The variant 'his-box' of the C18-Δ9-PUFA-specific elongase IgASE1 from *Isochrysis galbana* is essential for optimum enzyme activity

Baoxiu Qi*, Thomas C.M. Fraser, Claire L. Bleakley, Elisabeth M. Shaw, A. Keith Stobart, Colin M. Lazarus

School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK

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Abstract IgASE1, a C18-Δ9-polyunsaturated fatty acid-specific fatty acid elongase component from *Isochrysis galbana*, contains a variant histidine box (his-box) with glutamine replacing the first histidine of the conserved histidine-rich motif present in all other known equivalent proteins. The importance of glutamine and other variant amino acid residues in the his-box of IgASE1 was determined by site-directed mutagenesis. Results showed that all the variation in amino acid sequence between this motif in IgASE1 and the consensus sequences of other elongase components was required for optimum enzyme activity. The substrate specificity was shown to be unaffected by these changes suggesting that components of the his-box are not directly responsible for substrate specificity.

Key words: Fatty acid elongating activity; Site-directed mutagenesis; Specificity; *Isochrysis galbana*

1. Introduction

Much interest is currently being focused on the very long chain polyunsaturated fatty acids (PUFAs) arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) because of their involvement in early human retinal and brain development and disease prevention [1–5]. Their role as precursors of eicosanoids, a family of biological effectors involved in inflammatory responses, regulation of blood pressure and blood clotting, has also been established [6]. They are obtained either directly from the diet or synthesised from the ω6 and ω3 essential fatty acids linoleic acid (LA, C18:2n-6, Δ9,12) and α-linolenic acid (ALA, C18:3n-3, Δ9,12,15), respectively, by alternating desaturation and elongation reactions. Fatty acid elongation is a four-step process involving condensation, reduction, dehydration and a second reduction reaction, with substrate specificity residing in the rate-limiting condensation reaction [7]. This means that elongases with different substrate specificities can be assembled using variable condensing components and common dehydration and reduction components. Studies on the mouse *LCE* gene indicated that it encodes the condensation component of a long-chain fatty acid elongase [8], and related genes have been isolated from a number of sources including yeast [9,10], *Mortierella alpina* [11] human [12], nematodes [13] and moss [14]. The characterisation of a C18-Δ9-specific PUFA elongase condensation component from *Isochrysis galbana* [15], together with the isolation of Δ8 and Δ4 desaturases from *Euglena* [16] and *Thraustochytrium* [17], suggested that EPA and DHA may be synthesised from ALA by a so-called ω3-Δ8 pathway in these organisms [15]. In this pathway, which is shown in Fig. 1, ALA is first elongated by a C18-Δ9-specific fatty acid elongase to C20:3n-3 (ω3-eicosatrienoic acid (EtrA)), with further desaturations/elongations yielding C20:5n-3 (EPA) and finally C22:6n-3 (DHA).

Alignment of the amino acid sequences of fatty acid elongase condensation components reveals the presence of a highly conserved histidine-rich motif, HXXHH ('his-box') (Fig. 2). In IgASE1, however, glutamine (Q) replaces the first histidine in the his-box, and the significance of this substitution is unclear [15]. Similarly, alignment of most membrane-bound fatty acid desaturases reveals three highly conserved his-boxes, and these have been implicated in the binding of di-iron, a requirement for catalytic activity [18]. Site-directed mutagenesis of these histidine residues resulted in enzyme inactivation [19,20]. In the so-called 'front-end' desaturases from plants, animals and fungi, the first histidine in the third his-box is also substituted with glutamine. Site-directed mutagenesis of this glutamine in the Δ6-fatty acid desaturase from borage resulted in a complete loss of enzyme activity even when it was replaced by histidine [21].

We have now assessed the importance of the conserved histidine box present in the elongase component, particularly the H to Q substitution found in IgASE1 of *I. galbana*. Using a polymerase chain reaction (PCR)-based site-directed mutagenesis strategy the glutamine in the histidine box of IgASE1 was converted to histidine, alanine or phenylalanine. Further changes generated the HVYHH sequence found in GLELO1 from *Mortierella alpina*, which is specific for elongating the two Δ6-desaturated PUFAs, γ-linolenic acid (GLA, C18:3n-6) and stearidonic acid (STA, C18:4n-3) [11].

2. Materials and methods

2.1. Mutagenesis by PCR

The amplification of the IgASE1 coding region from an *I. galbana*

*Corresponding author. Fax: (44)-117-9257374.
E-mail address: baoxiu.qi@bristol.ac.uk (B. Qi).

Abbreviations: PUFA, polyunsaturated fatty acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; STA, stearidonic acid; EDA, ω6-eicosadienoic acid; EtrA, ω3-eicosatrienoic acid

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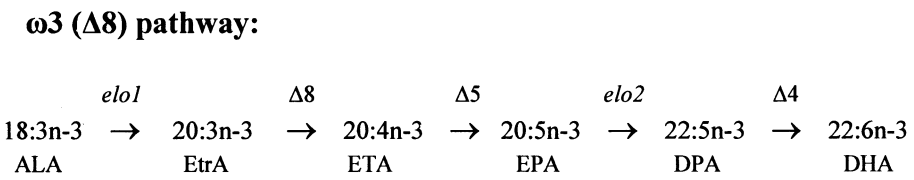

Fig. 1. Biosynthesis of EPA and DHA via the ω3 (Δ8) desaturation pathway by some microalgae. In this pathway, ALA is first elongated by a C18-Δ9-specific fatty acid elongase (elo1) to C20:3n-3 (EtrA). A Δ8 desaturase is required to add a double bond at the Δ8 position of the carbon chain to generate C20:4n-3 (ETA). Further desaturation by a Δ5 desaturase results in EPA (20:5n-3). DHA (C22:6n-3) is the product of one more elongation step (elo2) plus a Δ4 desaturation step.

cDNA library and its cloning into plasmid pCR2.1-TOPO (Invitrogen) is described in Qi et al. [15]. To change the his-box from QAFHH to HAFHH, first round PCRs were carried out with *Pfu* polymerase (Promega) using primer pairs M13 reverse (universal primer located on the vector) with GlnHisFor (5′-C TCC TTT CTC CAT GCC TTC CAC CAC-3′, and M13 forward with GlnHisRev (5′-GTG GTG GAA GGC ATG GAG AAA GGA G-3′). A second round PCR, containing 1 μl of each of the gel-purified first-round products, M13 forward and reverse primers and the Expand High Fidelity enzyme (Roche), was used to assemble the mutated IgASE1 coding region. The same techniques were used to change glutamine to alanine (QAFHH→AAFHH) or phenylalanine (QAFHH→FAFHH), alanine/phenylalanine to valine/tyrosine (QAFHH→QVYHH) and glutamine/alanine/phenylalanine to histidine/valine/tyrosine (QAFHH→HVYHH). The primers used are listed in Table 1. All PCR reactions were carried out under the following conditions: one initial denaturation step of 94°C for 3 min; 10 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 90 s; 20 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 93 s (increasing by 3 s in each successive cycle); one final extension step of 72°C for 6 min.

Second round PCR products were cloned using the TOPO TA cloning system (Invitrogen) and the desired base changes confirmed by sequencing. Modified IgASE1 coding regions were excised from the pCR2.1-TOPO vector using *Kpn*I and *Eco*RI and ligated into the corresponding restriction sites of the yeast expression vector pYES2 (Invitrogen), downstream of the *GAL1* promoter. Subsequent yeast transformation and feeding experiments were performed according to Qi et al. [15].

2.2. Fatty acid analysis

Yeast cells were pelleted, washed and dried under a stream of N_2. Total fatty acids were extracted and transmethylated with methanolic HCl. The fatty acid methyl esters were analysed by gas chromatography (GC) on a 30 m×0.25 mm fused silica DB-23 capillary column (J&W Scientific) using heptadecanoic acid as internal standard and quantified by flame ionisation detection (FID). The chromatograph was programmed for an initial temperature of 140°C for 5 min followed by a 20°C/min temperature ramp to 185°C and a secondary ramp of 1.5°C/min to 220°C. The final temperature was maintained for 2 min. Injector and detector temperatures were maintained at 230°C and 250°C respectively. The initial head pressure of the carrier gas (He) was 90 kPa; a split injection was used.

3. Results and discussion

3.1. Functional analysis of wild-type IgASE1

Plasmid pY2ASE1 contains the IgASE1 open reading frame (ORF) under the control of the *GAL1* promoter in the yeast expression vector pYES2. Transformed yeast cells harbouring this plasmid were grown on minimal medium supplemented with LA and ALA to compensate for the lack of endogenous substrates for the C18-Δ9-specific elongase. After 48 h the fatty acid elongation products ω6-eicosadienoic acid (EDA, 20:2n-6) and EtrA (20:3n-3) accumulated to 13.6 and 14.8 mol% of total fatty acids, representing some 55 and 48% conversion of C18:2n-6 and C18:3n-3 to C20:2n-6 and C20:3n-3, respectively (Table 2). These data are consistent with our previous assays [15], where we also showed no elongase activity with GLA (20:3n-6) and clearly demonstrate, therefore, that the *IgASE1* gene encodes a C18-Δ9-specific PUFA elongating activity.

3.2. Mutagenesis of the IgASE1 his-box

Glutamine in the IgASE1 his-box was replaced by histidine, alanine or phenylalanine, and the mutant proteins were assayed for activity in transformed yeast. Table 3 shows that all the substitutions resulted in lower elongase activity, although complete inactivation was never achieved. The greatest activity was obtained with the alanine substitution, where more than 70% of the original enzyme activity was retained. The phenylalanine substitution had the lowest (but still measurable) activity, whilst the histidine substitution resulted in an activity that was some 50% of the control value. The glutamine residue in the histidine box thus appears to be essential for optimum enzyme catalysis, although its substitution did not result in complete enzyme inactivation. This is in contrast to the effect of similar mutagenic changes to the glutamine residue in the third his-box of a Δ6 desaturase from borage, which resulted in complete loss of enzyme activity [21]. Stepwise mutagenesis was also performed to change residues in the IgASE1 his-box (QAFHH) to match those found in the consensus his-box sequence of several other PUFA-specific elongases, including that of the GLA-specific elongase GLELO1 from *Mortierella* (HVYHH) (Fig. 2). The double mutant, in which the middle two amino acids alanine and phenylalanine in the histidine box were converted to valine and tyrosine but with the glutamine remaining unchanged (QAFHH→QVYHH), reduced the activity to less than 30% of the wild-type activity. The triple mutant, in which the first three amino acids in the histidine box, QAF, were replaced by HVY (QAFHH→HVYHH), had less than 10% of the wild-type

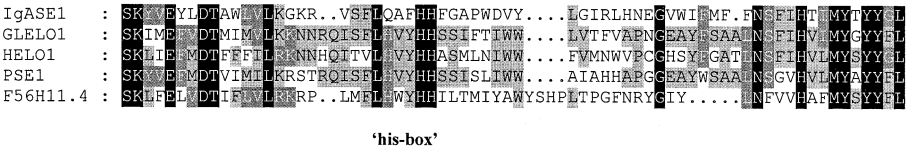

Fig. 2. Alignment of 'his-box' regions of the predicted protein sequence of *I. galbana IgASE1* [15] with sequences predicted from other known PUFA-specific elongating activity genes. *GLELO1* is from *Mortierella alpina* [11]; *HELO1* [12] is from human; *PSE1* is from *Physcomitrella patens* [14]; *F56H11.4* is from *Caenorhabditis elegans* [13].

Table 1
Oligonucleotide primers used in PCR reactions to construct site-directed mutants of IgASE1/pCR2.1-TOPO

Mutation	Oligonucleotide and amino acid sequences	His-box
Q→H	5'-C TCC TTT CTC CA**T** GCC TTC CAC CAC-3' S F L ***H*** A F H H	HAFHH
Q→A	5'- C TCC TTT CTC **GC**G GCC TTC CAC CAC-3' S F L ***A*** A F H H	AAFHH
Q→F	5'-GG GTC TCC TTT CTC **TTC** GCC TTC CAC CAC TTT G-3' V S F L ***F*** A F H H F	FAFHH
AF→VY	5'-TTT CTC CAG GTC **TA**C CAC CAC TTT G-3' F L Q ***V*** ***Y*** H H F	QVYHH
QAF→HVY	5'-TTT CTC CA**T** GT**C** TAC CAC CAC TTT G-3' F L ***H*** ***V*** ***Y*** H H F	HVYHH

The changed nucleotide bases are indicated by bold type, and the corresponding amino acids are indicated by bold, italic type and underlined. The mutated ORFs were confirmed by sequencing. The plasmids were cloned into pYES2 and the resulted constructs were used for yeast transformation and feeding experiments.

activity. These results suggest that interactions between the glutamine and its adjacent amino acids are important for maximising enzyme activity. The various his-box-mutated constructs were also expressed in the presence of numerous other exogenous fatty acid substrates including GLA, STA and EPA, but no elongation products were observed (data not presented).

The results suggest that the his-box is important for catalysis and that all the differences between the his-boxes of other elongases and IgASE1 are required for optimum enzyme activity. However, the elements required to regulate substrate specificity remain to be identified.

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Table 2
Elongation products of different fatty acid substrates supplied to yeast cells transformed with the empty vector pYES2 and pY2ASE1

Fatty acid	Mol% of total fatty acids					
	pYES2			pY2ASE1		
	−substrate	+LA (18:2n-6)	+ALA (18:3n-3)	−substrate	+LA (18:2n-6)	+ALA (18:3n-3)
16:0	27.0	27.0	24.7	24.5	23.2	22.2
16:1n-9	41.7	35.1	30.1	43.4	32.3	24.1
18:0	6.0	5.7	5.0	5.8	5.3	5.0
18:1n-9	25.3	22.3	18.4	26.2	14.6	18.1
18:2n-6*	–	10.0	–	–	11.0	–
18:3n-3*	–	–	21.8	–	–	16.3
20:2n-6	–	–	–	–	13.6	–
20:3n-3	–	–	–	–	–	14.8
Elongation (%)	0	0	0	0	55.3	47.6

Exogenous fatty acids supplied as substrates for elongation are indicated by an asterisk. The values given are expressed as mol% of total fatty acid methyl esters identified by GC and FID. In the case of elongated substrates, this is also expressed as a per cent elongation (product/product+substrate×100). Expression of the *IgASE1* transgene was induced by the addition of galactose to yeast cultures. Only C18 substrates with a double bond at the Δ9 position were elongated by IgASE1. All values are the means of triplicates from three separate experiments.

Table 3
Relative conversion of exogenously supplied LA (C18:2n-6) and ALA (C18:3n-3) by wild-type and mutant IgASE1 expressed in yeast

Mutation	IgASE1 (QAFHH)		GlnHis (Q→H)		GlnAla (Q→A)		GlnPhe (Q→F)		HeloGln (AF→VY)		HeloHis (QAF→HVY)	
Exogenous fatty acids	+18:2	+18:3	+18:2	+18:3	+18:2	+18:3	+18:2	+18:3	+18:2	+18:3	+18:2	+18:3
Conversion (%)	55	48	31	20	42	33	3.3	1	15	8.4	5.4	2.7
% IgASE1	100	100	61	42	76	69	6	2.1	27	17.5	9.8	5.6