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Evaluation of synthase and hemisynthase activities of glucosamine-6-phosphate synthase by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

Glucosamine-6-phosphate synthase (GlmS, EC 2.6.1.16) catalyzes the first and rate-limiting step in the hexosamine biosynthetic pathway, leading to the synthesis of uridine-5′-diphospho-*N*-acetyl-D-glucosamine, the major building block for the edification of peptidoglycan in bacteria, chitin in fungi, and glycoproteins in mammals. This bisubstrate enzyme converts D-fructose-6-phosphate (Fru-6P) and L-glutamine (Gln) into D-glucosamine-6-phosphate (GlcN-6P) and L-glutamate (Glu), respectively. We previously demonstrated that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) allows determination of the kinetic parameters of the synthase activity. We propose here to refine the experimental protocol to quantify Glu and GlcN-6P, allowing determination of both hemisynthase and synthase parameters from a single assay kinetic experiment, while avoiding interferences encountered in other assays. It is the first time that MALDI-MS is used to survey the activity of a bisubstrate enzyme.

The vast majority of enzyme reactions involve at least two substrates to afford more than one product. The mechanism, kinetics, and inhibition of these enzymes are more complicated to analyze than those of single substrate reactions. Most often they are studied under steady-state conditions measuring the rate of formation of only one of the products. However different results may be obtained depending on which of the products is considered. Glucosamine-6P synthase catalyzes the conversion of D-fructose-6-phosphate (Fru-6P)[1] into D-glucosamine-6-phosphate (GlcN-6P) using L-glutamine (Gln) as nitrogen donor (Scheme 1) [1]. It follows an ordered process to bind sequentially Fru-6P and Gln [2] and successively releases L-glutamate Glu (hemisynthase activity) and GlcN-6P (synthase activity).

Several methods were developed for monitoring GlcN-6P formation, such as a modified Morgan-Elson protocol [3–6], *o*-phthaldialdehyde derivatization [7], Ellman assay involving GlcN-6P *N*-acetyltransferase 1 (GNA1) coupling [8], radiometry [9,10] or MALDI-TOF mass spectrometry [11]. More recently, isothermal titration calorimetry (ITC) was used to determine the kinetic and thermodynamic parameters of GlmS activities under strictly identical experimental conditions [12]. Most of these assays suffer from interference, poor reproducibility, or sensitivity problems.

If the quantification of Glu was classically performed by UV or fluorescence methods [4,6] using glutamate dehydrogenase as coupling enzyme, this method has limits in screening inhibitors of the Fru-6P binding site, since glutamate production is not always coupled to the synthase activity [13]. Indeed compounds that inhibit the synthase reaction while activating [14] or having no effect on hemisynthase activity of Gln-dependent enzymes were reported [15], making the search for synthase-directed inhibitors a difficult task. As GlmS plays a crucial role in the cell wall elaboration of bacterial and fungal organisms, it was considered as a potential target for antibacterial and antifungal drugs [1,16,17]. It is thus absolutely crucial to be able to determine which part of the reaction can be altered during the process of inhibitor screening.

Despite a marked interest for assays monitoring simultaneously the different products of a multisubstrate enzyme, there is to our knowledge no example allowing such quantifications without requiring tags [18,19]. Our interest in the use of mass spectrometry

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[1] *Abbreviations used:* GlmS, glucosamine-6-phosphate synthase; Fru-6P, D-fructose-6-phosphate; Gln, L-glutamine; GlcN-6P, D-glucosamine-6-phosphate; Glu, L-glutamate (Glu); MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; THAP, trihydroxyacetophenone; 9-AA, 9-aminoacridine.

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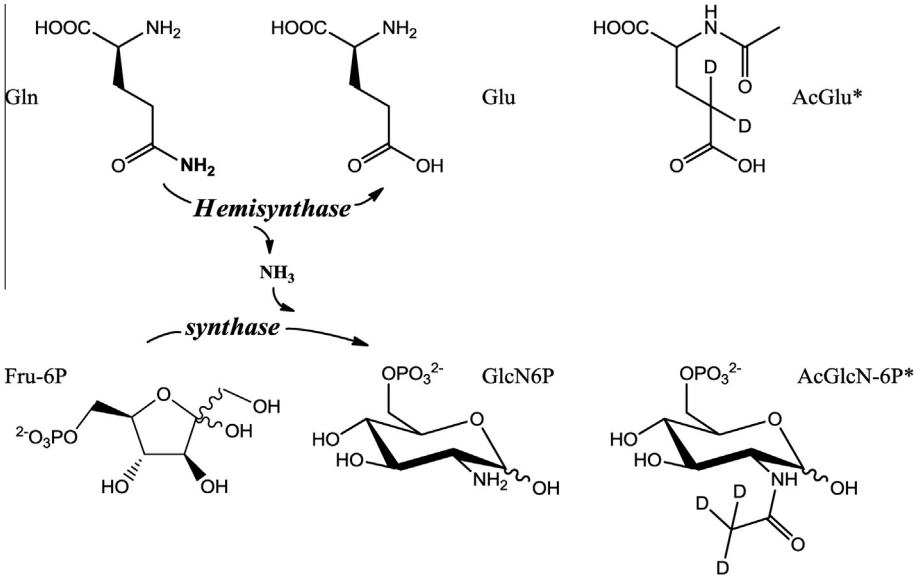

Scheme 1. Catalytic activity of GlmS and structures of the internal standard.

in enzymatic activity monitoring of the glucosamine-6-phosphate synthase [11] prompted us to investigate if such a strategy could be adapted to reach that goal. We previously demonstrated that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can be of great interest for monitoring enzyme-catalyzed production of GlcN-6P (synthase reaction) after *N*-acetylation. This derivation step was introduced to facilitate the discrimination by mass spectrometry between the formed GlcN-6P and the starting Fru-6P and led also to the use of a more accessible isotopic internal standard. Herein we report our attempts to extend this method for the simultaneous characterization of the hemisynthase and synthase activities by optimizing the sample preparation, especially the choice of the internal standard and the matrix.

Experimental

Chemicals

D-Glucosamine-6P, D-fructose-6P, L-glutamine, D,L-glutamate, acetic anhydride, 35% DCl solution in D_2O (99 at.% D), trimethylamine 33% ethanolic solution, TRIS (tris(hydroxymethyl)aminomethane), trihydroxyacetophenone, and 9-aminoacridine and solvents were purchased from Sigma-Aldrich. The 96-well PCR microplates and adhesive PCR film were purchased from Abgene. Water was purified by a Millipore water purification system. Glucosamine-6P synthase from *Escherichia coli* was obtained at a concentration of 6.6 mg ml^{-1} in phosphate buffer 50 mM, NaCl 150 mM, pH 7.2, with a specific activity of 7 U mg^{-1} according to the protocol reported by Obmolova et al. [20]. This GlmS solution was diluted just before use with TRIS buffer solution (20 mM, pH 7.2) in order to obtain a stock solution at a concentration of 0.04 µg/µl. NMR spectra were performed on Bruker Avance spectrometers operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR experiments and the chemical shifts are reported in parts per million relative to tetramethylsilane with the solvent resonance as the internal standard. Multiplicities were given as s (singlet); dd (doublets of doublet); m (multiplets), p (pintet). Coupling constants are reported as a *J* value in Hertz. High resolution mass spectroscopy (HRMS) data were conducted using a Water-Micromass mass spectrometer equipped with an ESI-TOF (electrospray-time-of-flight). HPLC analysis was performed on HILIC Kinetec column using Waters Alliance 2690 separation module equipped with mass spectrometer (Waters ZQ mass spectrometer with a single-quadrupole system and electrospray ionization), ELS detector (Waters 2420), and photodiode array detector (Waters 996). Melting points, measured in capillary tubes with a Büchi B-540 apparatus, are uncorrected.

Mass spectrometry

The trihydroxyacetophenone (THAP) matrix solution was freshly prepared prior to analysis at a concentration of 12 mg/ml in methanol/water (1/1, v/v). Solutions of 9-aminoacridine were freshly prepared either in methanol or in methanol/isopropanol (3/1, v/v) at three different concentrations (1, 5, and 10 mg/ml).

MS spectra were acquired on a Perspective Voyager DE-STR MALDI-TOF mass spectrometer (Perspective Biosystems, Framingham, MA, USA) equipped with a 337 nm pulse N_2 laser (20 Hz). Reflectron negative ion mode was used according to the following settings for THAP: accelerating voltage −20 kV, grid voltage 65% of accelerating voltage, extraction delay time of 100 ns, and low mass gate set at 160 Da. Each parameter was further optimized when using 9-aminoacridine as the matrix. The laser intensity was set just above the ionization threshold in order to obtain the best signal-to-noise ratio, mass resolution, and repeatability. Exact mass measurement was achieved using the signal of the deprotonated 9-aminoacridine at *m*/*z* 193.0766 as an internal calibrant. Experimental data retreatment consisting of rescaling, normalizing, baseline correction, and peak fitting using a Gaussian function was achieved using IGOR Pro 6 software (WaveMetrics, USA).

Internal standard synthesis

Synthesis of D-*N*-($^{13}C_2$)-acetylglucosamine-6P (AcGlcN-6P*) was performed as described elsewhere [11].

The *N*-acetyl-4,4-d^2-D,L-glutamate (AcGlu*) was obtained by acetylation of the known 4,4-d^2-D,L-glutamate hydrochloride [21] according to the following procedure. Acetic anhydride (2.20 ml, 23.0 mmol) was added dropwise to a cooled (0 °C) solution of 4,4-d^2-D,L-glutamate hydrochloride (2.16 g, 11.7 mmol) in water (16 ml) while maintaining the pH at 10 by the addition of 8 N NaOH solution. The mixture was stirred for 4 more hours at 0 °C before acidification (pH 3) by addition of concentrated HCl

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