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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

Florence Gaucher-Wieczorek, Vincent Guérineau, David Touboul *, Sophie Thétiot-Laurent, Franck Pelissier, Marie-Ange Badet-Denisot, Bernard Badet, Philippe Durand

Centre de recherche de Gif, Institut de Chimie des Substances Naturelles, CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

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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

* Corresponding author. Fax: +33 (0)1 69 07 72 47.
E-mail address: david.touboul@cnrs.fr (D. Touboul).

[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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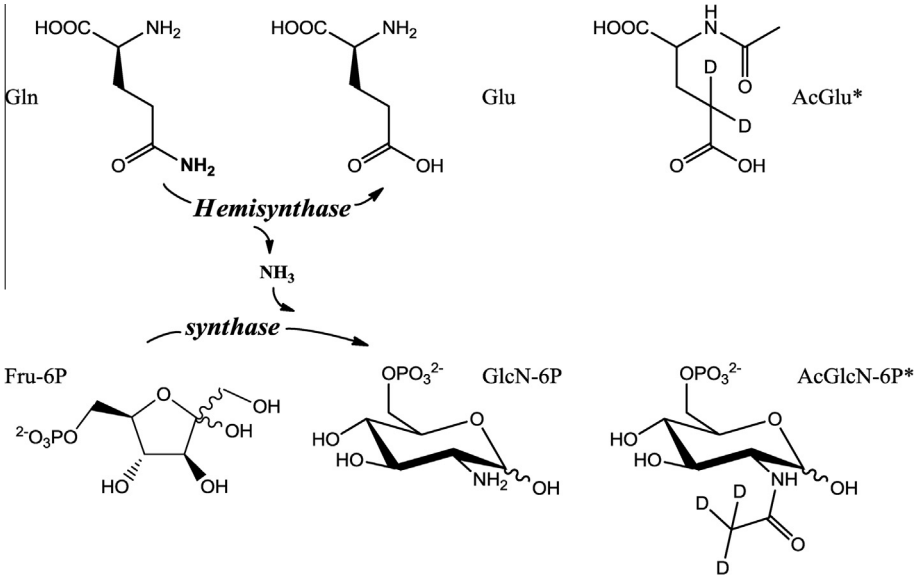

Scheme 1. Catalytic activity of GlmS and structures of the internal standard.

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 ^1H 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

(35% w/v). Water was evaporated under reduced pressure and coevaporated several times with methanol to give a white solid. The product was crystallized in water/ethanol mixture at 4 °C to give the sodium salt of acetylated glutamate AcGlu* as a white solid (1.56 g, 76%). Mp: 302-303 °C; ^{1}H (500 MHz, D_2O): δ 1.82 (dd, *J* = 9.0, 13.8 Hz, 1H), 1.98–2.02 (m, 1H), 1.99 (s, 3H), 4.07 (dd, *J* = 4.6, 9.0 Hz, 1H); ^{13}C (125 MHz, D_2O): δ 21.98, 28.40, 33.74(p), 55.33, 173.70, 179.02, 182.32; HRMS (ESI, *m*/*z*): calcd for $C_7H_8D_2NO_5$ $[M-H]^-$ 190.0685, found: 190.0681; LC-MS: A water solution of the solid (1 mg/ml) was acidified (pH 1) by addition of ion exchange resin AG 50x8 (H^+ form, 200–400 mesh) and filtrated before injection (5 µl), Rt = 3.1 min (see supplementary information).

Standard curves

Reactions were carried out in a 1.5 ml Eppendorf. For standard curve determination, 200 µl solutions of GlcN-6P and Glu were prepared in TRIS buffer solution (20 mM, pH 7.2) at final concentrations ranging from 0 to 750 µM. In order to reach a quantitative *N*-acetylation a two-step process was used. *Per*acetylation was first accomplished by treatment of the sample twice with 40 µl of a 0.5 M aqueous solution of trimethylamine and 20 µl acetic anhydride (10%, w/w) in dioxane for 2 h at room temperature. Selective hydrolysis of *O*-acetylated products was then performed by treatment with aqueous ammonia (26%, w/w, 10 µl) for 15 min. Standard solutions of AcGlu* and AcGlcN-6P* were finally added. Samples are mixed with matrix solution (1/9, v/v) and spotted on a Teflon-coated plate (AB Sciex) according to the dried-droplet method. For each condition, MS spectra were acquired in triplicate.

Enzyme assay

Enzyme assays were performed in a 96-well PCR microplate capped with adhesive PCR film and incubated in a PCR heating block (Mastercycler gradient; Eppendorf) for 20 min at 37 °C. For this purpose, 10 µl of the GlmS solution at 0.04 µg/µl was incubated with 40 µl of the saturating substrate at a concentration of 40 µM and increasing volume of a stock solution of the limiting substrate at a concentration of 20 mM. Tris buffer was added in order to reach a final volume of 200 µl.

The reaction was always initiated by the addition of GlmS at room temperature and was stopped by heating at 99 °C for 4 min followed by cooling at 4 °C for 5 min. The capped microplate was then centrifuged at 3000 tr min^{-1} for 5 min at 4 °C. The content of each well was transferred into 1.5 ml microtubes and treated as described for the standard curve. All experiments were carried out in TRIS buffer (20 mM, pH 7.2) with a total reaction volume of 200 µl.

Determination of the initial velocity phase

Initial velocity phase was determined using fixed concentrations of enzyme (0.002 µg/µl, 0.4 µg per well), Fru-6P (0.1 mM), and Gln (2 mM). Fru-6P is thus the limiting reactant and enzyme kinetic is followed by measuring GlcN-6P concentration at various time reactions (7, 10, 13, 16, 20, 30, and 40 min). AcGlcN-6P* is used as internal standard and GlcN-6P is determined using the standard curve.

Determination of kinetics parameters for hemisynthase and synthase activities

Michaelis constant K_m and maximal velocity V_{max} of Gln were determined by using a large excess of Fru-6P (4 mM) whereas the concentration of Gln was varied from 0 to 4 mM. The kinetic constants of Fru-6P were determined with excess of Gln (2 mM) whereas concentrations of Fru-6P were varied from 0 to 4 mM.

Results and discussion

Selection of internal standard and matrix for the determination of the calibration curves

With the aim of developing an assay for the simultaneous monitoring of hemisynthase and synthase activities of GlmS, we quantified the formed glutamate by MALDI-TOF-MS analysis using the method previously established for GlcN-6P. This latter was performed after *N*-acetylation of GlcN-6P using *N*-($^{13}C_2$) acetylglucosamine-6P as internal standard and trihydroxyacetophenone (THAP) as matrix. We have since improved the method by substituting 9-aminoacridine (9-AA) for THAP. This matrix was first described by the group of Hercules [22] as suitable for the analysis of low molecular weight compounds in the negative ion mode. In our case it led to very homogenous deposition exhibiting little interferences in the very low *m*/*z* range. Hence, we first envisaged using this matrix and 2,4,4-d^3-*N*-acetyl-D,L-glutamic acid as internal standard for the new assay. This compound was easily obtained by acetylation of commercially available 2,4,4-d^3-D,L-glutamic acid. However the overlap between the 2,4,4-d^3-*N*-acetyl-D,L-glutamate ion (*m*/*z* 191.0747) and a matrix interference probably corresponding to the [9-AA-3H]$^-$ ion (*m*/*z* 191.0609) makes it impossible to monitor enzyme activity in the negative ion mode. We thus decided to move to 4,4-d^2-*N*-acetyl-D,L-glutamic acid as internal standard. It was obtained in a two-step process from D,L-glutamic acid involving first a selective deuteration with aqueous DCl as reported by Ogrel et al. [21] followed by *N*-acetylation under basic conditions. Fig. 1 shows the MALDI-TOF-MS spectrum of Glu and GlcN-6P solution at 200 µM after acetylation reaction, addition of standard calibrants, and final mix with 9-AA in the negative ion mode. Signals corresponding to the deprotonated species are detected with reasonable signal-to-noise (S/N) ratio and without any interference due to the matrix or the buffer. In order to reach the best sensitivity and reproducibility, crystallization time was varied from 5 to 15 min and the ratio between sample and matrix solution (1 mg/ml in methanol) volumes was varied from 1/1 to 1/9 (v/v). Best results were obtained by minimizing crystallization time and for a 1/9 (v/v) ratio. In that case, homogenous samples on the MALDI plate were obtained leading to a decrease of the

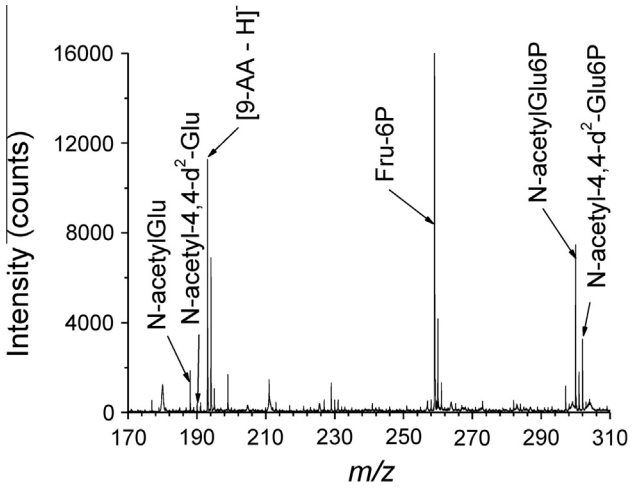

Fig.1. MALDI-TOF mass spectrum in the negative ion mode for a solution of AcGlu (500 µM) and AcGlcN-6P (500 µM) mixed with the two isotope-labeled calibrant in the presence of an excess of Fru-6P (2 mM).

interspectrum intensity variation. Moreover, the laser fluence was fixed just above the ionization threshold in order to reach the best repeatability [23].

To test the linearity of our method, two calibration curves were determined by using seven different concentrations of each analyte over one decade analyzed in triplicate and by plotting the ratio between light and heavy isotopes of AcGlu or AcGlcN-6P versus the initial concentration of Glu or GlcN-6P, respectively (Fig. 2). Linear responses were obtained with a coefficient of determination (R^2) greater than 0.985. Final calibration curves were also realized in the presence of a large excess of Gln and Fru-6P, i.e., 2 and 4 mM, respectively, corresponding to the initial conditions of the enzyme assay (Fig. 2). Slopes and intercepts were not dramatically modified and linear correlation coefficients remained higher than 0.985. This indicates a good robustness of the quantification method by MALDI-TOF-MS.

Determination of the initial velocity phase and Michaelis constants

The initial velocity phase was determined by dosing GlcN-6P formed by the action of GlmS (0.004 μg/μl, 0.4 μg per well) on Fru-6P (0.1 mM) and Gln (2 mM, in excess) after different incubation times (Fig. 3). Experiments showed that product concentration increases linearly with time until 20 min whereas a plateau is reached after 25 min. Thus the incubation time was fixed at 15 min for the following experiments.

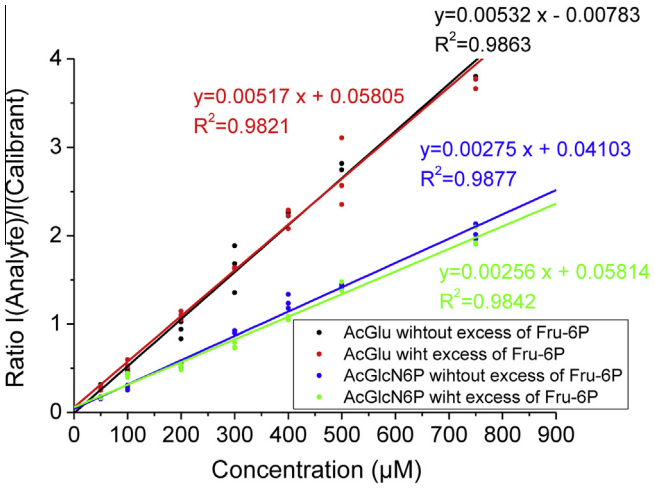

Fig.2. Calibration curve for AcGlu and AcGlcN-6P with or without an excess of Fru-6P.

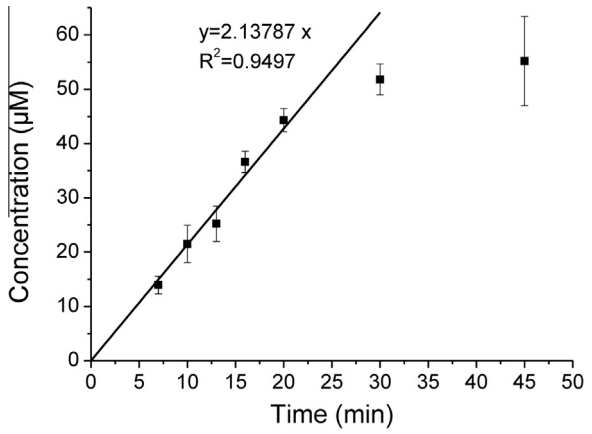

Fig.3. Concentration of GlcN-6P formed by the bioconversion of Fru-6P (0.1 mM) and Gln (2 mM) by GlmS (0.004 μg/μl) for different incubation times.

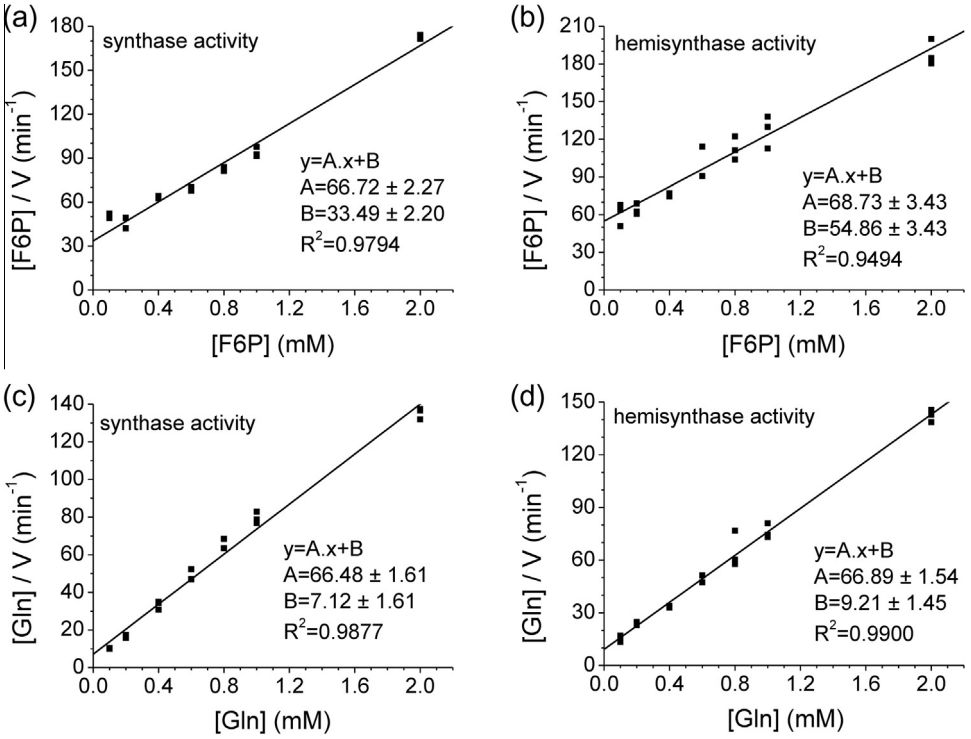

Fig.4. Hanes-Woolf plots for the synthase and hemisynthase activities. (a and c) Following formation of GlcN-6P at a fixed concentration of Gln (2 mM) and Fru-6P (4 mM), respectively; (b and d) following formation of Glu at a fixed concentration of Gln (2 mM) and Fru-6P (4 mM), respectively.

Table 1
Experimental and tabulated kinetic parameters for GlmS.

	Synthase	Literature [1]	Hemysynthase	Literature [1]
$K_m^{Fru\text{-}6P}$ (mM)	0.50 ± 0.05	0.25 – 0.99	0.80 ± 0.09	
$V_{max}^{Fru\text{-}6P}$ (μM min^{-1})	15.01 ± 0.51		14.59 ± 0.37	
k_{cat} (min^{-1})	1014 ± 35	480 – 930	985 ± 49	
K_m^{Gln} (mM)	0.11 ± 0.03	0.10 – 0.20	0.14 ± 0.02	0.2 – 0.3
V_{max}^{Gln} (μM min^{-1})	15.05 ± 0.36		14.96 ± 0.34	
k_{cat} (min^{-1})	1017 ± 25		1010 ± 23	931 – 1030

The first set of data was acquired at a fixed concentration of Fru-6P, i.e., 4 mM, whereas the concentration of Gln varied from 0 to 2 mM. GlcN-6P and Glu product concentrations were simultaneously measured after *N*-acetylation reaction and heavy isotope standard addition. This experiment allowed us to determine the kinetic parameters V_{max}^{Gln} and K_m^{Gln} for hemisynthase activity using a Hanes-Woolf representation which linearizes the Michaelis–Menten equation (Fig. 4). In the same way, the kinetic parameters $V_{max}^{Fru\text{-}6P}$ and $K_m^{Fru\text{-}6P}$ for synthase activity were determined from a set of data acquired at a fixed concentration of Gln, i.e., 2 mM, whereas concentrations of Fru-6P varied from 0 to 2 mM (Fig. 4). The values found with this MS-based assay (Table 1) are in very good agreement with reported data [1]. MALDI-TOF MS can thus be considered as a promising alternative to standard analytical methods for the kinetic parameter determination of multisubstrate enzymes.

Conclusion

We demonstrated for the first time that MALDI-TOF MS can be efficiently used for simultaneous monitoring the two products of a bisubstrate enzyme due to its selectivity and tolerance to a complex biological environment. It does not require complicated sample pretreatment procedures and even the enzyme reaction solution could be directly analyzed without chromatographic separation. Quantitative assay can be achieved using standards containing heavy isotopes (^2H and ^{13}C) by a careful control of sample preparation (choice of the matrix, solvent, crystallization time) and analysis (laser intensity, number of laser shots, etc.). Process automation will enable us to screen GlmS ligands and to separately characterize their effect on synthase and hemisynthase activities. This methodology could be easily applied to other multisubstrate enzyme reactions such as those catalyzed by kinases or methyltransferases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.04.033.

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