Analytical Biochemistry 423 (2012) 163–170

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

The first nonradioactive fluorescence assay for phosphatidylglycerol:prolipoprotein diacylglyceryl transferase that initiates bacterial lipoprotein biosynthesis

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ARTICLE INFO

Article history:
Received 8 December 2011
Received in revised form 17 January 2012
Accepted 19 January 2012
Available online 27 January 2012

Keywords:
Bacterial lipid modification
Prolipoprotein diacylglyceryl transferase
Peptide substrate
Coupled enzyme assay
Enzyme characterization

ABSTRACT

The unique and physiologically vital bacterial enzyme, prolipoprotein diacylglyceryl transferase (Lgt), which catalyzes the committed first step in the posttranslational transfer of diacylglyceryl group from phosphatidylglycerol to the prospective N-terminal cysteine of prolipoproteins, remains to be characterized for want of a simpler but equally sensitive nonradioactive assay. We, for the first time, report a coupled enzymatic fluorescence assay for Lgt using the de novo synthetic peptide substrate MKATK SAVGSTLAGCSSHHHHHH. The assay is based on the conversion of the by-product, glycerol-1-phosphate, to dihydroxyacetone using an alkaline phosphatase–glycerol dehydrogenase combination and estimating the fluorescence of the coupled reduction of resazurin to resorufin. The minimum amount of glycerol-1-phosphate, and hence the modified peptide, detected by this method is approximately 20 pmol, thereby making this assay a promising alternative to the radioactive assays. The assay is rapid, more convenient, less laborious, and suitable for purification and characterization of Lgt.

More than 15,000 posttranslationally modified lipoproteins have been either identified or predicted in both gram-positive and gram-negative bacteria. These lipoproteins perform essential functions in structural integrity, nutrient uptake, environmental signal transduction, cell division, sporulation, conjugation, protein secretion, adhesion, and antibiotic resistance [1]. They are synthesized as precursors with tripartite signal sequence, with a characteristic C-terminal "lipobox" (consensus sequence [LVI] [ASTVI] [GAS] [C]) [2]. During maturation, the invariant Cys sequentially undergoes diacylglyceryl modification catalyzed by phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt),[1] signal sequence cleavage at the modification site catalyzed by lipoprotein-specific signal peptidase (Lsp II), and N-acylation of the partially lipid-modified Cys catalyzed by apolipoprotein *N*-acyl transferase (Lnt) [3]. The resultant *N*-acyl-*S*-diacylglyceryl-Cys anchors bacterial lipoproteins to the cell membrane at the N-terminal end so that bulk of the protein efficiently functions in the aqueous compartment. Recently, the role of Sec (secretory) and TAT (twin arginine translocase) pathways in differentially recognizing the slow- and fast-folding lipoproteins, respectively, for lipid modification has been identified [4]. This unique adaptation at the hydrophobic–aqueous interface by bacteria has potential in protein engineering applications, including enzyme-linked immunosorbent assay (ELISA), liposomal integration, targeted drug delivery, and biosensors [5].

Of the three enzymes, Lgt is most significant because it catalyzes the first committed step of the unique and ubiquitous pathway seen only in bacteria. This enzyme, associated on the cytosolic side of the inner membrane, catalyzes the characteristic transfer of diacylglyceryl moiety from phosphatidylglycerol (PG) to the thiol group of lipobox Cys. The 31.6-kDa [6] enzyme is essential for the growth and viability of gram-negative organisms [7]. Lgt mutants of gram-positive bacteria were viable but showed physiological deficiencies and attenuated virulence [8–11]. In-depth study of the unique activity of Lgt has been hindered due to its low abundance, difficulty to overexpress by recombinant methods, poor stability, and lack of simpler nonradioactive assays. The original radioactive assay developed for Lgt using a synthetic peptide corresponding to the N-terminal 24 amino acids of the prototype prolipoprotein, the precursor of the most abundant murein lipoprotein, led to the discovery of Lgt, its preference for negatively charged phospholipid substrate, particularly PG, and the actual pathway [3]. The fate of glycerol-1-phosphate released in the Lgt reaction is not yet known, but homologues of glycerol-1-phosphate dehydrogenase (G1PDH) genes in gram-positive *Bacillus subtilis* predict the possible use of glycerol phosphate in the synthesis of glycerophospholipid backbone [12]. Comparison of primary

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[1] *Abbreviations used*: Lgt, prolipoprotein diacylglyceryl transferase; Sec, secretory; TAT, twin arginine translocase; PG, phosphatidylglycerol; G1PDH, glycerol-1-phosphate dehydrogenase; IMV, inverted membrane vesicle; OG, β-octyl glucopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; GDH, glycerol dehydrogenase; LB, Luria–Bertani; RFU, relative fluorescence units; WE, water extract; CPM, counts per minute; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2012.01.018

sequences of Lgt from phylogenetically distinct species of bacteria—*Escherichia coli, Salmonella typhimurium, Haemophilus influenza,* and *Staphylococcus aureus*—revealed a conserved 103-HGGLIG-108 motif, possibly involved in catalysis [6]. Site-directed mutagenesis of all the histidine residues and seven of the tyrosine residues and chemical modification of Lgt with diethyl pyrocarbonate (DEPC) implicated His103 and Tyr235 in Lgt activity [13]. The enzyme is very basic (deduced p*I* of 10.4), with an abundance of Arg residues in gram-negative organisms and an equal abundance of both Lys and Arg residues in gram-positive organisms.

The inability to precipitate hydrophilic peptide substrates and their diacylglyceryl-modified product by 90% acetone saturated with ammonium sulfate in the original peptide assay led to the development of a generic radioactive assay for Lgt employing a hydrophilic peptide synthesized de novo [14] and the differential paper-electrophoretic separation of PG (negatively charged and radioactive), unmodified substrate (positively charged and nonradioactive), and lipid-modified product (positively charged and radioactive) owing to their opposite charges. This assay led to the discovery that *E. coli* Lgt is superficially associated with the inner leaflet of inner membrane by hydrophobic interactions. Although low abundance of the enzyme and the inability to overexpress were major disadvantages in its isolation, water extraction of the inverted membrane vesicle (IMV) provided a convenient enriched source of the enzyme [14]. Very recently, it has been shown using engineered lipo-EGFP (enhanced green fluorescent protein) that Sec- or TAT-bound prolipoproteins could be the actual substrates of Lgt in vivo [4].

Both the radioactive assays indeed helped to unravel important characteristics of Lgt, but the serious disadvantages associated with radioactive assays have hindered the kinetic characterization and purification of this interesting enzyme. Therefore, we have developed a new, enzymatically coupled fluorescence assay for Lgt by estimating the by-product of the reaction, glycerol-1-phosphate. The newly developed nonradioactive assay is shown to be as sensitive and reliable as the radioactive assays.

Materials and methods

All fluorescence measurements were made with the BioTek Synergy HT multimode microplate reader at λ_{ex} = 550 ± 12.5 nm and λ_{em} = 590 ± 15 nm.

Reagents

The following chemicals were used in the study. The synthetic peptide MKATKSAVGSTLAGCSSHHHHHH was dissolved in 0.1 N HCl at 5 mg/ml concentration and stored in aliquots at −20 °C. PG, glycerol-1-phosphate, β-octyl glucopyranoside (OG), and resorufin were obtained from Sigma Chemical. NAD^+, NADH, glycerol, resazurin, diaphorase of *Clostridium* sp. (500 U), and alkaline phosphatase of calf intestinal mucosa (200 U) were purchased from Sisco Research Laboratories. [9,10] Palmitate (50 Ci/mmol) was purchased from GE Healthcare.

Buffers used in the study were as follows: TED buffer (20 mM Tris–HCl, pH 8.0, containing 5 mM ethylenediaminetetraacetic acid [EDTA] and 4 mM dithiothreitol [DTT]), TBS buffer (20 mM Tris–HCl, pH 8.0, containing 0.9% [w/v] NaCl), TED–NaCl buffer (20 mM Tris–HCl, pH 8.0, containing 5 mM EDTA, 4 mM DTT, and 0.5 M NaCl), 100 mM Tris–HCl buffer (pH 8.0), 100 mM sodium carbonate–bicarbonate buffer (pH 10.0), and 0.1 M sodium acetate buffer (pH 4.5).

Phosphatidylglycerol was solubilized in TED buffer containing 1% OG. Glycerol dehydrogenase (GDH) and diaphorase were dissolved in 100 mM Tris–HCl, and alkaline phosphatase was dissolved in 100 mM sodium carbonate–bicarbonate buffer. All of the enzyme reagents were stored at −20 °C. NAD^+, NADH, glycerol-1-phosphate, resazurin, and resorufin were dissolved in water and stored at −20 °C for approximately a month. PG was also extracted from *S. aureus.*

Design of fluorometric assay

The new fluorescence assay was designed based on the sensitive enzymatic estimation of glycerol-1-phosphate released in Lgt-catalyzed reaction. The sequential steps (Fig. 1) in the assay are as follows:

(i) Lgt catalyzes the transfer of diacylglyceryl moiety from PG to the thiol group of the invariable cysteine in the lipobox of the protein/peptide substrate, releasing the modified prolipoprotein/peptide and glycerol-1-phosphate;
(ii) glycerol-1-phosphate is cleaved in the presence of alkaline phosphatase into glycerol and inorganic phosphate (P_i);
(iii) the released glycerol undergoes dehydrogenation in the presence of NAD^+ and GDH, forming dihydroxyacetone and $NADH^+H^+$; and
(iv) the NADH is then coupled to diaphorase-catalyzed reduction of resazurin to resorufin (λ_{ex} = 550 nm and λ_{em} = 590 nm), a highly fluorescent product.

The resulting fluorescence will be directly proportional to the amount of glycerol-1-phosphate formed in the reaction catalyzed by Lgt and, hence, will reflect the Lgt activity.

Assay components

Partial purification of GDH from E. coli DH5α

The extraction and partial purification of GDH was carried out as described previously [15] but with alterations in the buffers used. *E. coli* DH5α was grown overnight in 1.5 L of Luria–Bertani (LB) medium, harvested by centrifugation, and washed twice with 0.9% NaCl. The pellet was resuspended in 0.1 M Tris–HCl (pH 7.5) and sonicated at 15,000 psi. The turbid supernatant was centrifuged at 12,000*g* for 45 min to obtain the cytosolic fraction, which was heated at 70 °C for 90 min to get enriched heat-stable protein preparation. After removing the denatured protein by centrifugation at 12,000*g* for 20 min, the enzyme present in the supernatant (because the enzyme is heat stable) was precipitated with 90% ammonium sulfate. The pellet was dissolved in 50 mM Tris–HCl (pH 7.5) to a final protein concentration of 10 mg/ml and dialyzed against the same buffer for 16 h at 4 °C to remove residual ammonium sulfate. The enzyme extract was then stored in aliquots at the final protein concentration of 5 mg/ml at −20 °C.

Kinetic characterization of GDH preparation

The activity of GDH was determined by measuring the absorbance of NADH at 340 nm during the enzymatic reaction (15 min at 37 °C) with 10 mM glycerol and 1 mM NAD^+ in 100 mM sodium carbonate–bicarbonate buffer (pH 10.0). The K_M of GDH for glycerol was measured by varying it from 25 to 500 mM with 1 mM NAD^+, and the K_M for NAD^+ was measured by varying it from 0.5 to 50 mM with 500 mM glycerol. Using a Lineweaver–Burk plot of the kinetic data, the K_M values of *E. coli* GDH for glycerol and NAD^+ were found to be 95 and 2.3 mM, respectively. The best preparations had a specific activity of 74 nmol/min/mg protein. The preparation was stable for approximately a week when stored at −20 °C.