Kinetic mechanism and order of substrate binding for *sn*-glycerol-3-phosphate acyltransferase from squash (*Cucurbita moschata*)

Matthew W. Hayman, Tony Fawcett, Antoni R. Slabas*

The University of Durham, Department of Biological Sciences, Science Laboratories, South Road, Durham DH1 3LE, UK

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Abstract ***sn*-Glycerol-3-phosphate acyltransferase (G3PAT, EC 2.3.1.15), a component of glycerolipid biosynthesis, is an important enzyme in chilling sensitivity in plants. The three-dimensional structure of the enzyme from squash (*Cucurbita moschata*), without bound substrate, has been determined [Turnbull et al. (2001) Acta Crystallogr. D 57, 451–453; Turnbull et al. (2001) Structure 9, 347–353]. Here we report the kinetic mechanism of plastidial G3PAT from squash and the order of substrate binding using acyl-acyl carrier protein (acyl-ACP) substrates. The reaction proceeds via a compulsory-ordered ternary complex with acyl-ACP binding before glycerol-3-phosphate. We have also determined that the reaction will proceed with $C_{4:0}$-CoA, $C_{6:0}$-CoA and $C_{12:0}$-ACP substrates, allowing a wider choice of acyl groups for future co-crystallisation studies.**

Key words: Glycerol-3-phosphate acyltransferase; Acyl-acyl carrier protein; Substrate binding; *Cucurbita moschata*

1. Introduction

Glycerol-3-phosphate-1-acyltransferase (G3PAT; EC 2.3.1.15) catalyses the biosynthesis of 1-acyl-glycerol-3-phosphate, the initial reaction in the biosynthesis of both glycerolipids and storage triacylglycerols. The enzyme exists in both soluble and membrane bound forms [3]. Soluble G3PAT, located in the chloroplast, uses acyl-acyl carrier protein (acyl-ACP) as the natural substrate and is of particular interest due to its demonstrated role in chilling resistance [4]. Following analysis of the fatty acid composition of lipids from chilling-resistant and chilling-sensitive plants it was noted that resistant plants had a higher content of unsaturated fatty acids at position *sn*-1 of the glycerolipids. Membranes containing lipids with a high percentage of unsaturated fatty acids have a lower phase transition temperature, from the liquid to the crystalline state, in comparison to those rich in saturated fatty acids. As a consequence, lowering the temperature in chilling-sensitive plants would give rise to membrane damage at a higher temperature than in chilling-resistant plants. It was hypothesised that the molecular basis of chilling resistance in plants could be due to different substrate selectivities of the G3PAT between chilling-resistant and -sensitive plants, with resistant plants preferentially incorporating unsaturated fatty acids at position *sn*-1 [4]. Chloroplast G3PATs have been isolated from several plant species and substrate-selective and non-selective forms of the enzyme identified. cDNAs for both selective (*Arabidopsis thaliana*) and non-selective (*Cucurbita moschata*) G3PATs have been overexpressed in tobacco, the former resulting in increased chilling resistance and the latter in increased chilling sensitivity [4]. These experiments demonstrated that the substrate selectivity of GPATs can alter both the chilling sensitivity and acyl composition of the glycerolipids by preferentially selecting certain acyl-ACPs from the available pool of metabolites. Similar results have been obtained by overexpressing the membrane bound G3PAT from *Escherichia coli* in plants [5]. Whilst there may be other contributing factors to chilling sensitivity, dependent on the species involved [6], it is clear that in both tobacco and cyanobacteria membrane fluidity is important (reviewed in [7]).

Despite the importance of these enzymes we have little insight into the molecular basis of substrate selectivity. The amino acid sequence of G3PATs from chilling-resistant and -sensitive plants is highly conserved [1,2], complicating a mutagenesis-based approach. Additionally, selectivity assays have often been performed using the substrate analogue acyl-CoA to circumvent difficulties in the synthesis of the physiological substrate acyl-ACP.

Understanding the molecular basis of substrate selectivity would be greatly aided by direct visualisation via X-ray crystallography of an acyl-ACP:enzyme complex. We have previously reported the crystal structure of G3PAT from squash. However, no enzyme:substrate complex crystals have been obtained. Progress in making such crystals would be accelerated by understanding not only the order of binding of the substrate but the acyl chain length which the enzyme will utilise, as longer chain acyl-substrates require detergent to solubilise them. In this study we have synthesised various acyl-ACP substrates and used them in studies to determine the kinetic mechanism of G3PAT. The order of substrate binding and the stoichiometry of an acyl-ACP:enzyme complex have been determined in addition to the range of acyl-chain lengths which the enzyme will utilise.

*Corresponding author. Fax: (44)-191-374 2417.
E-mail address: a.r.slabas@durham.ac.uk (A.R. Slabas).

Abbreviations: ACP, acyl carrier protein; G3PAT, glycerol-3-phosphate acyltransferase (EC 2.3.1.15); LPA, lysophosphatidic acid

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2. Materials and methods

2.1. Production and purification of G3PAT proteins

Squash G3PAT protein was produced in *E. coli* BL21 (DE3) containing the pET 24a vector system. Cells were lysed in 20 mM Tris–HCl pH 7.4 using three freeze/thaw cycles in dry ice/ethanol and ice/water baths [8] and samples were centrifuged at 150 000 × *g* for 1 h at 4°C. G3PAT protein was purified from the supernatant via anion-exchange chromatography (Q-Sepharose), eluted with a gradient of lysis buffer containing 0–2 M NaCl. Enriched fractions containing G3PAT activity were analysed via sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and densiometric scanning to assess purity and quantify G3PAT protein levels. A highly purified protein preparation contained a single band of 41 kDa demonstrated on SDS–PAGE.

2.2. Preparation of recombinant E. coli ACP

Recombinant *E. coli* apo-ACP was produced as described by Fawcett et al. [9]. Apo-ACP was converted to holo-ACP in a reaction containing potassium phosphate buffer 50 mM pH 7.3, 10 mM $MgCl_2$, 2 mM EDTA, 333 μM apo-ACP, 666 μM co-enzyme A and 100 μg holo-ACP synthetase in a total volume of 1.5 ml. The reaction was incubated at 37°C for 3 h before buffers and salts were exchanged for de-ionised water using a desalting column (Amersham Pharmacia Biotech PD10). The product was freeze-dried and stored at −80°C. The identity of the ACPs was validated and quantified by visualisation of the mobility on 18% native polyacrylamide gels containing 0.5 M urea [10].

2.3. Synthesis of radiolabelled acyl-ACPs

Acyl-ACPs were synthesised enzymatically, as described previously [11], with slight modification. The synthesis mixture consisted of 100 mM Tris–HCl pH 8.0, 400 mM LiCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM ATP, 1% Triton X-100, 33 μM holo-ACP, 25 μM [1-^{14}C]lauryl or [1-^{14}C]palmitic or [9,10-^{3}H]oleic acid at a specific activity of 55 Ci/mol and 5.0 U/ml acyl-ACP synthetase [12,13]. The reaction was performed in a total volume of 5.0 ml and incubated at 30°C for a minimum of 10 h. Radiolabelled acyl-ACPs were purified via anion exchange and hydrophobic interaction chromatography. The sample was eluted from a Q-Sepharose column with a gradient of 0–2 M NaCl in 20 mM Tris–HCl pH 7.0 and from an octyl-Sepharose column with a gradient of 0–40% *iso*-propyl alcohol in 20 mM *N*-ethyl morpholine acetate buffer pH 7.0.

2.4. Standard G3PAT assay

Assays were performed in a total volume of 320 μl 250 mM HEPES buffer pH 8.0, 5.0 mg/ml bovine serum albumin (BSA) and variable G3P and acyl-ACP concentrations as given in the text. Assays were started with the addition of 195 ng of G3PAT. 80 μl aliquots were removed at 0 min (before addition of enzyme), 1, 2 and 3 min after initiation and mixed with 710 μl of chloroform/methanol (1:1) and 280 μl of 0.2 M H_2PO_4 in 1 M KCl. After centrifugation for 15 min at 13 000 rpm, 250 μl of the lower phase was dried in a vacuum centrifuge, resuspended in 280 μl of methanol and mixed with 4 ml Ecoscint A. Samples were counted in a liquid scintillation counter to determine the rate of incorporation of radiolabelled acyl-ACP into lysophosphatidic acid (LPA).

2.5. Assay to determine binding of acyl-ACP and G3P substrates to G3PAT

Assays were performed to assess the binding of each substrate to the enzyme in the absence of the other substrate. In a total volume of 200 μl 250 mM HEPES pH 8.0, 100 pmol of radiolabelled [9,10-^{3}H]18:1ACP or [1-^{14}C]G3P (both at 55 Ci/mol) were incubated with 50 pmol of G3PAT for 5 min. Following centrifugation through a Millipore Ultrafree® MC filter unit (30 000 Da molecular weight cut-off) for 5 min at 5000 × *g*, the sample volume in both compartments was readjusted to 200 μl with 250 mM HEPES pH 8.0 and transferred to 4 ml Ecoscint A prior to counting in a liquid scintillation counter. Radiolabelled substrate bound to G3PAT was retained by the membrane in the upper compartment, unbound substrate passed through the membrane into the lower compartment. Assays were performed in triplicate.

2.6. Assays with short chain acyl-CoA substrates

$C_{4:0}$-CoA and $C_{6:0}$-CoA substrates were purchased from Sigma. Assays were carried out in 250 mM HEPES NaOH pH 8.0 containing 5.0 mg/ml BSA with 33 μM [1-^{14}C] G3P (55 Ci/mol) and 0.1 mM acyl-CoA, in a total volume of 20 μl. Assays were initiated by the addition of 16.25 ng of G3PAT and terminated after 5 min by the addition of 40 μl of chloroform/methanol (1:1). The samples were loaded onto a thin layer chromatography (TLC) plate and separated for 3 h in butanol:acetic acid:water 5:2:3 to isolate LPA from unreacted [1-^{14}C]G3P. The LPA spot was scraped into 1 ml methanol, vortexed and added to 4 ml Ecoscint A. Samples were counted in a liquid scintillation counter.

3. Results and discussion

3.1. Characterisation of recombinant squash G3PAT

Squash G3PAT was expressed in *E. coli* BL21 (DE3) as an N-terminally truncated protein. We have deliberately used this protein in these studies as the full-length protein did not crystallise and all structural data has been obtained on the truncated form. The protein has an N-terminal sequence of MASHSRKFLDV, with the first three residues coded for by vector DNA.

G3PAT purified from squash has apparent K_ms of 1.1–1.7 μM for acyl-ACP and 17–320 μM for G3P, dependent on the type of acyl-ACP used as substrate [14]. The recombinant protein used in our assays displays similar kinetic constants: K_ms of 3.0–3.4 μM for acyl-ACP and 117–143 μM for G3P were observed.

3.2. Determination of range of substrate utilisation by G3PAT

As we are interested in obtaining co-crystals of G3PAT with the bound acyl-ACP substrate, we decided to investigate the minimal chain length of the acyl group which the enzyme would use. This is important as the acyl group influences the critical micelle size, substrate solubility and potential requirements for detergents. Assays were performed using [1-^{14}C]G3P with unlabelled acyl-CoAs and acyl-ACPs (Table 1). From these data it can be seen that $C_{4:0}$-CoA and $C_{6:0}$-CoA can be used as substrates for this enzyme. However, acylation rates are significantly lower with the shorter acyl chain-length substrates; $C_{4:0}$-CoA and $C_{6:0}$-CoA are used at approximately 5 and 10%, respectively, of the rate with C≥16-CoAs. $C_{12:0}$-ACP is used at approximately one third of the rate of the longer acyl chain-length substrates, C≥16-ACPs.

Table 1
G3PAT reaction velocities with various acyl-CoA and acyl-ACP substrates

	Velocity
$C_{4:0}$CoA	5.1 ± 0.4
$C_{6:0}$CoA	11 ± 1.1
$C_{16:0}$CoA	120 ± 4.3
$C_{18:1}$CoA	111 ± 4.7
$C_{12:0}$ACP	28 ± 3.2
$C_{16:0}$ACP	83 ± 1.9
$C_{18:1}$ACP	81 ± 2.8

Velocity is presented as pmol LPA formed per min per mg G3PAT under the following conditions: acyl-CoAs, 100 μM acyl-CoA and 33 μM G3P. Acyl-ACPs, 2 μM acyl-ACP and 300 μM G3P. Assays were performed in triplicate and results are presented as mean values ± 1S.E.M.

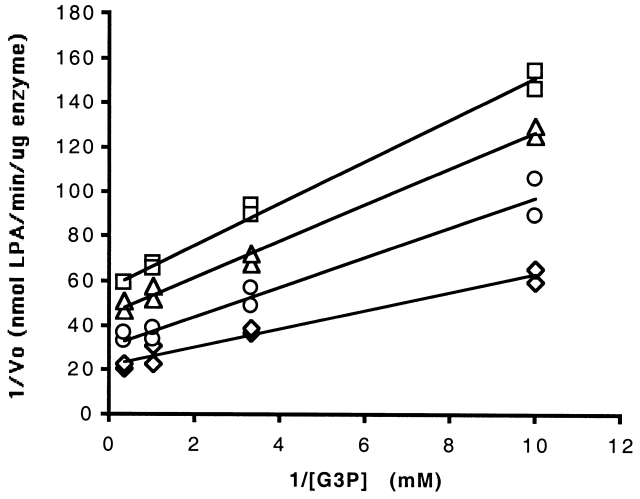

Fig. 1. Determination of squash G3PAT kinetic mechanism. Initial velocity was recorded in assays using varying G3P concentrations at fixed, non-saturating concentrations of acyl-ACP (2 μM (□), 3 μM (△), 5 μM (○), 10 μM (◇)). Assays were performed in duplicate. Linear regression shows that the series of lines converge, demonstrating that the enzyme mechanism is sequential rather than substitution.

3.3. Determination of reaction mechanism

To determine whether G3PAT proceeds via a sequential or ternary complex reaction mechanism, a series of assays using fixed, non-saturating levels of acyl-ACP (2, 3, 5 and 10 μM) and varying concentrations of G3P (0.1, 0.3, 1.0 and 3.0 mM) were carried out. The double reciprocal plots produced from these assays indicated a series of lines which were converging (Fig. 1). When fixed, non-saturating levels of G3P were used and the concentration of acyl-ACP varied, a similar converging pattern of lines was recorded (data not shown). These patterns indicate that the squash G3PAT reaction occurs via a ternary complex reaction mechanism.

3.4. Product inhibition studies to determine order of substrate binding

To determine whether the G3PAT ternary complex reaction mechanism is compulsory or random ordered the reaction products, ACP and LPA, were used as reaction inhibitors. Analysis of the results was performed as described by Cleland [15]. When acyl-ACP was varied in the presence of LPA (Fig. 2B) and G3P varied in the presence of ACP (Fig. 2C) and LPA (Fig. 2D), mixed patterns of inhibition were observed. However, when acyl-ACP was varied in the presence of ACP (Fig. 2A) competitive inhibition was observed, indicating that the mechanism is a compulsory-order ternary complex with acyl-ACP binding first.

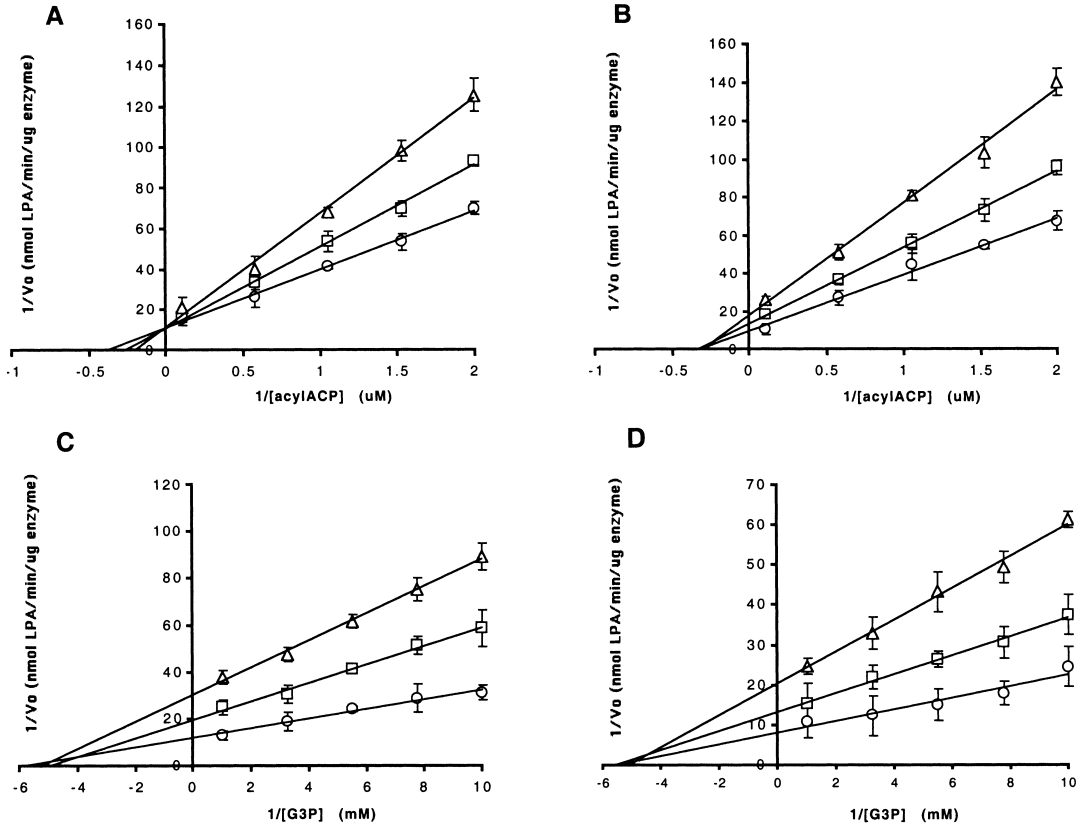

Fig. 2. Determination of the order of substrate binding by product inhibition. Assays were performed with varying concentrations of one substrate at fixed, non-saturating concentrations of the second substrate in the absence of product (○) and in the presence of 0.2 mM (□) and 0.4 mM (△) product. The plots are double reciprocal using (A) varying acyl-ACP concentrations at 300 μM G3P at different fixed concentrations of ACP; (B) varying acyl-ACP concentrations at 300 μM G3P at different fixed concentrations of LPA; (C) varying G3P concentrations at 2 μM acyl-ACP at different fixed concentrations of ACP and (D) varying G3P concentrations at 2 μM acyl-ACP at different fixed concentrations of LPA. Assays were performed in triplicate and presented as mean values (±1S.E.M.).

Table 2
Differential binding of acyl-ACP and G3P substrates to G3PAT

	+G3PAT		−G3PAT	
	acyl-ACP	G3P	acyl-ACP	G3P
Total pmoles of substrate in upper compartment	47.4 ± 0.48	1.01 ± 0.11	0.11 ± 0.02	0.09 ± 0.01
Moles of substrate bound per mole of enzyme	0.95 ± 0.01	0.02 ± 0.002	n/a	n/a

100 pmol of radiolabelled 18:1ACP or G3P were incubated with or without 50 pmol of G3PAT enzyme in the absence of the second substrate and spun through a 30 000 Da molecular weight cut-off membrane. Substrate bound to G3PAT did not pass through the membrane and was retained in the upper compartment. Assays were performed in triplicate and results are presented as mean values ± 1S.E.M.

3.5. G3PAT substrate binding assays

In order to supplement the evidence supporting a compulsory substrate binding order, binding studies were conducted using acyl-ACP and G3P substrates. Each substrate was incubated with the enzyme in the absence of the other substrate before separation of unbound substrate from enzyme-bound substrate in an ultrafiltration unit. With no G3PAT present, more than 99% of both substrates passed through the membrane. With G3PAT present, the enzyme bound acyl-ACP in a ratio of approximately 1 mol of substrate to 1 mol of enzyme and G3P did not bind to the enzyme in a significant molar ratio (Table 2). This is in agreement with earlier evidence (Section 3.4) that the substrate order is compulsory with acyl-ACP binding first.

3.6. Implications for future studies

In this study the kinetic mechanism of G3PAT has been investigated and kinetic proof obtained that acyl-ACP binds before and in the absence of glycerol-3-phosphate. This has been directly tested using ultrafiltration columns to separate enzyme:substrate complexes from unbound substrate. It was shown that acyl-ACP will form a 1:1 complex with the enzyme in 250 mM HEPES buffer at pH 8.0. We have also shown that the enzyme is capable of using short chain acyl-ACP and acyl-CoA substrates. In addition to giving insight into the mechanism of the enzyme, these studies are important in determining the direction of future attempts to obtain enzyme:substrate complex crystals. These would allow us to directly investigate the basis of substrate selectivity. It is now apparent that the acyl-thiol substrate binds first and that a much wider range of substrates can be utilised in attempts to obtain enzyme:substrate complexes. Had the G3P bound first, it would become highly unlikely that one could obtain an acyl-ACP complex with the active enzyme, due to catalytic turnover and product release. The pH and ionic conditions used in these experiments can act as a guideline in the preparation of enzyme:substrate complexes. The likelihood of obtaining complexes is increased, not only by using shorter chain acyl-ACP substrates but by diffusion of short chain acyl-CoAs into pre-existing crystals. Approaches are also available for photoaffinity labelling G3PAT with suitable acyl-CoA or acyl-ACP probes to locate the thiol binding site, as has been accomplished with the FadR lipid transcription factor from *E. coli* [16]. Knowledge of substrate binding order is critical for further investigations of the acyl binding site and the basis of substrate selectivity in G3PAT enzymes.

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