.9 |
| CpDes12[152V,206A] | 11.6 ± 0.90 | 10.8 ± 0.95 | 3.15 ± 0.54 | 54.6 ± 4.2 | 22.4 ± 1.9 | 0.411 ± 0.002 | 68.2 |
| CpDes12[152V,206A] | 19.1 ± 0.32 | 8.3 ± 0.71 | 0.12 ± 0.01 | 30.5 ± 1.5 | 1.42 ± 0.05 | 0.047 ± 0.002 | 7.7 |
| CpDes12 wild type | 24.8 ± 0.29 | 0.11 ± 0.01 | 0.23 ± 0.01 | 1.35 ± 0.06 | 67.5 ± 1.8 | 50.0 ± 2.1 | 1.0 |
| CpDesX[152V] | 24.5 ± 0.65 | 0.84 ± 0.06 | 1.46 ± 0.05 | 8.54 ± 0.21 | 63.5 ± 1.0 | 7.4 ± 0.2 | 0.0149 |
| CpDesX[206V] | 27.0 ± 0.82 | 0.73 ± 0.02 | 0.47 ± 0.02 | 4.28 ± 0.08 | 39.3 ± 0.9 | 9.2 ± 0.3 | 0.184 |
| CpDesX[152V,206V] | 23.6 ± 1.6 | 1.8 ± 0.23 | 0.21 ± 0.02 | 7.78 ± 0.50 | 10.7 ± 0.4 | 1.4 ± 0.1 | 0.028 |

*%TFA, weight percent of the total fatty acids.*
strates and products) was observed on 18:2–9c,12c (50%), followed by 18:3–6c,9c,12c, 20:2–11c,14c, 20:3–8c,11c,14c, and 20:4–5c,8c,11c,14c.

Structural Determinants of Substrate Specificity and Regioselectivity—Although CpDes12 and CpDesX possess quite different substrate specificity and regioselectivity, these two desaturases share striking similarity in their primary structure and hydrophobicity profiles. Hydrophobicity profiles have been previously used to support a general topological model containing four transmembrane domains (11, 14). With this topological model in mind it is informative to inspect amino acid sequence alignments of plant and fungal desaturases, including the bifunctional desaturases (Fig. 4). Of particular interest are those amino acids that are conserved above the alignment indicate the two amino acid positions chosen for the site-directed mutagenesis.

The replacement of the entire C-terminal domain of CpDes12 by that of CpDesX also resulted in significant increase of the ratio of Δ\(^{15}\) over Δ\(^{12}\) desaturation. This was apparently the result of opposing changes in both Δ\(^{12}\)- and Δ\(^{15}\)-desaturase activities.

To confirm whether similar regioselective switch can be observed with CpDesX, a reciprocal muta-
genesis experiment was performed with CpDesX by replacing the two critical amino acid residues identified above with the corresponding counterparts in CpDes12 (Table 2). In the wild-type CpDesX, the $\Delta^{15}/\Delta^{12}$ desaturation ratio is high (50.0 ± 2.1). A single substitution from isoleucine to valine at position 152 (CpDesX[I152V]) decreased the $\Delta^{15}/\Delta^{12}$ ratio significantly (7.4 ± 0.2, ∼15% of the native enzyme), and a single substitution from alanine to valine at position 206 (CpDesX[A206V]) reduced the ratio to the similar degree (9.2 ± 0.3, ∼18% of the native enzyme). Substitution at both positions in CpDesX (CpDesX[I152V,A206V]) resulted in the ratio being further decreased (1.4 ± 0.1, only ∼3% of the native enzyme). In the single CpDesX mutants, the effects appeared to be mainly on $\Delta^{12}$-desaturase activity. However, in the double mutant, opposing changes in both $\Delta^{12}$- and $\Delta^{15}$-desaturase activities affected the $\Delta^{15}/\Delta^{12}$ desaturation ratio.

To verify whether these amino acid substitutions in CpDesX and CpDes12 also affect their desaturase activities on $\omega^3$-polyunsaturated fatty acids, yeast strain INVSc1-expressing mutant enzymes were grown in minimal medium supplemented with 18:3–6c,9c,12c, 20:3–8c,11c,14c, or 20:4–5c,8c,11c,14c, and $\omega^3$-desaturase activities were measured relative to that of the native CpDes12 enzyme (Fig. 5). Although conversion efficiencies for the various substrate differed (see supplemental Table S1), when these were normalized to 100% for wild-type CpDesX, a clear trend, which parallels the results of Table 2, could be observed. For single amino acid substitutions of CpDesX, $\omega^3$-desaturase activities dropped 40–60% for the substrates tested, and the effect of double mutation was more severe, with a decline of ∼85% relative to the native enzyme activity. In reciprocal fashion, mutation of CpDes12 raised $\omega^3$-desaturase activities on the tested substrate, although the effect of substitution at position 152 was not particularly strong.

**DISCUSSION**

In this study, we report the isolation of two cDNAs corresponding to the fatty acid desaturase genes CpDes12 and CpDesX from *C. purpurea*, an infectious phytopathogen of many grasses. Although the two protein sequences encoded by the cDNAs share very high sequence similarity (87% identity at the amino acid level), they possess distinct substrate specificities and regioselectivities. CpDes12 is a $\Delta^{12}$-desaturase mainly acting on 16C and 18C monounsaturates, whereas CpDesX possesses both $\Delta^{12}$- and $\Delta^{15}$-desaturase activities on 16C and 18C fatty acids and $\omega^3$-desaturase activity on 18C and 20C $\omega^6$-polyunsaturates. To our knowledge, this is the first example of a desaturase that carries out such wide catalytic activities. Recently, a number of bifunctional desaturases denoted as $\Delta^{12}/\omega^3$ or $\Delta^{12}/\Delta^{15}$ enzymes have been reported. A group of bifunctional desaturases identified from the Ascomycota species *F. graminearum*, *F. moniliforme*, *M. grisea*, and *N. crassa* have $\omega^3$-desaturase activity on $\omega^6$-polyunsaturates with 18C to 20C chains, and $\Delta^{12}$-desaturase activity on 18:1–9c. However, no desaturase activity was reported for 16C fatty acids (27). In addition, a bifunctional desaturase identified from protozoan *Acanthamoeba castellanii* (29) has $\Delta^{12}$- and $\Delta^{15}$-desaturase activities on 16C and 18C fatty acids, but no $\omega^3$ activity on $\omega^6$-polyunsaturates with chain length longer than 18 carbons. CpDesX possesses activities combining these two types of desaturases, i.e. comparable $\omega^3$-desaturase activity toward C18 to C20 $\omega^6$-polyunsaturates as observed in fungal bifunctional desaturases, as well as $\Delta^{12}$- and $\Delta^{15}$-desaturase activities for sequential conversion of 16:1–9c to 16:2–9c,12c, then to 16:3–9c,12c,15c, as observed for the protozoan desaturase.

The high similarity of the primary sequences of CpDes12 and CpDesX provides an opportunity for us to localize distinct catalytic properties to individual regions or amino acid residues. Previous studies have shown the importance of conserved histidine residues (9, 11) and their proximal residues (12, 13) as well as membrane-spanning helices and C-terminal regions for the functionality and substrate specificity (14). By using site-directed mutagenesis we found that two residues are critical for defining the regioselectivity of fungal membrane-bound fatty acid desaturases. One residue is located at position 152, three amino acids upstream of the first histidine box, and the other residue is at position 206, 11 amino acids downstream of the second histidine box. When plotted on a membrane topological model for membrane-bound desaturases, both of these residues map to, or near to, the predicted cytoplasmic face of the membrane (Fig. 4A). When the two residues in CpDes12 were simultaneously substituted by the equivalents in CpDesX, the ratio of $\Delta^{15}/\Delta^{12}$ desaturation on 18-carbon fatty acids was increased by >68-fold relative to that in the native CpDes12. Thus, the double mutant acquired high $\Delta^{15}$-desaturase activity on 18:2–9,12, as well as $\omega^3$-desaturase activity on other $\omega^6$-polyunsaturates with 18C to 20C chain length. In a reciprocal experiment where the two residues in CpDesX were substituted by the equivalents at CpDes12, the $\Delta^{15}/\Delta^{12}$ desaturation ratio and $\omega^3$ activity were dramatically reduced.

The evidence presented regarding *C. purpurea* genes encoding membrane-bound fatty acid desaturases, in combination with information from other studies, provides some very interesting insights into the structure-function-evolution relationships among desaturases. This is particularly true regarding substrate specificity and regioselectivity and its evolution in...
these enzymes. For substrate specificity, the features that are relevant are chain length, position, and geometry of double bonds and head group (where this is typically a glycerol ester or CoA thioester). For regioselectivity, the position of the incipient double bond depends variously on its position relative to (i) a head group binding domain, (ii) a double bond binding domain, (iii) the active site, and (iv) a methyl terminus domain. An important aspect of the model that borrows from the situation for soluble fatty acid desaturases is that the methyl terminus domain may be part of a fatty acyl binding pocket, which, depending on the structure, either provides little restriction of the active site-to-methyl-end distance, or in the other extreme, limits the distance to some absolute maximum (giving rise to $\omega^3$ regioselectivity, for example). Thus, for $\Delta^\alpha$ and $\nu + 3$ desaturases, there may be little restriction of binding at the methyl terminus. On the other hand, for plant and nematode $\omega^3$-desaturases, the methyl terminus domain may be the most important determinant of regioselectivity. In any case, the regioselectivity appears to be influenced by more than one putative binding domain giving rise to primary and secondary preferences.

From both structural and evolutionary points of view, the switch between $\nu + 3$ ($\Delta^{12}$) and $\nu + 3$ ($\omega^3$) appears to be relatively facile. The site-directed mutagenesis data show that even a single specific amino acid change can have a very significant change in regioselectivity. This is particularly true of position 206, for which a very conservative change (valine versus alanine) causes a significant alteration in the regioselectivity of CpDes12 and CpDesX in a reciprocal fashion.

From an evolutionary point of view, as has been pointed out previously (27, 29, 35), enzymes capable of $\omega^3$ desaturation appear to have evolved independently in a number of fungal lineages. The phylogenetic analysis is consistent with these events resulting from gene duplication of a $\nu + 3$ ($\Delta^{12}$) gene giving rise to the evolution of an additional desaturase with $\nu + 3$ ($\omega^3$) activity (the bifunctional enzymes) or in some cases, ultimately $\omega^3$ ($\nu + 3$) (e.g. the "classic" $\omega^3$-desaturase of Mortierella alpina). This appears to be paralleled by a single gene duplication event giving rise to plant extraplastidic $\omega^3$ ($\nu + 3$) desaturases. Thus, the data are consistent with the existence of an ancestral $\nu + 3$ ($\Delta^{12}$) desaturase prior to the divergence of the kingdoms, which gave rise in multiple lineages to $\nu + 3$ ($\omega^3$) (bifunctional) and $\omega^3$ ($\nu + 3$) (classic $\omega^3$) desaturases. Further-

### Table 4

| Primary regioselectivity | Secondary regioselectivity | Common name(s) | Example(s) | Diagnostic substrate(s) | Reference |
|--------------------------|---------------------------|----------------|------------|-------------------------|-----------|
| $\nu + 3$ ($\Delta^\alpha$) | Plant extraplastidic $\Delta^{12}$ | Arachis hypogaea FAD2 | 19:1–10c | (34) |
| $\nu + 3$ ($\Delta^\alpha$) | Fungal bifunctional $\Delta^{12}/\Delta^\alpha$ | C. purpurea CpDes12 | 16:1–9c | (35) |
| $\nu + 3$ ($\omega^3$) | Fungal $\omega^3$-desaturase | M. alpina $\omega^3$-desaturase | 16:1–11c | (36) |

- The plant extraplastidial $\Delta^{12}$ desaturases are the "classic" $\nu + 3$ desaturases, where the secondary regioslectivity is in parentheses (see Table 4).
- The plant extraplastidial $\omega^3$-desaturases (FAD3s) would be classified as $\omega^3$ ($\nu + 3$) enzymes, because they primarily reference the methyl group of substrates, including 18:1–9c (8), but they have a strong preference for $\omega^3$ substrates. The C. elegans $\omega^3$-desaturase is also in the $\omega^3$ ($\nu + 3$) class.
- The plant extraplastidial $\omega^3$-desaturases (FAD3s) would be classified as $\omega^3$ ($\nu + 3$) enzymes, because they primarily reference an existing double bond, but have a strong preference for $\omega^3$ desaturation (see Table 4). Similarly, the classification of CpDesX as a $\nu + 3$ ($\omega^3$) desaturase is supported by the two successive $\nu + 3$ desaturations of both 16:1–9c and 18:1–9c and a secondary preference for $\omega^3$ desaturation.
more, it is possible to speculate that, given the very close structural similarity of $v + 3 (\Delta^{12})$ and $v + 3 (\omega^3)$ desaturases exemplified by C. purpurea, the $v + 3 (\omega^3)$ enzymes are likely intermediates in the evolution of $\omega^3 (v + 3)$ desaturases.

Acknowledgments—We thank Mark Smith and Jitao Zou (Plant Biotechnology Institute, Canada) for reviewing the manuscript.

REFERENCES

1. Shanklin, J., and Cahoon, E. B. (1998) Annu. Rev. Plant Physiol Plant Mol. Biol. 49, 611–641
2. Sperling, P., Ternes, P., Zank, T. K., and Heinz, E. (2003) Prostaglandins Leukot. Essent. Fatty Acids 68, 73–95
3. Los, D. A., and Murata, N. (1998) Biochim. Biophys. Acta 1394, 3–15
4. Yadav, N. S., Wierzbicki, A., Aegerter, M., Caster, C. S., Perez-Grau, L., Kinney, A. J., Hitz, W. D., Booth, J. R., Jr., Schweiger, B., Stecca, K. L., Allen, S. M., Blackwell, M., Reiter, R. S., Carlson, T. J., Russell, S. H., Feldmann, K. A., Pierce, J., and Browse, J. (1993) Plant Physiol. 103, 467–476
5. Hitz, W. D., Carlson, T. J., Booth, J. R., Jr., Kinney, A. J., Stecca, K. L., and Yadav, N. S. (1994) Plant Physiol. 105, 635–641
6. Fujimori, K., Anamnart, S., Nakagawa, Y., Sugioka, S., Ohta, D., Oshima, Y., Yamada, Y., and Harashima, S. (1997) FEBS Lett. 413, 226–230
7. Meesapyodsuk, D., Reed, D. W., Savile, C. K., Buist, P. H., Ambrose, S. J., and Covello, P. S. (2000) Biochemistry 39, 11948–11954
8. Reed, D. W., Schafer, U. A., and Covello, P. S. (2000) Plant Physiol. 122, 715–720
9. Avelange-Macherel, M. H., Macherel, D., Wada, H., and Murata, N. (1995) FEBS Lett. 361, 111–114
10. Diaz, A. R., Mansilla, M. C., Vila, A. J., and de Mendoza, D. (2002) J. Biol. Chem. 277, 48099–48106
11. Shanklin, J., Whittle, E., and Fox, B. G. (1994) Biochemistry 33, 12787–12794
12. Broun, P., Shanklin, J., Whittle, E., and Somerville, C. (1998) Science 282, 1315–1317
13. Broadwater, J. A., Whittle, E., and Shanklin, J. (2002) J. Biol. Chem. 277, 15613–15620
14. Libisch, B., Michaelson, L. V., Lewis, M. J., Shewry, P. R., and Napier, J. A. (2000) Biochem. Biophys. Res. Commun. 279, 779–785
15. Mey, G., Oeser, B., Lebrun, M. H., and Tuzdynski, P. (2002) Mol. Plant Microbe Interact. 15, 303–312
16. Mantle, P. G., and Nisbet, L. J. (1976) J. Gen. Microbiol. 93, 321–334
17. Berben, G., Dumont, J., Gilliquet, V., Bolle, P. A., and Hilger, F. (1991) Yeast 7, 475–477
18. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003) Nucleic Acids Res. 31, 3497–3500
19. Felsenstein, J. (1989) Cladistics 5, 164–166
20. Page, R. D. (1996) Comput. Appl. Biosci. 12, 357–358
21. Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1990) BioTechniques 8, 528–535
22. Luthria, D. L., and Sprecher, H. (1993) Lipids 28, 561–564
23. Claros, M. G., and von, H. G. (1994) Comput. Appl. Biosci. 10, 685–688
24. Krogh, A., Larsson, B., von, H. G., and Sonnhammer, E. L. (2001) J. Mol. Biol. 305, 567–580
25. Tusnady, G. E., and Simon, I. (1998) J. Mol. Biol. 283, 489–506
26. Araiz, M., Mitsuoka, H., Ikeda, M., Xia, J. X., Kikuchi, T., Satake, M., and Shimizu, T. (2004) Nucleic Acids Res. 32, W390–W393
27. Damude, H. G., Zhang, H., Farrall, L., Ripp, K. G., Tomb, J. F., Hollerbach, D., and Yadav, N. S. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 9446–9451
28. Covello, P. S., and Reed, D. W. (1996) Plant Physiol. 111, 223–226
29. Cahoon, E. B., and Kinney, A. J. (2004) J. Biol. Chem. 279, 12495–12502
30. Sayanova, O., Haslam, R., Guschina, I., Lloyd, D., Christie, W. W., Harwood, J. L., and Napier, J. A. (2006) J. Biol. Chem. 281, 36533–36541
31. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Larcroute, F., and Cul- lin, C. (1993) Nucleic Acids Res. 21, 3329–3330
32. Heilmann, I., Pidkowich, M. S., Girke, T., and Shanklin, J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10266–10271
33. Stukey, J. E., McDonough, V. M., and Martin, C. E. (1990) J. Biol. Chem. 265, 20144–20149
34. Schwartzbeck, J. L., Jung, S., Abbott, A. G., Mosley, E., Lewis, S., Pries, G. L., and Powell, G. L. (2001) Phytochemistry 57, 643–652
35. Zhang, S., Sakuradani, E., Ito, K., and Shimizu, S. (2007) FEBS Lett. 581, 315–319
36. Sakuradani, E., Abe, T., Iguchi, K., and Shimizu, S. (2005) Appl. Microbiol. Biotechnol. 66, 648–654