Mutagenesis of squash (Cucurbita moschata) glycerol-3-phosphate acyltransferase (GPAT) to produce an enzyme with altered substrate selectivity


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Abstract

In an attempt to rationalize the relationship between structure and substrate selectivity of glycerol-3-phosphate acyltransferase (GPAT, 1AT, EC 2.3.1.15) we have cloned a number of cDNAs into the pET overexpression system using a PCR-based approach. Following assay of the recombinant enzyme we noted that the substrate selectivity of the squash (Cucurbita moschata) enzyme had altered dramatically. This form of GPAT has now been crystallized and its full three-dimensional structure elucidated. Since we now have two forms of the enzyme that display different substrate selectivities this should provide a powerful tool to determine the basis of the selectivity changes. Kinetic and structural analyses are currently being performed to rationalize the changes which have taken place.

Introduction

We have recently been working towards elucidation of the structure of glycerol-3-phosphate acyltransferase (GPAT) from plant sources. This has involved the cloning of appropriate cDNAs into the pET expression system of Escherichia coli, using PCR-based procedures, for overproduction of the enzyme. The primary sequence of GPAT from plant species is highly conserved, so precluding an easy assessment of individual residues that could be important in substrate selectivity. One way forward would be to systematically mutate all the residues which are different, individually and in combination, and assay the enzyme for changes in substrate selectivity. This type of approach has been used in converting the oleate Δ^{12}-desaturase into a hydroxylase and vice versa [1]. Another way would be by the introduction of fortuitous errors during PCR. In order to take advantage of the latter approach highly sensitive assays for substrate selectivity must be available. Assay conditions have been established which use competitive conditions between 16:0- and 18:1-acyl carrier proteins (ACPs) at physiological concentrations of substrate. Concentrations of acyl-ACP and glycerol 3-phosphate in vivo fall in the range 1–5 μM [2] and 70–300 μM [3] respectively and the pH in the plastid varies between 7.4 in the dark to 8.0 in the light. The results of the assays with both the wild-type GPAT and a PCR-mutated form of the enzyme are reported.

Materials and methods

cDNAs for Arabidopsis and squash were cloned using PCR-based techniques. The clones were transformed into E. coli and expressed to a high level using the bacteriophage T7 promoter/T7 polymerase and isopropyl β-D-thiogalactoside (IPTG) induction. Standard procedures were used in all cases [4,5]. Cells were disrupted by freeze-thaw and recombinant proteins were expressed to approx. 20% of the total soluble protein. GPATs were purified via MonoQ ion-

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Abbreviations used: ACP, acyl carrier protein; GPAT, glycerol-3-phosphate acyltransferase; IPTG, isopropyl β-D-thiogalactoside; LPA, lysophosphatidic acid.

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exchange chromatography with elution using a linear salt gradient. Apo-ACP was prepared in an overproducing E. coli strain and purified to homogeneity by ion-exchange chromatography. Purified apo-ACP was used to produce holo-ACP via the action of recombinant halo-ACP synthetase. Recombinant acyl-ACP synthetase was used to convert holo-ACP to acyl-ACP using either [3H]18:1 or [14C]16:0 fatty acids radio-labelled at 55 Ci/mol. Acyl-ACP was recovered by ion exchange, followed by octyl-Sepharose chromatography [6,7]. Standard assays for GPAT using acyl-ACPs were performed in a reaction mixture containing 250 mM Hepes buffer, pH 8.0, 5 mg/ml BSA, 0.3 mM glycerol 3-phosphate and 1.0 μM of each acyl-ACP in a final volume of 180 μl. Incorporation of the acyl group into lysophosphatic acid (LPA) was determined following solvent partitioning of aliquots of the reaction mixture and counting of the organic phase in a liquid scintillation counter. Linear initial velocities were observed in all cases.

Results and discussion

Recombinant GPAT was expressed in E. coli at high levels. Figure 1 shows a typical SDS/PAGE gel following induction of the enzyme and its purification to homogeneity via chromatography. During the course of our studies several individual PCR reactions were performed to make the overproduced squash construct. These were individually ligated into the pET vector system and subsequently expressed. The substrate selectivity of the wild-type enzyme is shown in Figure 2, together with the selectivity of the Arabidopsis enzyme and a mutant squash enzyme. It can be clearly seen that the mutant squash enzyme has altered substrate selectivity. Wild-type squash uses 18:1- and 16:0-ACP at comparable rates. The mutant squash enzyme has a ratio of 18:1-ACP/16:0-ACP usage of approx. 4.5:1 and now behaves more like the selective Arabidopsis GPAT, which selects for 18:1-ACP over 16:0-ACP with a ratio of roughly 3.2:1.

Since the squash enzyme has been crystallized and we now have two forms of the enzyme that have different substrate selectivities it should be possible to rationalize the reasons for these differences following further structural and kinetic analyses. These are currently underway.

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References

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