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Specific Protein Regions Influence Substrate Specificity and Product Length in Polyunsaturated Fatty Acid Condensing Enzymes

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ABSTRACT: We describe a condensing enzyme from *Pythium irregulare* (PirELO) that shows highest activity on the 18-carbon, Δ-6 desaturated fatty acids, stearidonic acid and γ-linolenic acid. However, this enzyme is also capable of elongating a number of other fatty acids including the 20-carbon, Δ-5 desaturated fatty acid eicosapentaenoic acid. Surprisingly, a *Phytophthora infestans* condensing enzyme (PinELO) with very high homology to PirELO did not show activity with 20-carbon fatty acids. A series of chimeric proteins for these two enzymes were constructed to investigate the influence of different regions on substrate and product length. The substitution of a region from near the center of PirELO into PinELO resulted in an enzyme having EPA-elongating activity similar to that of PirELO. Only eight amino acids differed between the two proteins in this region; however, substitution of the same region from PinELO into PirELO produced a protein which was almost inactive. The addition of a small region from near the N-terminus of PinELO was sufficient to restore activity with GLA, indicating that amino acids from these two regions interact to determine protein structure or function. Predicted topology models for PirELO and PinELO placed the two regions described here near the luminal−proximal ends of the first and fourth/fifth transmembrane helixes, at the opposite end of the condensing enzyme from four conserved regions thought to form a catalytic ring. Thus, protein characteristics determined by specific luminal−proximal regions of fatty acid condensing enzymes have a major influence on substrate specificity and final product length.

Fatty acids with lengths of 20 carbons or more are known as very long chain fatty acids. The formation of these fatty acids involves the elongation of shorter fatty acids via a multistep reaction catalyzed by a set of four enzymes, namely, a condensing enzyme (also known an elongase), a β-keto-reductase, a dehydratase, and an enoyl-reductase (*1*). The condensing enzyme, which is thought to determine the substrate specificity of the overall reaction, catalyzes the condensation of malonyl-CoA with an acyl CoA precursor. This reaction represents the rate limiting step in fatty acid elongation (*2*). The yeast very long chain fatty acid condensing enzymes ELO2p and ELO3p (also known as Fen1p and Sur4p, respectively) are encoded by ELO2 and ELO3 (*3*). While ELO2p and ELO3p seem to have overlapping activities and can both elongate saturated fatty acids to a final length of 24C, ELO3p is required for the final conversion of 24:0 to 26:0.

Certain mammalian elongases with homology to the yeast enzymes are capable of elongating polyunsaturated fatty acids (*4*, *5*). However, very long chain polyunsaturated fatty acids such as arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), and docosahexaenoic acid (22:6n-3) are not synthesized de novo in mammals, but require precursor essential fatty acids such as linoleic (18:2n-6) or linolenic (18:3n-3) acid. Even when levels of precursor fatty acids are adequate, the synthesis of these fatty acids in the body may be inefficient, particularly in the case of DHA, and dietary supplementation is thought to be beneficial (*6*). In humans, very long chain polyunsaturated fatty acids have been shown to be involved in normal brain and eye development (*7*, *8*), maintenance of eye health (*9*), and reducing the risk of cardiovascular diseases (*10*). EPA[1] and DHA are produced in high levels in certain microorganisms, particularly marine microorganisms, which serve as a food source for fish. Consequently, fish and fish oil represent the main sources of EPA and DHA in human diets. With fish stocks declining worldwide, there has recently been a great deal of interest in producing these nutritionally important fatty acids in plants (*11*−*14*).

The major fatty acids in oilseed crops usually include some combination of LA, ALA, and oleic (18:1n-9) acids. The pathway most often used for the transgenic metabolic engineering of very long chain polyunsaturated fatty acids involves the Δ6-desaturation of LA and ALA, producing γ-linolenic acid (18:3n-6) and stearidonic acid (18:4n-3), which are then elongated. The elongation of these 18C Δ6-desaturated fatty acids therefore represents an early and critical step in the biosynthesis of plant-based very long chain polyunsaturated fatty acids. Numerous enzymes catalyzing this activity have been isolated from various species of fungi, lower plants, and algae (*12*, *15*−*18*). Generally, these enzymes are capable of elongating both the ω-6 fatty acid GLA and the ω-3 fatty acid SDA, forming dihomo-γ-linolenic acid (20:3n-6) and eicosatetraenoic acid (20:4n-3), respectively.

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[1]Abbreviations: ARA, arachidonic acid; EPA, eicosapentaenoic acid; LA, linoleic acid, ALA; linolenic acid; GLA, γ-linolenic acid; SDA, stearidonic acid; DGLA, dihomo-γ-linolenic acid; PinELO, *Phytophthora infestans* elongase; PirELO, *Pythium irregulare* elongase.

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While some of these elongases are very specific for 18C chain substrates, others are also capable of accepting longer chain fatty acids, albeit with lower activities. Examples of condensing enzymes which elongate GLA and SDA, but not EPA, include the Δ6-elongase from the moss *Physcomitrella patens* (*19*) and the polyunsaturated fatty acid elongase from *Caenorhabditis elegans* (*20*). Similarly, Meyer et al. (*17*) identified elongases from the algae *Thalassiosira pseudonana* and *Ostreococcus tauri* which are specific for Δ6-desaturated 18C fatty acids and have no activity with the Δ5-desaturated 20C fatty acids EPA and ARA. Conversely, the same group identified related elongases from the fish *Oncorhynchus mykiss* and the frog *Xenopus laevis* that accept both 18C and 20C fatty acids as substrates. However, all four of these condensing enzymes are most active on SDA, with somewhat lower activity on GLA. When these enzymes were placed in an unrooted phylogram containing 29 functionally characterized elongases, the algal elongases specific for the elongation of Δ6-desaturated 18C polyunsaturated fatty acids fell in a separate clade than the chordate enzymes with multiple regioselectivities. Nevertheless, an alignment of protein sequences including the 18C-specific and the less specific enzymes did not result in the identification of regions which could account for the differences in chain-length specificity.

Elongases are integral membrane proteins having seven transmembrane helixes. In the yeast ELO2p/ELO3p enzymes, the luminal−proximal region of the sixth transmembrane helix has been shown to be important in determining fatty acid chain length (*21*). Specifically, the location of a lysine residue within this region determines whether the enzyme is capable of elongating unsaturated fatty acids to a length of 24 versus 26 carbons. However, because the long chain saturated/monounsaturated fatty acid elongating yeast enzymes share only limited sequence homology with the polyunsaturated fatty acid elongases from animals and protists, it is quite conceivable that different regions in these two types of enzyme are involved in determining substrate specificity and product chain length.

The specificity of the elongase reaction can be particularly important in plant-based production of very long chain polyunsaturated fatty acids since nonspecific activity may lead to undesirable mixtures of side-products. Furthermore, the elongase reaction appears to act as a bottleneck in the biosynthesis reaction. A clearer understanding of the contributions of specific protein regions to the activity and specificity of condensing enzymes will be useful in engineering enzymes with higher levels of the desired activity. To date, the high degree of sequence variance at the amino acid level among polyunsaturated fatty acid elongases has made it difficult to pinpoint areas that affect substrate specificity or final product length.

Here, we have analyzed polyunsaturated fatty acid condensing enzymes from the fungi *Pythium irregulare* and *Phytophthora infestans*. Although these enzymes have a high level of identity at the protein level, they differ in activity. While the elongase from *P. irregulare* is quite promiscuous with regard to substrate, the *P. infestans* enzyme shows much higher substrate specificity. We identified two luminal proximal regions that influence chain length specificity in these polyunsaturated fatty acid elongases, neither of which overlapped with the area involved in this function in the yeast elongases.

MATERIALS AND METHODS

Isolation of a Pythium irregulare Elongase. *Pythium irregulare* cells were grown in potato dextrose broth (Sigma Chemical Co., St. Louis, MO), and total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Single stranded cDNA was synthesized by reverse transcription with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). This cDNA was used as a template for PCR using a number of primers designed on the basis of sequence information from internal regions of a *P. infestans* elongase (*22*) that shows activity on 18-carbon, Δ-6 desaturated fatty acids. A product of the expected size was produced with primers nPiF4 and nPiR4 (primer sequences are given in Supporting Information Table 1). This PCR product was gel purified and sequenced. On the basis of the sequence of this internal product, primers were designed to obtain the 3′ and 5′ ends of the cDNA, and a Marathon cDNA Amplification Kit (BD Biosciences, CLONTECH) was used for 3′ and 5′ RACE according to the manufacturer's instructions. The full-length cDNA was amplified with primers PiFLF and PiFLR, using PfuUltra high-fidelity DNA polymerase (Stratagene, La Jolla, CA).

Generation of Chimeric Proteins. To create the chimeric proteins, overlapping fragments were generated by PCR from the *P. infestans* or *P. irregulare* genes. These fragments were mixed and used to generate the primary full-length chimeric sequences by PCR. For example, for chimeric protein 1, primers PiFLF and PHMR were used to generate a PCR fragment from the 5′ end of the *P. irregulare* cDNA clone, and primers PhyFLR and PHMF were used to generate a fragment from the 3′ end of the *P. infestans* cDNA. These fragments were mixed and amplified with PiFLF and PhyFLR to give a PCR product including the entire coding region, which was cloned into the yeast expression vector pYES 2.1/V5-His-TOPO (Invitrogen, Carlsbad, CA). Fragments from specific regions of primary chimeric proteins were generated in the same way, mixed, and used to generate secondary chimeric proteins by PCR. All clones were sequenced prior to expression studies.

Yeast Expression Studies. Clones in the yeast expression vector pYES2.1/V5-His-TOPO were transformed into the yeast (*Saccharomyces cerevisiae*) strain INVSc-1 (Invitrogen, Carlsbad, CA), and transformants were selected on dropout base (DOB; MP Biomedicals, Irvine, CA) plates which included a dropout supplement lacking uracil (CSM-URA; MP Biomedicals, Irvine, CA). Individual colonies were cultured overnight at 28 °C in DOB liquid media lacking uracil, then collected by centrifugation, washed once in induction media (0.67% yeast nitrogen base, 2% galactose, and 1% raffinose plus uracil dropout supplement), and resuspended at an OD_{600} of 0.3 in fresh induction media supplemented with 0.1% tergitol and 0.25 mM EPA or GLA. After a further 24 h of growth at 28 °C, cells were harvested by centrifugation and washed twice with sterile water. Fatty acids were extracted and transmethylated as previously described (*23*). Fatty acid methyl ester samples were analyzed on an Agilent 6890N gas chromatograph equipped with a DB-23 column (30 m × 0.25 mm) with a 0.25 μm 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, and held for 10 min. GC-MS analysis of fatty acid methyl esters was performed with an Agilent 6890N gas chromatograph and an Agilent 5973 mass selective detector.

Prediction of Membrane Topology. The OCTOPUS (http://octopus.cbr.su.se/) program (*24*) was used to predict membrane topology, and results were compared with predictions obtained using the MEMSAT3 (*25*) and PRODIV-TMHMM (*26*) methods.

Table 1: Percent Conversion of Fatty Acid Substrates by PirELO and PinELO

fatty acid[a]	PirELO[b]	PinELO	control[c]
GLA	20.7 ± 1.1%[d]	17.4 ± 0.3%	0.4 ± 0.4%
SDA	35.0 ± 3.5%	27.8 ± 1.7%	1.3 ± 0.1%
ARA	6.8 ± 0.3%	0.8 ± 0.8%	0
LA	10.9 ± 1.5%	5.2 ± 0.6%	0.8 ± 0.3%
ALA	20.0 ± 1.1%	13.1 ± 0.5%	1.2 ± 0.2%
20:1n-9	18.2 ± 3.7%	3.8 ± 2.2%	5.3 ± 1.2%
EPA	8.8 ± 1.3%	0.9 ± 0.8%	0

[a]Exogenous fatty acids were supplied at a concentration of 250 μM. [b]Full-length cDNAs were cloned in the pYES2.1 vector and expressed in yeast. [c]Empty pYES2.1 vector. [d]Conversion level was calculated as [product/(substrate+product)] × 100. Data are expressed as mean ± SD. Data are representative of at least three experiments.

RESULTS

Isolation and Characterization of a Pythium irregulare Fatty Acid Condensing Enzyme. The sequence of a *P. infestans* elongase cDNA (GenBank accession number EA070845; called PinELO from hereon in) with activity on 18-carbon, Δ-6 desaturated fatty acids was used to design primers which allowed us to isolate a highly homologous *P. irregulare* cDNA, which we named PirELO (GenBank accession number GN042638). The full-length coding region of PirELO was 843 base pairs in length, giving a putative translation product of 281 amino acids, compared to the 278 amino acid product of the PinELO gene. The additional amino acids were located in the extreme C-terminal region of the PirELO protein. The two putative translation products had an identity level of 77%, and the coding regions of these two genes also shared approximately 77% identity at the nucleotide level.

As was expected on the basis of its homology to PinELO, PirELO was capable of elongating the Δ-6 desaturated 18 carbon fatty acids GLA and SDA when expressed in yeast (Table 1). Surprisingly, however, PirELO was also capable of elongating a number of other fatty acids, including EPA. When yeast cells carrying PirELO were fed EPA, a new peak with the same retention time as that of docosapentaenoic acid (22:5n-3) was observed (Figure 1A); the identity of this peak was confirmed as DPA using GC-MS analysis (Figure 1B). A number of condensing enzymes capable of elongating GLA are also able to elongate EPA, and we thought it likely that PinELO would also have this activity, given its high homology to PirELO. However, when PinELO was expressed in yeast, no detectable elongation of EPA occurred (Figure 1A). To investigate the cause of this difference, a number of chimeric proteins were constructed by substituting portions of PirELO into PinELO and vice versa.

Identification of Regions of PirELO Involved in the Elongation of 20 Carbon Fatty Acids. Chimeric proteins 1 and 2 represent fusions engineered at approximately the midpoint of PinELO and PirELO (Figure 2A). When the N-terminal half of the protein consisted of amino acids from PinELO and the C-terminal half amino acids from PirELO, conversion levels for GLA and EPA were similar to those observed with PirELO, suggesting that the amino acids required for EPA elongation were located in the C-terminal half of PirELO (Chimera 2, Figure 2A). Conversely, when the N-terminal half of the protein consisted of amino acids from PirELO and the C-terminal half of amino acids from PinELO (Chimera 1), the trace level of elongation observed was similar to that occurring with the empty pYES vector.

A series of 23 chimeric proteins were constructed to delineate the regions from these proteins that were required for the elongation of GLA and/or EPA. Figure 2B shows the chimeric proteins that allowed us to identify the regions of PirELO that were required for EPA extension. When only the last 56 amino acids originated from PirELO, only the GLA extension (as was typical for PinELO) was observed (Chimera 6, Figure 2B). However, when amino acids 137−225 from PirELO were substituted into the PinELO protein, the activity of the chimeric protein resembled PirELO, with EPA elongation occurring (Chimera 10, Figure 2B). Activities observed with chimeric proteins 13 and 14 indicated that substitution of amino acids 137−181 inclusive from PirELO was sufficient to achieve EPA elongation. Furthermore, although chimeric proteins 13 and 14 shared a high level of homology and GLA was elongated to DGLA more efficiently by chimeric protein 13 than by chimeric protein 14 (Figure 2B), further extension of DGLA to 22:3n-6 was only observed with chimeric protein 14 (data not shown). This was consistently observed with all chimeric proteins; only those chimeric proteins showing PirELO-like activity (i.e., extension of EPA) produced a detectable level of 22:3n-6 when cells were supplemented with GLA.

Chimeric protein 14 carries only a 45 amino acid region from PirELO, and of these 45 amino acids, only 11 are different between the two proteins. We attempted to further narrow down the region contributing to the elongation of EPA, as shown in Figure 2C. Substitution of only the first 22 amino acids from this region into PinELO resulted in a protein which did not extend EPA (Chimera 17, region A), while substitution of either the middle 11 (Chimera 19, region B) or the last 12 amino acids (Chimera 18, region C) allowed relatively low levels of EPA elongation (approximately 2−2.5%). Thus, amino acids from within both regions B and C appear to be required for EPA extension. Eight of the amino acids within regions B and C are variable between PinELO and PirELO.

Finally, chimeric protein 23, containing PirELO amino acids from regions B and C only, was constructed and expressed in yeast. With this construct, the conversion level with GLA reached slightly over 13% and reached over 5% with EPA, confirming that regions B and C were critical for EPA elongation.

Identification of Regions from PinELO Required for Activity on GLA. Since the chimeric protein consisting of the N-terminal half of PinELO and the C-terminal half of PirELO (Chimera 2, Figure 2A) represented a protein with activity similar to that of PirELO, it seemed likely that the reciprocal chimeric protein would have activity resembling that of PinELO. In fact, the reciprocal protein (Chimera 1, Figure 2A) was almost inactive. This implied that certain amino acids from the N-terminal half of PirELO are unsuitable for maintaining PinELO-type activity in the presence of the C-terminal PinELO amino acids. Therefore, we next considered which amino acids from PinELO were required to provide elongation of GLA without associated EPA elongation. Figure 2D shows the chimeric proteins which were diagnostic for determining which PinELO amino acids are required to produce an active protein capable of elongating GLA.

Substitution of only the first 55 amino acids from PirELO into the PinELO protein resulted in an inactive protein (Chimera 3), implying that amino acids from this N-terminal region of PinELO are necessary to produce an active protein in the presence of PinELO amino acids 137−278. Conversely, substitution of amino acids 225−281 from PirELO appeared to have little effect

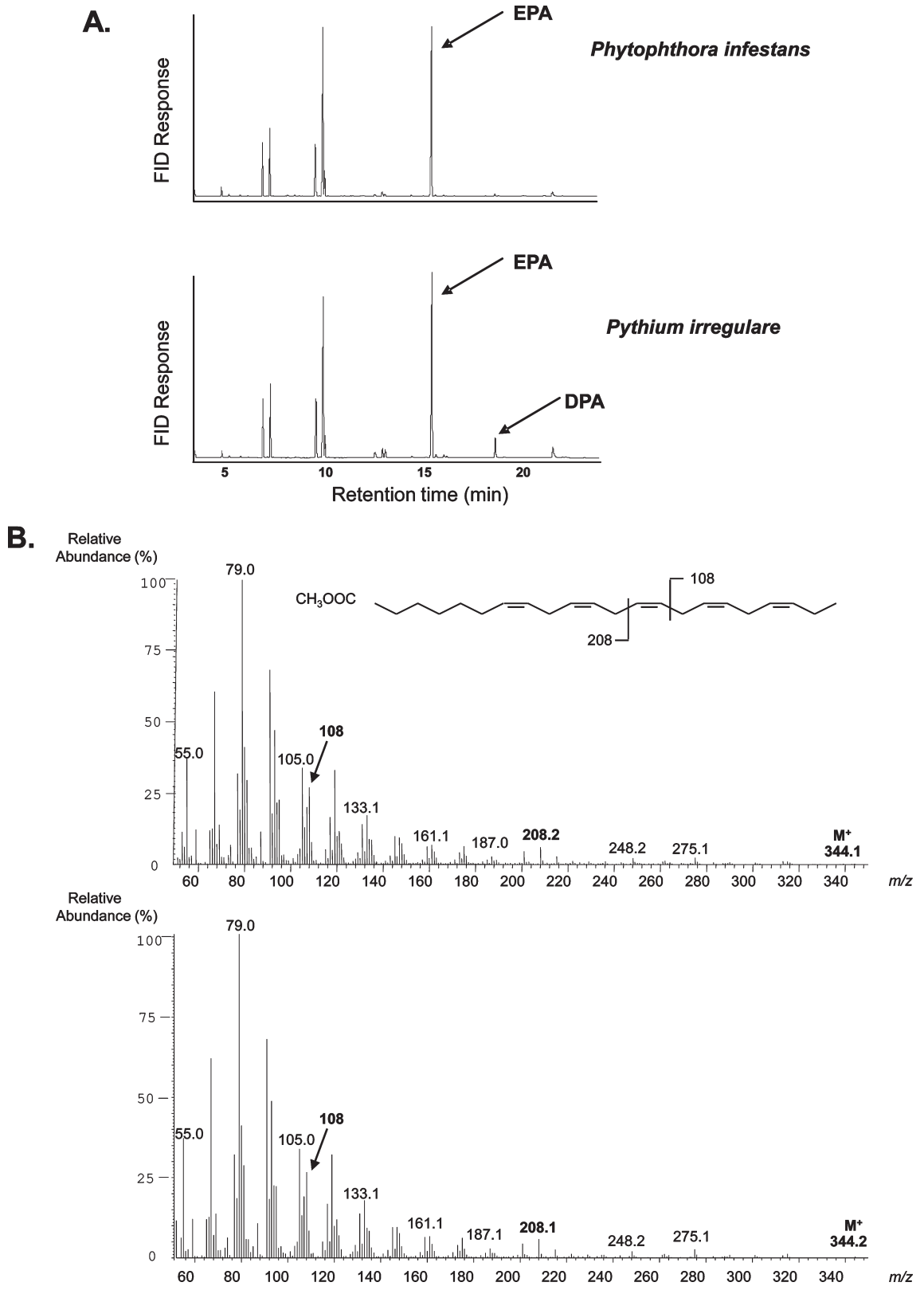

FIGURE 1: Elongase activities of PinELO and PirELO. (A) Expression of PinELO (*Phytophtera infestans*) and PirELO (*Pithium irregulare*) cDNAs in yeast. Exogenous EPA (20:5n-3) was supplied at a concentration of 250 μM. Chromatograms show the fatty acid methyl ester profiles of yeast expressing full-length elongase cDNAs from *P. infestans* and *P. irregulare* in pYES2.1. (B) Electron impact spectra generated by GC/MS for the fatty acid methyl esters from the additional peak found in the yeast sample expressing PirELO (top panel) compared to the known standard, DPA (22:5n-3) (bottom panel).

(Chimera 6), and GLA elongation reached over 15%, suggesting that the PirELO amino acids from the C-terminus are not involved in the observed inactivation of the PinELO enzyme. Similarly, substitution of PirELO amino acids 55−137 into PinELO produced a protein (Chimera 8) having activity comparable to that of PinELO, with GLA extension reaching over 18%, but with only trace amounts of EPA extension being detected. Therefore, PirELO amino acids 55−137 do not appear to be involved

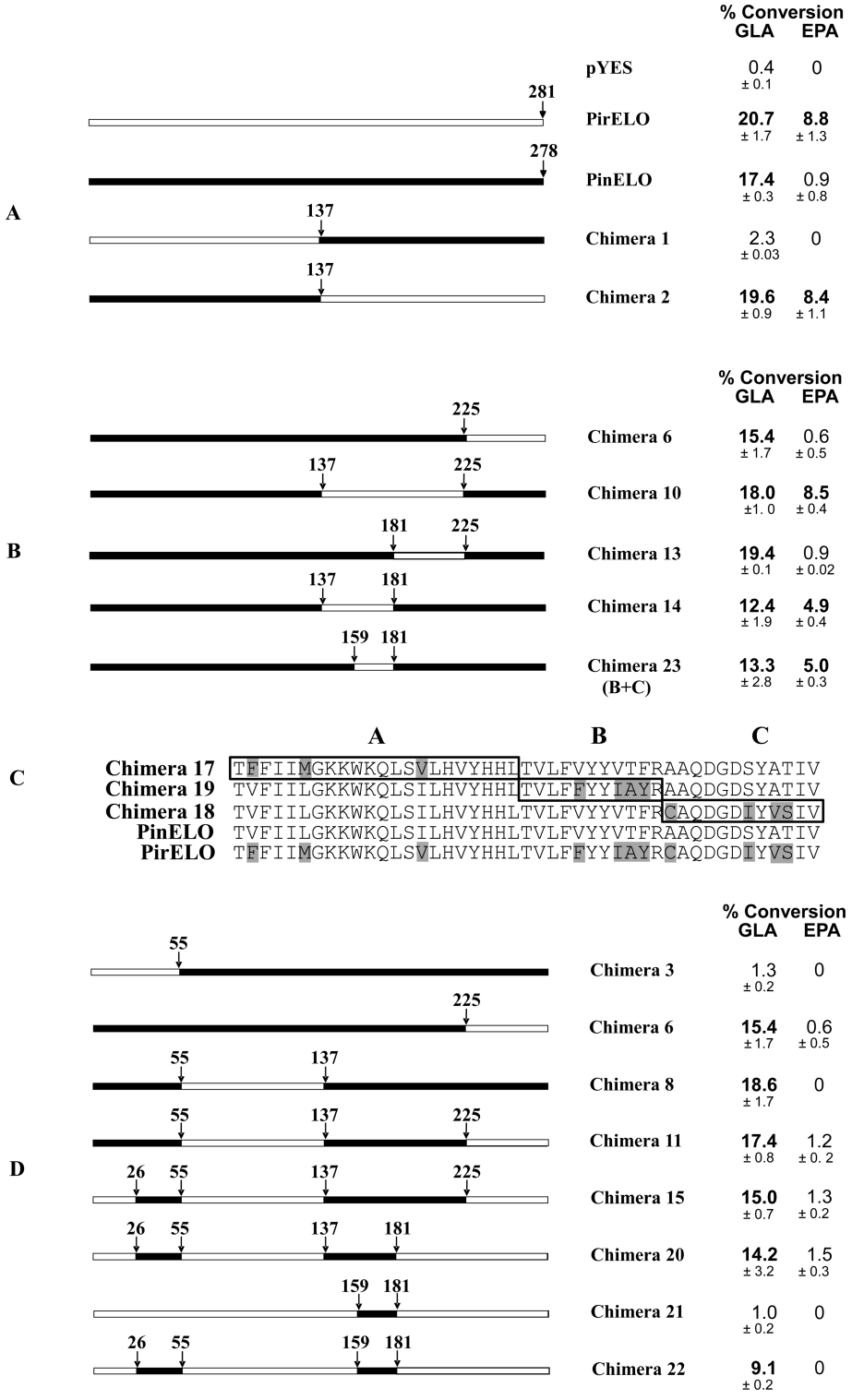

FIGURE 2: Elongation of GLA and EPA by chimeric proteins constructed between PinELO and PirELO. Arrows indicate the amino acid at the position at which chimeras were created. Exogenous fatty acids were supplied at a concentration of 250 μM. Conversion levels are representative of at least three experiments. (A) Elongation of GLA and EPA by PirELO, PinELO, and chimeric proteins engineered at the midpoint of these elongases. (B) Chimeras diagnostic for determining the regions involved in the elongation of EPA by PirELO. (C) Amino acids required for EPA elongation. Shaded amino acids represent amino acids from PirELO that differ from the amino acids at the corresponding positions in PinELO. Only amino acids 137−181 of each protein are shown; the remaining amino acids of chimeras 17, 18, and 19 are identical to those found in PinELO. (D) Chimeras diagnostic for determining the regions of PinELO required for GLA elongation.

in the observed inactivation of PinELO. This was confirmed by substituting these PirELO amino acids into chimeric protein 6, producing chimeric protein 11, in which PinELO-type activity was maintained. While the substitution of PirELO amino acids 26−55 into chimeric protein 11 produced an almost inactive protein (not shown), substitution of amino acids 1−26 resulted in an enzyme showing GLA elongation but very little EPA elongation (Chimera 15), and the further substitution of PirELO amino acids 181−225 into chimeric protein 15, producing chimeric protein 20, did not seem to affect activity. Although bifunctional

elongation of EPA and GLA can be achieved with only amino acids 137−181 from PirELO in a PinELO background (Chimera 14, Figure 2B), it appears that the presence of this region from PinELO necessitates the further inclusion of PinELO amino acids 26−55 to produce an active enzyme. In the region between positions 26−55, only 5 amino acids from PinELO are different from those in PirELO. One or more of these five amino acids must be critical for the GLA elongation observed in PinELO.

Finally, substitution of only amino acids 159−181 from PinELO (representing regions B and C described above) into PirELO resulted in an inactive protein (Chimera 21, Figure 2D). When PinELO amino acids 26−55 were substituted into chimeric protein 21, creating chimeric protein 22, activity was partially restored, and the GLA conversion level increased to about 9%. Although substitution of the PinELO amino acids from region BC results in inactivation of the PirELO protein and the addition of the 18 amino-acid region from near the N-terminal region results in regained activity, the level of activity did not reach that observed with chimeric protein 20, and one or more of the remaining three amino acids that are variable between chimeric proteins 20 and 22 may also have an influence on activity levels.

Similarly, differences in activity have also been observed between highly homologous condensing enzymes from yeast. The yeast elongase Sur4p is capable of processive elongation of 20 carbon substrates to produce 26 carbon fatty acids, whereas the highly homologous elongase Fen1p elongates acyl-CoA substrates to 22 and to a lesser extent 24 carbons (*21*). A six-amino acid region was determined to be important in the processive elongation observed with Sur4p, and particularly, the location of a lysine residue within this amino acid block determined the chain length of fatty acids produced by these proteins. However, the critical blocks of amino acids identified in PirELO and PinELO are found at different positions than the amino acid stretch that is critical for determining chain length in the yeast ELO protein. Pairwise alignments of PinELO or PirELO with the yeast elongases indicate that about 19−21% of amino acids are identical and that about 30% of amino acids are conserved, but the highly conserved amino acids are generally grouped in specific regions. In alignments between the yeast and *Pythium* or *Phytophthora* proteins, a gap in the PinELO and PirELO proteins occurs where the critical 17 amino acid region from Sur4p is found, and few amino acids similar to amino acids in region BC are found in the corresponding region of Sur4p (data not shown).

Topology Analysis of PinELO and PirELO and Comparison with Yeast Enzymes. Topology analysis of the yeast elongase Sur4p indicated that the 17 amino acid region of Sur4p including the lysine residue responsible for determining product chain length was located near the luminal end of the sixth transmembrane helix (*21*). In the mammalian ELOp family, four highly conserved sequences (KXXEXXDT, HXXHH, HXXMYXYY, and TXXQXXQ) (*27*) are found at the cytosolic-proximal regions of transmembrane helices 3, 4, 5, and 6, respectively. These motifs are thought to influence enzyme activity, and Denic and Weissman (*21*) suggest that these regions may be organized into a catalytic ring. To determine the relative location of the critical sites in PinELO and PirELO, topology predictions for these enzymes were performed using the Octopus program (http://octopus.cbr.su.se/). Results from this program were similar to those obtained using the MEMSAT (*25*) and PRODIV-TMHMM (*26*) prediction methods (Supporting Information Figure 1). These predictions indicated that the BC region with a role in the elongation of fatty acids such as EPA and DGLA to their 22C counterparts was located near the luminal regions of transmembrane helices 4 and 5 (Figure 3). The additional 18-amino acid region in PinELO that is critical for elongation of GLA was located at the luminal region near transmembrane helix 1. The luminal−proximal location of residues influencing substrate specificity and product length in the PinELO/PirELO enzymes and in the Sur4p/Fen1p enzymes provides further evidence that the three-dimensional structures of these enzymes are important in determining substrate specificity and in regulating the final length of products.

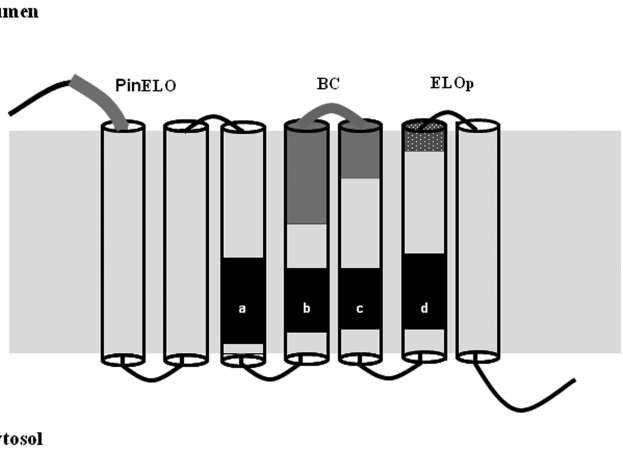

FIGURE 3: Predicted topology model for PirELO. The approximate locations of region BC and the 18 amino acid region from PinELO required for GLA elongation in the presence of PinELO amino acids from BC are indicated by gray boxes. The stippled gray box indicates the approximate location of a region found in the yeast condensing enzymes Sur4p and Fen1p that is critical for determining substrate length (*21*), with the position of lysine residues within this region influencing progressive elongation of substrates. Black boxes indicate the positions of motifs which are conserved among mammalian ELO-type elongases (*27*). a, KXXEXXDT; b, HXXHH; c, HXXMYXYY; d, TXXQXXQ. Motif a (KXXEXXDT) is not perfectly conserved in PirELO.

DISCUSSION

Here, we identified a condensing enzyme from *Pythium irregulare* that shared a high level of homology with a similar enzyme from *Phytophthora infestans*, yet differed in substrate specificity and final fatty acyl-CoA product length. This presented an ideal opportunity for investigating how these differences were conditioned at the amino acid level. Although a wide array of condensing enzymes having vastly different substrate specificities have been isolated, such enzymes generally differ substantially in amino acid sequence. For example, the yeast ELOps, which elongate saturated and monounsaturated fatty acids, share less than 30% identity with the polyunsaturated fatty acid elongating enzymes PinELO and PirELO. With such large differences at the protein level, the simple substitution of a region from one elongase into another often does not give us the expected corresponding change in activity. One reason for this is demonstrated by the results obtained here; although substitution of the BC region from PirELO into PinELO conditioned the elongation of EPA, substitution of the same region from PinELO into PirELO resulted in an inactive protein, and PinELO amino acids from another region were also required to act in concert with this region for the restoration of enzyme activity. Thus, while it may be possible to identify motifs indicative of a particular activity, substitution of these motifs into another enzyme will not necessarily result in the same activity.

Condensation of a fatty acyl-CoA substrate with malonyl-CoA produces a chain that is two carbons longer than the original acyl-CoA. Denic and Weissman (*21*) found that while neither of the yeast condensing enzymes Sur4p nor Fen1p was able to accept fatty acids with a length greater than 20 carbons, Sur4p was able to produce fatty acids of 26 carbons in length. This was thought to be due to the processive activity of Sur4p; that is, more than one reaction cycle could occur per association/disassociation with the acyl-CoA substrate, leading to the addition of four or six carbons. Conversely, the BC region from PirELO appears to influence the ability of this enzyme to accept 20 carbon fatty acids such as EPA, as well as influencing the two step elongation of 18 carbon fatty acids such as SDA and GLA to 22:3n-6 and 22:4n-3. On the basis of the experiments performed here, it was not possible to determine if the multistep elongation of GLA to 22:4n-6 is actually due to PirELO having a degree of processive activity or if DGLA is released by the elongase complex before being used as a substrate in a second round of elongation.

Perhaps even more interesting than the ability of the amino acids from region BC of PirELO to influence extension of longer-chain fatty acids when substituted into PinELO is the drastic loss of elongase activity conditioned by the substitution of the corresponding fatty acids from PinELO into PirELO, a loss in activity which is greatly ameliorated by also substituting five PinELO-specific amino acids from near the luminal end of transmembrane helix 1 into the protein. The influence of amino acid motifs from the luminal or luminal−proximal regions of PirELO and PinELO on substrate specificity and final product length provides support for the hypothesis of a molecular caliper mechanism for determining product length put forward by Denic and Weissman (*21*), who suggested that the acyl-CoA fatty acid substrate extends across the membrane during elongation, with the CoA group oriented toward the cytosol, in proximity with a putative catalytic ring, while the methyl end is oriented toward the lumen during elongation. The results observed here, in combination with Denic and Weismann's results, suggest that amino acids toward the luminal end of the protein may also form an interacting ring structure involved in determining the chain lengths of substrates and products. However, the homology between the unsaturated/monounsaturated versus polyunsaturated elongating enzymes is quite limited; in a comparison of 256 elongase homologues from 56 genomes, enzymes capable of elongating monounsaturated and saturated fatty acids, such as the yeast proteins, fell quite clearly into a separate clade than proteins capable of elongating polyunsaturated fatty acids (*28*). This low homology, along with the bending inherent in the structure of polyunsaturated fatty acids, may suggest why the exact location of amino acids involved in controlling chain lengths are quite different between the polyunsaturated elongases and the yeast enzymes.

The results from the experiments described here using two very similar elongases should provide a valuable starting point when considering the rational design of condensing enzymes with activity on polyunsaturated fatty acids.

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SUPPORTING INFORMATION AVAILABLE

Primer sequences and topology predictions. This material is available free of charge via the Internet at http://pubs.acs.org.

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