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Characterization of an isopentenyl diphosphate isomerase involved in the juvenile hormone pathway in *Aedes aegypti*

Miguel E. Diaz, Jaime G. Mayoral, Horacio Prietap, Marcela Nouzova, Crisalejandra Rivera-Perez, Fernando G. Noriega

Department of Biological Sciences, Florida International University, 11200 SW 8th St., Miami, FL 33199, USA

**Abstract**

Isopentenyl diphosphate isomerase (IPPI) is an enzyme involved in the synthesis of juvenile hormone (JH) in the corpora allata (CA) of insects. IPPI catalyzes the conversion of isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP); afterward IPP and DMAPP condense in a head-to-tail manner to produce geranyl diphosphate (GPP); this head-to-tail condensation can be repeated, by the further reaction of GPP with IPP, yielding the JH precursor farnesyl diphosphate. An IPPI expressed sequence tag (EST) was obtained from an *Aedes aegypti* corpora-allata corpora cardiaca library. Its full-length cDNA encodes a 244-aa protein that shows a high degree of similarity with type I IPPIs from other organisms, particularly for those residues that have important roles in catalysis, metal coordination and interaction with the diphosphate moiety of the IPP. Heterologous expression produced a recombinant protein that metabolized IPP into DMAPP; treatment of DMAPP with phosphoric acid produced isoprene, a volatile compound that was measured with an assay based on a solid-phase micro extraction protocol and direct analysis by gas chromatography. *A. aegypti* IPPI (AaIPPI) required Mg\(^{2+}\) or Mn\(^{2+}\) but not Zn\(^{2+}\) for full activity and it was entirely inhibited by iodoacetamide. Real-time PCR experiments showed that AaIPPI is highly expressed in the CA. Changes in AaIPPI mRNA levels in the CA in the pupal and adult female mosquito corresponded well with changes in JH synthesis (Li et al., 2003). This is the first molecular and functional characterization of an isopentenyl diphosphate isomerase involved in the production of juvenile hormone in the CA of an insect.

1. Introduction

Isoprenoids are a large family of organic compounds derived from C5 isoprene units. They are present in Archaea, Bacteria and Eukarya and have essential roles in signal transduction, electron transport, photosynthesis and regulation of development and reproductive cycles (Sacchettini and Poulter, 1997; Ramos-Valdivia et al., 1997; Satterwhite, 1985; Spurgeon and Porter, 1981; Bonano et al., 2001). An important building block of all isoprenoids is the compound isopentenyl pyrophosphate (IPP) (Ramos-Valdivia et al., 1997). Almost all organisms synthesize IPP by the condensation of three units of acetyl-CoA through the mevalonate pathway (MVA) (Ramos-Valdivia et al., 1997). In contrast, in some prokaryotes and plant plastids, IPP production is accomplished via the 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) (Rohmer, 1999).

The synthesis of isoprenoids includes the conversion of IPP to the electrophile dimethylallyl pyrophosphate (DMAPP), catalyzed by isopentenyl diphosphate isomerase (IPPI). IPPI generates DMAPP by the isomerization of the carbon–carbon double bond of IPP via the stereoselective antarafacial transposition of hydrogen (Durbecq et al., 2001). There are two types of IPPIs, type I is found in almost all eukaryotes while type II has only been observed in prokaryotes (Ramos-Valdivia et al., 1997). The optimal functioning of IPPI type I requires a divalent metal cation (Mg\(^{2+}\) or Mn\(^{2+}\)) (Durbecq et al., 2001). Zinc has also been identified as an essential cofactor for the activity of recombinant IPPI type I from *Escherichia coli* (Carrigan and Poulter, 2003). Type II IPPI requires a reduced flavin coenzyme in addition to a divalent metal cation (de Ruyck et al., 2008).

In the corpora allata (CA) of insects, IPPI is involved in the synthesis of juvenile hormone (JH) (Bellés et al., 2005; Goodman and Granger, 2005); IPP and DMAPP can condense in a head-to-tail manner to produce geranyl diphosphate (GPP). This type of head-to-tail condensation can be repeated by the further reaction...
of GPP with IPP, yielding the JH precursor farnesyl diphosphate (Goodman and Granger, 2005). In insect species, IPPIs have been only partially characterized from extracts of the silkworm Bombyx mori larva (Koyama et al., 1985). We cloned an IPPI expressed in the CA of the mosquito Aedes aegypti (Noriega et al., 2006). Heterologous expression produced a recombinant protein that metabolizes IPP into DMAPP. The protein requires Mg2+ or Mn2+ but not Zn2+ for full activity and is entirely inhibited by alkylation of the catalytic cysteine residue by iodoacetamide. Real time PCR experiments showed that IPPI mRNA levels in the CA match the changes in JH synthesis.

2. Material and methods

2.1. Insects

A. aegypti of the Rockefeller strain were reared at 28 °C and 80% relative humidity under a photoperiod of 16 h light: 8 h dark. Mated adults were offered a cotton pad soaked in 3% sucrose solution. The cotton pad sucrose-fed adults are referred to as sugar fed. Four-day-old female mosquitoes were fed porcine blood equilibrated to 37 °C. Adenosine triphosphate was added to the blood meal to a final concentration of 1 mM immediately before use (Noriega et al., 1999).

2.2. Chemicals

Isoprene was purchased from Sigma–Aldrich (St Louis, MO). IPP and DMAPP were obtained from Echelon Biosciences (Salt Lake City, UT).

2.3. Identification of the A. aegypti IPP isomerase cDNA

An IPPI expressed sequence tag (EST) was obtained from an A. aegypti corpora-allata + corpora cardiaca library, constructed and sequenced as previously described (Noriega et al., 2006). The IPPI EST sequence was queried against the A. aegypti database at VectorBase (Lawson et al., 2009); it revealed a single identical sequence (Accession number: AAEL06144). The A. aegypti IPPI (AaIPPI) cDNA was PCR-amplified from cDNA obtained from the thorax of mosquitoes (containing the corpora allata) using the following two primers:

Forward: AaIPPI-F, 5'-CATATGTCTCTTCTGGCTCGCTT-3'
Reverse: AaIPPI-R, 5'-GCCGCCGTCAAAATCTGTFCAGATTCTCGTTG-3'

2.4. Expression and purification of recombinant AaIPPI

The coding region of the AaIPPI cDNA was ligated into the pET-28a(+) expression vector (Novagen, Gibbstown, NJ). E. coli strain BL21 (DE3) were transformed with the construct and expressed as previously described (Mayoral et al., 2009). Recombinant protein containing a C-terminal His-tag was purified with a cobalt column (Pierce, Rockford, IL) and desalted with a PD-10 column (Amersham, Pharmacia Piscataway, NJ) (Mayoral et al., 2009). The purified protein was concentrated (225 ng/μL) using Centricon YM-10 centrifugal filters (Millipore, Billerica, MA), aliquoted and stored at −20 °C until use.

2.5. IPPI enzymatic assay

IPPI activity was determined by conversion of DMAPP to isoprene by treatment with phosphoric acid, followed by gas chromatographic (GC) analysis of isoprene (Fisher et al., 2001; Bruggemann and Schmitzler, 2002) (Fig. 1). We modified previously described methods by using a solid-phase micro extraction (SMPE) protocol to adsorb the volatile isoprene for direct GC analysis. A sample containing 93 μL of assay buffer (0.4 M Tris, 1 mM DTT, 10 mM MgCl2, pH 8.0), 5 μL of IPP (1 μg/μL) and 2 μL of the recombinant AaIPPI (200 ng/μL) was incubated at 35 °C for 1 h and the reaction was terminated by the addition of 30 μL of phosphoric acid (14.8 M). After incubation the solution was transferred to a 2-mL glass vial sealed with a silicone septum vial (Agilent) and the needle of the holder carrying the SPME fiber was introduced through the septum. The SPME holder and the 30 μm divinylbenzene-carboxen-polydimethylsiloxane (DVD-CAR-PDMS) fibers used to absorb isoprene were supplied by Supelco (Belmont, PA). The vial was placed on a heating block at 50 °C and the fiber was exposed to the atmosphere in the vial (Supplementary Fig. 1). After 1 h of incubation at 50 °C, the fiber was retracted into the needle. The holder was removed and transferred to the GC equipment. The needle was then introduced into the injection port of the GC equipment preheated to 200 °C; isoprene was immediately desorbed from the fiber and the GC run started.

GC determinations of isoprene were performed on a Trace Gas Chromatography Ultra apparatus (Thermo Electron Corporation, Waltham, MA) equipped with a non-polar capillary column DB-5MS (30 m × 0.25 mm i.d.; film thickness 0.25 μm) (Agilent, Santa Clara, CA) and a flame ionization detector. The output was recorded using a ChromQuest version 4.1 data system. Helium was employed as the gas carrier at a flow rate of 1 mL/min. The injector and detector temperatures were 200 °C and 230 °C, respectively. The column oven temperature was isothermally held at 30 °C. The amounts of isoprene detected were expressed as GC peak areas.
2.6. RNA extraction and Real Time-PCR

Corpora allata—corpora cardiac complexes (CA—CC) (20 pairs per replicate) were dissected in a drop of sterile DNA-RNAse free phosphate buffered saline (PBS). Total RNA was isolated using RNA-binding glass powder as previously described (Noriega and Wells, 1993). RNA samples were treated with RNAse I using DNA-free™ kit (Ambion, Austin, TX, US) according to the manufacturer recommendations. Reverse transcription was carried out using the SuperScript™ III first strand synthesis kit. Real-time PCR was performed in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, US) using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems). The primers and probes for the house keeping gene 60S ribosomal protein rpL32 (Accession number: AAELO03396) and the AaIPPI gene are included in Supplementary Table 1. Primer/probes were synthesized by Applied Biosystems and reactions were carried out in 20 μl volume according to the manufacturer’s recommendations for Custom TaqMan® Gene Expression Assays. Reactions were run in triplicate using 1 μl of cDNA per reaction. Standard curves to quantify relative gene copy number were made from ten-fold serial dilutions of plasmids containing rpL32 or AaIPPI (from 300,000 to 3 copies of a plasmid per reaction). Real-time data were collected by 7300 System SDS Software and analyzed in Microsoft Excel. Transcript levels were normalized with rpL32 mRNA levels in the same sample. Relative transcript levels are expressed as a number of copies of transcript per 10,000 copies of rpL32.

2.7. Phylogenetic analysis

IPPI sequences were obtained from GenBank and VectorBase (Lawson et al., 2009) databases and used for the alignments and phylogenetic analysis. We aligned the IPPI sequences using ClustalW (Higgins et al., 1994). A Maximum-Likelihood tree was build using MEGA software version 5.1 (Tamura et al., 2011) with a bootstrapping of 1000 (Felsenstein, 1985). Partial deletion method was selected for the gap/missing data in the software.

2.8. Modeling A. aegypti IPPI

The tertiary structure model of the AalIPPI was created using the protein structure homology-modeling server Swiss-Model v.8.05 (Arnold et al., 2006; Schwede et al., 2003) using as template the Human IPPI (2i6kA) that has 46.6% identity with AaIPPI. The QMEAN6 (Benkert et al., 2011) score obtained for the model obtained was 0.720, the secondary structure agreement 80.0% and the solvent accessibility agreement 82.3%.

3. Results

3.1. Molecular characterization of A. aegypti IPPI

A single orthologue gene to the AalIPPI EST was found in the genome of A. aegypti (VectorBase) (Lawson et al., 2009). The IPPI gene is located on supercontig 1.191, and it is composed of three exons interrupted by two introns with lengths of 4158 and 9351 bp. It encodes a 244 amino acid protein with an estimated molecular weight of 28.5 kDa. Analysis of the AalIPPI sequence revealed the presence of a typical Nudix hydrolase superfamily domain (Nudix: NUCleoside Diphosphate linked to some other moiety X) (Fig. 2).

IPPI orthologues were searched in other species of insects. An alignment of the AalIPPI with related insects is shown in the Supplementary Fig. 2. A cladogram of the phylogenetic relationship of the insect’s IPPI sequences was generated (Supplementary Fig. 3).

3.2. Establishment of an improved protocol to measure IPPI activity

We developed a convenient and simple IPPI activity assay based on the non-enzymatic acidic hydrolysis of DMAPP, which generates the volatile compound isoprene (Fisher et al., 2001). Our modification was to introduce the use of solid-phase micro extraction (SPME) to adsorb the volatile isoprene that can be later directly quantified by GC (Fig. 1). Optimal conditions for hydrolysis were explored; a temperature of 50 °C was chosen because at this temperature isoprene was very effectively adsorbed by the SPME fiber. Higher temperatures could also be used, but heating the sample over 50 °C caused spontaneous hydrolysis of IPP and unwanted production of isoprene. At 50 °C formation and adsorption of isoprene derived from DMAPP was very effective and generation of isoprene from IPP was minimalized (Supplementary Fig. 4). Analysis of different reaction times showed that most of the isoprene formed from DMAPP was recovered within 1 h. Using these conditions three standard curves were constructed by non-enzymatic hydrolysis of mixtures containing 1) increasing...
3.3. AaIPPI activity in the presence of cofactors and inhibitors

To analyze the effect of metal cofactors, Mg$^{2+}$ in the form of MgCl$_2$ was added to samples to obtain final concentrations of 5, 10 and 20 mM. There was a 2-fold increase in activity when 10 mM was added; increasing the concentration to 20 mM also stimulated AaIPPI activity but it was less pronounced (Fig. 3). Buffer without metal cofactor was used as control (0.4 M Tris, 1 mM DTT, pH 8.0). The same process was repeated with Mn$^{2+}$ in the form of MnCl$_2$ to produce concentrations of 0.02, 0.05, 0.1 and 0.2 mM. There was a 2.5-fold increase in activity when 0.1 mM was added; increasing the concentration to 0.2 mM also stimulated AaIPPI activity but it was less marked (Fig. 3). The effect of Zn$^{2+}$ as a cofactor was analyzed with the addition of ZnCl$_2$ to produce concentrations ranging from 50 µM to 10 mM (50 µM, 100 µM, 200 µM, 1 mM, 5 mM and 10 mM). Addition of Zn$^{2+}$ had no effect on AaIPPI activity. The 0.1 mM Mn$^{2+}$ samples yielded the highest rate of IPP isomerization, thus this concentration was chosen to examine the effect of the irreversible inhibitor iodoacetamide. The inhibitor was added to the sample reactions at concentrations between 1 µM and 5 mM. The inhibitor was very effective; 1 µM iodoacetamide caused a relative inhibition of ~ 70%, with complete inhibition at 100 µM (Fig. 4).

3.4. Kinetic properties of recombinant AaIPPI

Kinetic constants were measured for purified recombinant AaIPPI. A plot of the initial velocity of DMAPP production versus IPP concentration was hyperbolic (Fig. 5). The values for $K_m$ and $V_{max}$ were calculated by non-linear regression analysis. A Lineweaver–Burk double reciprocal plot was derived from transformation based upon nonlinear regression analysis (Supplementary Fig. 5). The $K_m$ was 276.8 µM and $V_{max}$ was 0.6 µmol of substrate (IPP) converted to product (DMAPP) per minute per mg of enzyme. The equilibrium constant, $K_{eq} = [IPP]/[DMAPP] = 0.6$ was uniform over the logarithmic phase of the enzymatic reaction (Fig. 5).

3.5. Tissue distribution and developmental expression of AaIPPI mRNA

Real time PCR was used to analyze the expression of AaIPPI mRNA in adult female and male tissues. AaIPPI mRNA expression was higher in the female CA–CC. Lower levels of mRNA transcripts were detected in the ovary, hindgut and brain of the female, as well as the testis and accessory glands of the male (Fig. 6). AaIPPI mRNA was also detected in the female heart, midgut, fat body and Malpighian tubules. In addition, the levels of the AaIPPI transcripts were studied in the CA–CC during female pupal and adult development. In the female CA–CC, the AaIPPI mRNA levels were low in the early pupae, started increasing 6 h before adult eclosion and reached a maximum 24 h after female emergence. Blood feeding resulted in a decrease in transcript levels. The pattern of changes of AaIPPI mRNA resembled the changes in JH biosynthesis (Fig. 7).
4. Discussion

4.1. Molecular and functional characterization of an insect IPPI

We have molecularly and functionally characterized an A. aegypti IPPI involved in the synthesis of JH in the CA. An IPPI expressed sequence tag (EST) was obtained from an A. aegypti corpora-allata + corpora cardiaca library, constructed and sequenced as previously described (Noriaga et al., 2006). The AaIPPI 244 amino acid sequence is slightly longer than the 182 amino acid long IPPI from E. coli, considered to represent the basic IPPI core necessary for IPP isomerization (Durbecq et al., 2001). The extra 62 amino acid present in A. aegypti IPPI are mostly an N-terminus extension. Analysis of the AaIPPI sequence revealed the presence of a typical Nudix hydrolase superfamily domain. Members of the Nudix family are found in a wide variety of organisms and they are capable of hydrolyzing organic pyrophosphates. Nudix family proteins are characterized by the conserved motif, G-X3-A-X2-R-R/K-U-X2-E-L-G-U (Fig. 2) (Bonano et al., 2005). The Nudix motif forms a loop–α helix–loop structure which functions as a versatile Mg$$^{2+}$$-binding and catalytic site (Lin et al., 2008). This motif is part of a shared z/β sandwich or Nudix fold containing additional regions which differ, depending on the detailed mechanism and specificity of the enzyme (Mildvan et al., 2005; Lin et al., 2008). AaIPPI exhibits a compact globular z/β structure very similar to that described for human IPPI (Zheng et al., 2007) (Supplementary Fig. 6). AaIPPI shows a high degree of similarity with IPPIs from other organisms, especially in two important motifs that have been associated with IPPI type I functionality: a TNCACSHPL motif containing a conserved cysteine residue and a WGEHEIDY motif that contains a conserved glutamate residue (Fig. 2) (Zheng et al., 2007). The antifaracial stereochemistry of the isomerization is consistent with an active site containing two residues located on opposite faces of the allyl moiety in IPP; both residues are indispensable to accomplish the protonation/deprotonation steps during IPP isomerization; in the sequence of AaIPPI these two residues have been identified as C$$^{305}$$ and E$$^{156}$$, located face-to-face close to the bottom of the active site pocket. W$$^{253}$$ and Y$$^{268}$$ are two additional active site residues with important roles in catalysis that are located at the bottom of the deeply buried active site cavity. These four residues are highly conserved among the insect IPPIs (Supplementary Fig. 2). E. coli type I IPPI requires two divalent metal atoms for activity. In its native resting form, E. coli type I IPPI seems to be a zinc metalloprotein, but this zinc can be replaced by a Mn$$^{2+}$$. Alternatively, IPPI activity has been challenging because substrate (IPP) and product (DMAPP) are isomers, excluding the possibility of using spectrophotometric assays. The most common assay for IPPI is a radiometric technique based on the acid lability of DMAPP and 14C radiolabeled precursors (Satterwhite, 1985). Alternatively, IPPI activity has been determined by acidic hydrolysis of DMAPP, which is converted into isoprene, a volatile compound
that can be analyzed in the headspace of a vial by adsorption in alumina and subsequent desorption in the GC equipment (Lehning et al., 1999; Fisher et al., 2001; Bruggemann and Schnitzler, 2002). We developed a novel method that takes advantage of the differential liability of DMAPP under acidic conditions as compared with IPP, as well as the efficacy of SPME fibers to adsorb the volatile isoprene (Hyspler et al., 2000; Ignea et al., 2011). The method is much simpler and the use of the fiber avoids the necessity of a GC instrument equipped with a headspace auto sampler or similar devices in the injector to get desorption of adsorbed isoprene (Bruggemann and Schnitzler, 2002).

The SPME-GC protocol was employed to assay the effects of three metal cofactors and an irreversible inhibitor on the AaIPPI. For every IPPI type I studied, addition of Mn\(^{2+}\) results in a stronger activation than addition of Mg\(^{2+}\) (Ramos-Valdivia et al., 1997; Zheng et al., 2007). AaIPPI activity was also stimulated by addition of Mn\(^{2+}\) or Mg\(^{2+}\), with Mn\(^{2+}\) having a stronger activation than Mg\(^{2+}\). The maximum activity was detected at 100 \(\mu\)M Mn\(^{2+}\), a value similar to the optimum concentration of manganese for full activity of a partially purified IPPI from \(B.\) \(mori\) (Koyama et al., 1985). Interestingly, the enzyme’s catalytic activity did not reach a plateau after passing an optimal cofactor concentration threshold, but its activity decreased at higher concentrations of cofactor. This observation may be explained through the formation of a complex between the metal cofactor and the substrate, thus the availability of the substrate is reduced with increasing concentrations of cofactor, as reported in other enzymes requiring metal cofactors (Hallcher and Sherman, 1980). A stimulatory effect of Zn\(^{2+}\) on IPPI has so far only described for \(E.\) coli type I (Lee and Poulter, 2006). Our studies showed that Zn\(^{2+}\) is not a required cofactor for mosquito IPP.

Iodoacetamide has been previously described as a robust inhibitor of IPPIs (Reardon and Abeles, 1986); with the mechanism of inhibition occurring from protonation of the thiol group of the catalytic cysteine residue (Reardon and Abeles, 1986). It was also very effective on AaIPPI, with complete inhibition at 0.1 mM, a concentration in the range previously described for other IPPIs (Ramos-Valdivia et al., 1997). IPPIs are critical enzymes very well conserved during evolution, with identical structure and functional features of members of the family described in other groups of organisms. Changes in IPPI mRNA levels in the CA in the pupa and adult female mosquito correspond well with the changes in JH synthesis, suggesting that AaIPPI transcript fluctuations are at least partially responsible for the changes of JH biosynthesis observed.

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Appendix A. Supplementary Data

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References


including heme, ubiquinone, dolichol and isopentenyl adenine (Bellés et al., 2005). In addition, isoprenoids from the mevalonate pathway are involved in the prenylation of membrane-bound proteins that play an important role in cell signaling: as well as in synthesis of defensive secretions, pheromones and JH (Bellés et al., 2005). AaIPPI transcripts were expressed in all adult female mosquito tissues evaluated; suggesting that AaIPPI might be involved in many metabolic pathways. The higher levels of IPPI mRNA were detected in the CA; because transcript levels are expressed as the ratio IPPI/ribosomal proteins, and the CA has very low cell number when compared to other tissues, the remarkable differences in the IPPI/rip32 ratios between CA and the rest of the tissues should not be misinterpreted as AaIPPI strict specificity of expression in the CA (Nouzova et al., 2011). Transcript levels were also markedly elevated in the reproductive tissues of female and male. IPPI mRNA levels are expressed almost exclusively in the CA of \(B.\) \(mori\) 4th instar larvae, with relative low levels in other tissues (Kinjo et al., 2007).

We found a good correlation between AaIPPI mRNA expression in the CA and JH synthesis (Li et al., 2003). AaIPPI mRNA levels were very low in the CA of early pupae and JH synthesis is suppressed during pupae development. In adult females, CA AaIPPI transcript levels and JH synthesis reached maximum values during the first day after eclosion. In addition, changes in AaIPPI transcripts in CA of sugar-fed and blood-fed female mosquito were also in good agreement with the changes in JH synthesis in the CA (Li et al., 2003). Studies on \(B.\) \(mori\) also showed a good agreement between JH biosynthesis and expression of IPPI in the CA of larvae, pupae and adult female (Kinjo et al., 2007).

In summary, this is the first functional characterization of an isopentenyl diphosphate isomerase involved in the production of juvenile hormone in the CA of an insect. AaIPPI has the typical structure and functional features of members of the family described in other groups of organisms. Changes in IPPI mRNA levels in the CA in the pupa and adult female mosquito correspond well with the changes in JH synthesis, suggesting that AaIPPI transcript fluctuations are at least partially responsible for the changes of JH biosynthesis observed.


