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Kinetic measurements of phosphoglucose isomerase and phosphomannose isomerase by direct analysis of phosphorylated aldose–ketose isomers using tandem mass spectrometry

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Abstract

A mass spectrometry based method for the direct determination of kinetic constants for phosphoglucose isomerase (PGI) and phosphomannose isomerase (PMI) is described. PGI catalyzes the interconversion between glucose-6-phosphate (Glc6P) and fructose-6-phosphate (Fru6P) and PMI performs the same function between mannose-6-phosphate (Man6P) and Fru6P. These two enzymes are essential in the pathways of glycolytic or oxidative metabolism of carbohydrates and have been considered as potential therapeutic targets. Traditionally, they are assayed either by spectrophotometric detection of Glc6P with one or more coupling enzymes or by a colorimetric detection of Fru6P. However, no suitable assay for Man6P has been developed yet to study the reaction of PMI in the direction from Fru6P to Man6P. In the work presented herein, a general assay for the isomeric substrate-product pair between Glc6P and Fru6P or between Man6P and Fru6P was developed, with the aim of directly studying the kinetics of PGI and PMI in both directions. The 6-phosphorylated aldose and ketose isomers were distinguished based on their corresponding tandem mass spectra (MS^2) obtained on a quadrupole ion trap mass spectrometer, and a multicomponent quantification method was utilized to determine the composition of binary mixtures. Using this method, the conversion between Fru6P and Glc6P and that between Fru6P and Man6P are directly monitored. The equilibrium constants for the reversible reactions catalyzed by PGI and PMI are measured to be 0.3 and 1.1, respectively, and the kinetic parameters for both substrates of PGI and PMI are also determined. The values of K_M and V_{max} for Fru6P as substrate of PMI are reported to be 0.15 mM and 7.78 μmol/(min mg), respectively. All other kinetic parameters measured correlate well with those obtained using traditional methods, demonstrating the accuracy and reliability of this assay.

Keywords: Phosphoglucose isomerase; Phosphomannose isomerase; Kinetic measurements; Aldose–ketose isomers; Tandem mass spectrometry

1. Introduction

The aldose–ketose isomerases catalyze the interconversion of isomeric aldo and keto sugars by causing the migration of a hydrogen between the C1 and C2 position [1]. They are further classified into two groups according to their action on free or phosphorylated monosaccharide substrates. Those working on phosphorylated monosaccharides are essential in the pathways of glycolytic or oxidative metabolism of carbohydrates [1–3]. For example, phosphoglucose isomerase (PGI) catalyzes the interconversion between fructose-6-phosphate (Fru6P) and glucose-6-phosphate (Glc6P), the second step in glycolysis, and is a housekeeping enzyme of metabolism. It is also important for gluconeogenesis, membrane protein glycosylation, and the pentose phosphate pathway [4]. Due to the differences between human and parasitic glycolytic pathways and because of the dependence of the parasite on glycolysis, PGI has been studied as a potential target for the design of novel inhibitors against parasites [5,6]. Regulation of PGI activity is also important because a deficiency in PGI activity in human leads to non-spherocytic

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hemolytic anemia [7,8], and high levels of PGI activity are measured in the sera of patients with certain cancers [9].

Phosphomannose isomerase (PMI), another important enzyme in this class, catalyzes the interconvertion of Fru6P and mannose-6-phoshate (Man6P). In mammalian cells, PMI is responsible for channeling Man6P into the glycolytic pathway through its conversion to Fru6P [10]. In all eukaryotes and prokaryotes, this reaction is the initial committed step for the supply of the activated mannose donor guanosine diphosphate D-mannose, which is required for the biosynthesis of many mannosylated structures, including glycoproteins, glycolipids, and cell-wall components in microorganisms such as fungi [11–15]. Therefore, PMI seems a likely target for drugs against fungi [15,16], whose infection can lead to serious illness and death in immunosuppressed individuals. Recent research indicates that a deficiency of PMI is responsible for a human disease, congenital disorder of glycosylation 1b (CDG-1b) [17,18]. CDG-1b results from hypoglycosylation of serum and other glycoproteins, and is diagnosed with congenital hepatic fibrosis and protein-losing enteropathy.

Because of their biological significance as summarized above, activity assays and mechanistic studies of PGI, PMI and other related phosphorylated aldose–ketose isomerases have been of interest to researchers for several decades. Thus far, PGI has been mainly assayed by two methods. The first one is by colorimetric estimation of Fru6P in a chemical stop assay in the forward direction [19]; the second method, which is used more frequently, is by a continuously recording spectrophotometric assay of Glc6P in the reverse direction with glucose-6-phosphate dehydrogenase as the coupling enzyme [20]. Likewise, Dyson and Noltmann [21] described a convenient continuously recording pH-stat assay for Fru6P, utilizing phosphofructokinase as the indicator enzyme. For PMI, all of the previous kinetic assays were realized by monitoring the conversion from Man6P to Fru6P in the forward direction, either by the spectrophotometric method together with two coupling enzymes, glucose-6-phophate isomerase and glucose-6-phosphate dehydrogenase [22], or by the discontinuous colorimetric assay for keto sugars when desired conditions would interfere with the spectrophotometric measurement [23]. Recently, Tureček and co-workers [24] reported an affinity capture and elution/electrospray ionization (ESI) mass spectrometry based assay for PMI. In their method, the product isomer Fru6P was subjected to another coupling enzyme, yeast transketolase, to alter its mass and thus differentiate it from the substrate isomer Man6P by mass spectrometry. Kinetic constants have not yet been determined in the direction from Fru6P to Man6P because of the lack of a convenient assay for Man6P. Although the continuous nature of the coupling enzyme assay is an advantage, the major limitation is the requirement for highly purified coupling enzymes. In addition, potential errors in kinetic and mechanism studies exist due to the interference by the large number of intermediates formed in the sequence of reactions or by the coupling enzymes themselves. The colorimetric assay on the other hand, is limited by its time consumption and high background. Given the above reasons, a general and direct assay for phosphorylated aldo–keto sugars would be advantageous to study the kinetics and mechanisms of isomerases.

Direct quantitative analysis of phosphorylated monosaccharides has been reported using high performance anion-exchange chromatography combined with a pulsed amperometric detection [25]. With this method, Fru6P can be separated from Glc6P or Man6P, and the assay sensitivity is in the micro-molar range. However, the total assay time, over 50 min for each run, is quite long for a kinetic assay. Our laboratory has been working on developing different mass spectrometry based assays to directly study steady state kinetics and mechanisms of various enzyme systems [26–33]. Using single ion monitoring to follow the formation of products during the reaction process, the kinetic parameters of a sulfurtransferase [27,33] and a phosphotransferase [29] have been successfully measured. A similar methodology was applied to follow the process of a multi-enzyme system, in which reaction intermediates and products at different stages were monitored simultaneously based on their masses [32]. To study enzymatic reactions where substrates and products are isomers is more challenging for mass spectrometry based assays. Recently, we reported a methodology to quantify positional isomers of phosphorylated monosaccharides using ion/molecule reactions and FT-ICR mass spectrometry [28]. This assay was successfully applied to study kinetics of phosphoglucomutase, which interconverts of two positional isomers, glucose-1-phosphate and glucose-6-phosphate [31]. In the current study, a novel mass spectrometry based assay was developed to differentiate the isomeric aldose and ketose 6-phosphates by their MS^2 spectra. Similar to those described previously for quantification of phosphorylated positional isomers [28] and diastereomers of hexosamines [34], *N*-acetylhexosamines [35] and sulfated disaccharides [36,37], a multicomponent quantification method was utilized to determine the composition of binary mixtures. Using this strategy, the equilibrium constants of the reversible reactions catalyzed by PGI and PMI were measured, and their kinetic parameters, K_M's and V_{max}'s for both directions were obtained, with those for PMI in the direction from Fru6P to Man6P being the first values reported.

2. Experimental

2.1. Materials

Glucose-6-phosphate, mannose-6-phosphate and fructose-6-phosphate were all purchased from Sigma Chemical Company (St. Louis, MO). Yeast phosphoglucose isomerase (in lyophilized form) and *Escherichia coli* phosphomannose isomerase (as a suspension in ammonium sulfate) were also provided by Sigma (St. Louis, MO). These enzymes were directly used after dilution for kinetic assays without any fur-

ther purification. All other chemicals are of analytical grade and all solvents are of HPLC grade.

2.2. Instrumentation

All experiments were performed on a Thermo Finnigan (San Jose, CA) LCQ classic quadrupole ion trap mass spectrometer equipped with an electrospray ionization source in the negative ion mode. The samples were injected through a 5 μL injection loop and delivered at a flow rate of 20 μL/min by using a Harvard syringe pump. The capillary temperature and the spray voltage were kept at 200 °C and 3.6 kV, respectively. For MS^2 experiments, the precursor ions were selected using an isolation width of 1 Da, and activated at 27% normalized collision energy for 50 ms unless noted otherwise. The chromatogram in the Qual Browser program (Thermo Finnigan) was used to monitor the delivery and ionization of each sample. The relative abundance of each product ion was determined as the integration peak area obtained from the extracted ion chromatogram of each ion divided by the integration peak area obtained from the corresponding total ion chromatogram. The integration was realized through the ICIS algorithm on the Qual Browser program, and the same integration parameters were used for all chromatograms. Each sample was injected three times and the average value was used in the subsequent calculations.

2.3. Quantification

A procedure similar to those reported previously [28,31] was used here to quantify phosphorylated aldose–ketose isomers. Briefly, the percent total ion currents of each diagnostic ion (*m*/*z* 169 and 199 for this system) were measured for the two isomers, a 1:1 mixture of the two and any unknown mixture. These values were then applied to a two-equation, two-unknown system as shown below:

$$aI_{169,K} + bI_{169,A} = I_{169} \quad (1)$$

$$aI_{199,K} + bI_{199,A} = I_{199} \quad (2)$$

here, *a* and *b* calculated from Eqs. (1) and (2) represent the raw percentages for the ketose isomer and the aldose isomer, respectively. The constants $I_{169,K}$ and $I_{169,A}$ refer to the percent total ion current measured of the *m*/*z* 169 ion for pure ketose-6-P and pure aldose-6-P standards, respectively. Similarly, $I_{199,K}$ and $I_{199,A}$ refer to the corresponding values measured of the *m*/*z* 199 ion for the two standards. I_{169} and I_{199} are the values measured for an unknown mixture of the two isomers. Through this system of equations, the raw percentage of each isomer, *a* and *b*, can be calculated. In order to determine the final composition of a mixture, two additional normalizations are introduced as before [28,31]. The first normalization takes into account the bias effect of the instrumental response towards each isomer, which may come from a slight difference in ionization efficiency. To correct for this bias effect, a 1:1 mixture is analyzed, and the calculated values of *a* and *b* for this mixture are divided by the actual percentage (0.5) in order to obtain the normalization factors for each isomer, i.e., $N1_K = a(1{:}1)/0.5$; $N1_A = b(1{:}1)/0.5$. The calculated *a* and *b* for other mixtures are then divided by $N1_K$ and $N1_A$ to obtain a' and b', i.e., $a' = a/N1_K$ and $b' = b/N1_A$. The second normalization ensures that %K + %A = 100%; therefore the final percentages a'' and b'' can be expressed as: $a'' = a'/(a' + b')$; $b'' = b'/(a' + b')$.

2.4. Enzyme kinetic assay

For kinetic measurements of PGI and PMI, different concentrations of either substrate were prepared in 50 mM NH_4OAC, pH 7.4 at room temperature. The reaction was initiated by adding aliquots of enzyme solution of known concentration. At different time intervals, aliquots were withdrawn from the reaction mixture and quenched into three volumes of MeOH. For those solutions with a total concentration higher than 100 μM, an appropriate volume of 1:1 MeOH:H_2O solution was added to dilute the concentration to within 100 μM. The quenched solutions were then delivered to the LCQ mass spectrometer to determine their compositions as described above. These data were then converted to reaction velocities as will be documented in the following section. To obtain kinetic parameters, experimental data were fitted to theoretical equations using Grafit software. All values are reported as the average of at least three replicate experiments.

3. Results and discussion

3.1. Differentiation of 6-phosphorylated aldose–ketose isomers through the use of MS^2 spectra

The structures of 6-phosphorylated monosaccharides investigated in this study are shown in Fig. 1, and their corresponding MS^2 spectra are shown in Fig. 2. Upon collision induced dissociation, several product ions are observed in addition to the precursor ion at *m*/*z* 259: the ions at *m*/*z* 241 and 223 correspond to loss of one and two water molecules, respectively. The ions at *m*/*z* 199, 169 and 139 result from cross ring cleavage with loss of $C_2H_4O_2$, $C_3H_6O_3$, and $C_4H_8O_4$. Ions corresponding to phosphate species, $H_2PO_4^-$ (*m*/*z* 97) and PO_3^- (*m*/*z* 79) were also observed when scanning to a lower mass range (data not shown). Comparing the MS^2 spectra of ketose-6-phosphate (Fru6P) with that of aldose-6-phosphate (Glc6P and Man6P), an obvious difference was observed in the relative abundance of the product ion at *m*/*z* 199 versus that at *m*/*z* 169: for Fru6P, the ion at *m*/*z* 169 is far more predominant than the ion at *m*/*z* 199. Formations of the *m*/*z* 169 and 199 ions for each phosphorylated monosaccharide are indicated in Fig. 1. Consequently, the *m*/*z* 199 and 169 ions can be taken as the diagnostic ions for the aldose-6-phosphate and ketose-6-phosphate isomer, respectively, and thus a binary mixture containing Glc6P and Fru6P or Man6P

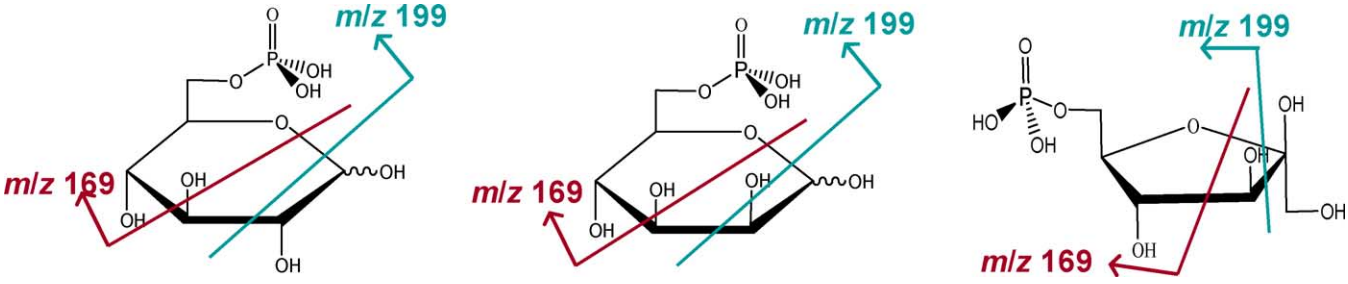

Fig. 1. Structure and dissociation pathway of the three phosphorylated mannosaccharide.

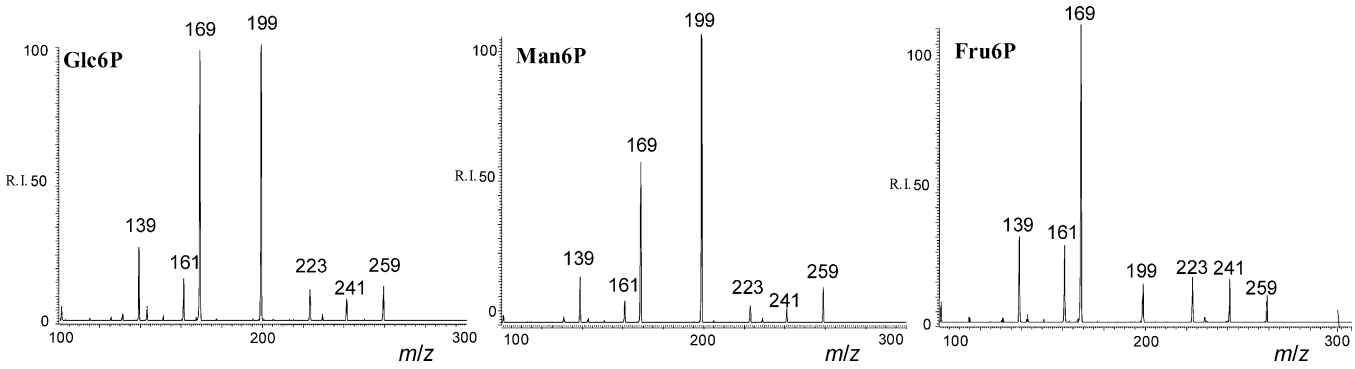

Fig. 2. MS^2 spectra of the three phosphorylated mannosaccharide, instrumental conditions are described in Section 2.

and Fru6P can be quantified for the purpose of direct kinetic analysis of PGI and PMI.

Since differentiation between the 6-phosphorylated aldose–ketose isomers was realized through collision-induced dissociation, the influence of dissociation efficiency on the relative abundance of each diagnostic product ion was investigated. For that purpose, the three isomers were analyzed at different dissociation efficiencies as determined by relative collision energy (CE) and activation time (AT). According to the results shown in Table 1, there was little variation in the relative abundance of the two diagnostic ions (I_{169}/I_{199}) with collision energy and activation time under the conditions tested. This indicates that the distinction between the aldose and ketose isomers obtained through CID was not affected to an apparent extent by the dissociation efficiency. Furthermore, the reproducibility of MS^2 spectra in terms of percent total ion current for each diagnostic ion was also investigated with the three standard compounds. It was found that under the same collision energy and activation time (see Section 2), the product ion distribution of the resulting MS^2 spectra was very reproducible. As listed in Table 2, with a relative collision energy of 27% and an activation time of 50 ms used, the standard deviations in percent total ion current for each diagnostic ion remain less than 2% for all three isomers. Therefore, arbitrary experimental conditions of 27% collision energy and 50 ms activation time were chosen and remained the same throughout the following experiments.

Table 1
MS^2 spectra variation at different dissociation conditions

	I_{169}/I_{199}		
	Glc6P	Man6P	Fru6P
CE = 24%, AT = 30 ms	0.901	1.644	0.125
CE = 26%, AT = 30 ms	1.086	1.873	0.148
CE = 28%, AT = 30 ms	1.087	1.933	0.151
CE = 28%, AT = 50 ms	0.999	1.779	0.141
CE = 28%, AT = 100 ms	0.940	1.622	0.131

3.2. Quantification of binary mixtures between 6-phosphorylated aldose–ketose isomers

To demonstrate the quantification accuracy for biological samples, a series of mock mixtures composed of Glc6P/Fru6P or Man6P/Fru6P with different compositions at 50 μM total concentration were prepared in the same buffer conditions as would be used for enzymatic analysis. These mock mixtures were analyzed on the LCQ and the measured percent total

Table 2
Reproducibility of product ion distribution for 6-phosphorylated monosaccharide

Samples		Percent total ion current			Average	S.D.
		1	2	3		
Glc6P	% *m/z* 169	31.91	31.43	31.98	31.77	0.3
	% *m/z* 199	33.36	34.39	34.24	34.00	0.55
Man6P	% *m/z* 169	25.12	24.63	24.99	24.91	0.25
	% *m/z* 199	46.58	45.69	47.22	46.50	0.77
Fru6P	% *m/z* 169	46.45	44.63	46.78	45.95	1.16
	% *m/z* 199	7.55	7.74	6.79	7.36	0.5

Table 3
Quantification of mock mixtures

	% *m/z* 169	% *m/z* 199	Percentage after normalization		Difference (calculated − actual)
			a′	*b*′	
Fru6P:Glc6P					
5:95	32.47	32.66	4.5	95.5	−0.5
10:90	33.55	30.22	12.3	87.7	2.3
25:75	34.62	28.98	17.4	82.6	−2.6
45:55	38.62	22.62	40.5	59.5	0.5
55:45	39.73	16.53	59.6	40.4	−0.4
75:25	41.87	11.73	77.6	22.4	−2.4
90:10	43.10	8.86	88.7	11.3	−1.3
95:5	43.86	7.30	94.9	5.1	−0.1
Fru6P:Man6P					
5:95	26.34	45.14	6.4	93.6	1.4
10:90	27.36	43.95	11.1	88.9	1.1
25:75	29.12	40.35	21.3	78.7	1.3
45:55	32.03	33.88	38.4	61.6	−1.6
55:45	36.92	25.22	61.0	39.0	1.0
75:25	41.41	16.46	81.0	19.0	1.0
90:10	43.85	11.41	91.7	8.3	1.7
95:5	44.68	9.19	96.2	3.8	1.2

ion currents of the two diagnostic ions and the calculated compositions of these mixtures are shown in Table 3. It can be seen that the calculated Fru6P percentages were quite close to the actual ones with an absolute deviation of less than 3% for both Glc6P/Fru6P and Man6P/Fru6P systems.

All of the above mock mixtures tested were made to the same concentration of 50 μM. However, in the case of enzyme kinetic measurements, the concentration of the substrate is varied in a range wide enough to determine the value of the Michaelis–Menten constant, K_M. Therefore, it is important to test whether the assay described above can also be applied to mixtures with different total concentrations. Table 4 lists the percents of total ion current for the ions at *m/z* 199 and 169 obtained for a series of 10:90 Fru6P:Glc6P mixtures and 20:80 Fru6P:Man6P mixtures with the concentration varied from 5 to 100 μM (values refer to the concentration of total isomers in buffer before dilution with MeOH, see Section 2). Also tabulated in Table 4 are the calculated percentages for each mixture. Although the absolute values of percent total ion current for the two diagnostic ions decrease at lower concentration, the ratio of the two remains constant throughout this concentration range, and therefore the percentage calculated through the two-equation, two-unknown system is quite precise. The data in Table 4 indicate that for the 10:90 Fru6P:Glc6P samples, there is a standard deviation of less than 0.3% among the calculated values of Fru6P% at different concentrations, and the average difference from the actual value is 2.2%. Comparably, for the 20:80 Fru6P:Man6P samples, a standard deviation of 1.1% and an average difference of 1.6% for values of Fru6P% were obtained. The

Table 4
Quantification of mock mixtures with different concentration

Concentration (μM)	Percent of total ion current		$R = I_{199}/I_{169}$	Calculated % of Fru6P
	m/z 169	*m/z* 199		
10:90 Fru6P:Glc6P				
5	26.46	18.96	0.72	12.6
10	37.36	27.19	0.73	12.1
20	38.04	27.97	0.74	11.7
50	37.48	27.28	0.73	12.1
100	38.81	28.11	0.72	12.2
S.D.			0.01	0.29
Av. diff.				2.2
20:80 Fru6P:Man6P				
5	24.76	35.92	0.69	22.3
10	25.38	36.31	0.70	22.0
20	27.99	40.21	0.70	19.8
50	29.96	43.00	0.71	21.7
100	29.49	42.05	0.70	22.4
S.D.			0.004	1.1
Av. diff.				1.6

quantification method is thus demonstrated to be unaffected by the concentration of the mixture from 5 to 100 μM under optimized conditions.

3.3. Measurements of equilibrium constants

The value of K_{eq} for the reversible reaction catalyzed by PGI as shown in Eq. (3) has been reported previously to be 0.30–0.32 both by direct measurement of the equilibrium concentrations of Glc6P and Fru6P with the methods discussed earlier and by calculation from the Haldane relationship [38]. For the conversion between Man6P and Fru6P catalyzed by PMI as shown in Eq. (4), the value of K_{eq} was calculated to be 1.03 as the equilibrium product–substrate concentration ratio for the forward reaction [1]. The concentration of Fru6P was measured by colorimetric assay, and that of Man6P was estimated as the difference between the initial Fru6P concentration and the Fru6P concentration at equilibrium. Since the composition of the equilibrium mixture can be easily and directly determined using our methodology, the measurements of K_{eq} for both reactions catalyzed by PGI and PMI were conducted and compared with previous values.

$$\text{Glc6P} \underset{\text{r}}{\overset{\text{f}}{\rightleftarrows}} \text{Fru6P} \quad (3)$$

$$\text{Man6P} \underset{\text{r}}{\overset{\text{f}}{\rightleftarrows}} \text{Fru6P} \quad (4)$$

For this purpose, several experiments were performed, in which the initial substrates were altered such that the forward and reverse reaction were each monitored individually. Subsequent to this, an equal molar mixture of the two substrates was also analyzed. If the equilibrium is reached, the same final mixture composition would be obtained regardless of the initial substrates used. On the other hand, if the reaction stops before equilibrium due to the loss of enzyme activity, different initial substrates would generate different final mixture compositions. For PGI, three different reaction mixtures were prepared: one containing 1 mM Glc6P, one with 1 mM Fru6P and one containing a mixture of 0.5 mM Glc6P and 0.5 mM Fru6P. Similarly, three reaction mixtures were prepared for PMI in the same substrate compositions as for the PGI reactions. Adding PGI and PMI to the corresponding reaction mixtures provides a final concentration of 80 and 320 ng/mL, respectively. After reacting overnight, the mixtures were quenched, and analyzed on the LCQ to obtain mixture compositions. Using the above quantification methodology, the percentages of each isomer (a'' and b'') in the equilibrium mixture were determined and are listed in Table 5. K_{eq} was therefore calculated as the corresponding ratio between the two values. As shown in Table 5, the values of K_{eq} for PGI and PMI calculated by the three different measurements agree very well with each other, which demonstrate the precision of the equilibrium condition. An average K_{eq} value of 0.31 ± 0.01 was calculated for PGI and that for PMI was calculated to be 1.10 ± 0.04. Both agree very well with those values reported previously [1,38], which attests to its accuracy.

3.4. K_M and V_{max} measurements for both substrates of PGI

With percentages for each isomer determined using the above quantification methodology in the progress of the enzymatic reactions, the steady-state kinetics of reactions catalyzed by PGI and PMI could be studied. Beginning with either isomer at an initial substrate concentration of S_0, the reaction is quenched after a reaction time t, and analyzed to obtain the percentages of both isomers (a'' and b'') in the reaction mixture. The product concentration, P, is calculated by multiplying S_0 and the calculated percentage of the product isomer. For PGI, the value of K_{eq} in the forward reaction is approximately 0.3, which means that the reverse direction is thermodynamically more favorable. It is, therefore, easier to control the initial velocity state condition when Fru6P is used as the initial substrate. Before the K_M and V_{max} parameters can be determined, a time curve (P versus t) was recorded for both the lowest (0.05 mM) and highest (1.6 mM) concentrations of Fru6P (data not shown). Based on that result, the appropriate quenching time (within linear region of the time curve) was chosen for every substrate concentration to ensure initial velocity conditions. To calculate the values of K_M and V_{max}, the same methodology as described previously for the kinetic assay of PGM was employed, in which the average velocity was plotted against the average substrate concentration

Table 5
Data for K_{eq} measurements of PGI and PMI

PGI	From 1 mM Glc6P	From 1 mM Fru6P	From 0.5 mM Glc6P + 0.5 mM Fru6P
Fru6P% (a'')	23.64	22.82	24.05
Glc6P% (b'')	76.36	76.18	75.95
K_{eq} (a''/b'')	0.30	0.31	0.32
PMI	**From 1 mM Man6P**	**From 1 mM Fru6P**	**From 0.5 mM Man6P + 0.5 mM Fru6P**
Fru6P% (a'')	52.57	52.95	51.23
Man6P% (b'')	47.43	47.05	48.77
K_{eq} (a''/b'')	1.11	1.13	1.05

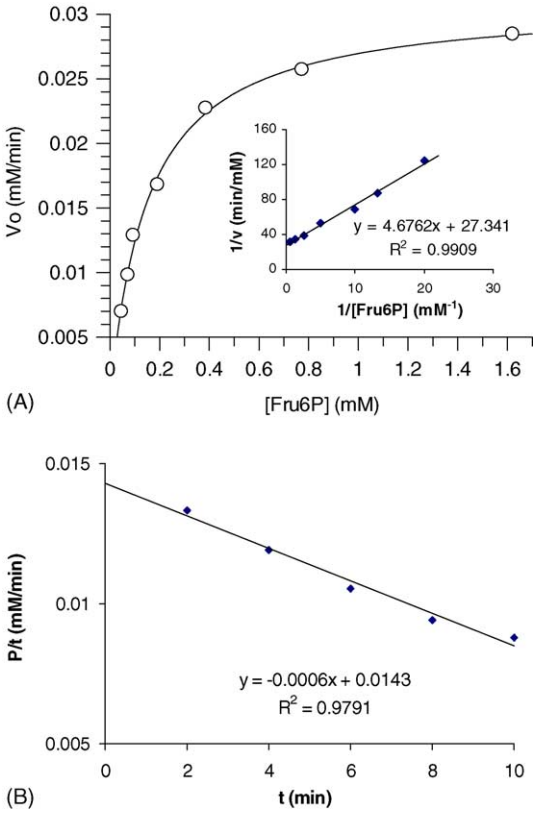

Fig. 3. Kinetic measurement of PGI. (A) Saturation plot of Fru6P with Lineweaver–Burk plot shown in the inset, Fru6P concentration was varied from 0.05 to 1.6 mM, final PGI concentration was 167 ng/mL; (B) P/t vs. t plot for Glc6P, Glc6P concentration was 2 mM, final PGI concentration was 117 ng/mL.

according to the following equation:

$$\bar{v}_0 = \frac{V_{\max} \times \bar{S}}{K_M + \bar{S}} \quad (5)$$

where the average velocity $\bar{v}_0$ could be approximated as P/t, and $\bar{S}$ is the arithmetic average substrate concentration over the course of the reaction. The saturation plot for PGI with Fru6P as the initial substrate is shown in Fig. 3A with the corresponding Lineweaver–Burk plot as the inset. By fitting the data with Eq. (5) using Grafit, the value of K_M for Fru6P was calculated to be 0.147 ± 0.006 mM, which agrees with the reported literature values of 0.15 mM [39–41]. $V_{\max}$ in the reverse direction was calculated to be 35.4 ± 2 μM/min. With the final enzyme concentration of 167 ng/mL, the specific activity is 212.6 ± 12.6 μmol/(min mg), which agrees with the value of 200 μmol/(min mg) obtained with the UV-coupling enzyme assay carried out in our laboratory (data not shown).

To measure the K_M and $V_{\max}$ in the forward reaction with Glc6P as the initial substrate, the following Haldane relationship was utilized:

$$K_{eq} = \frac{V^f_{\max} K_{M(Fru6P)}}{V^r_{\max} K_{M(Glc6P)}} \quad (6)$$

According to the Haldane relationship, $K_{M(Glc6P)}$ can be determined providing all the other four parameters are known. $V^r_{\max}$, $K_{M(Fru6P)}$ and K_{eq} have already been determined as described above. In order to measure $V^f_{\max}$, a saturated solution of Glc6P (2 mM) was used as substrate with appropriate PGI added to initiate the reaction. Aliquots were withdrawn from the reaction mixture at certain time intervals, quenched with MeOH, and analyzed as before to obtain values of P at different t. As suggested by Alberty and Koerber [42], $V^f_{\max}$ can be estimated as the intercept of a regression line on the P/t versus t plot. In our case, five P/t values were calculated at the very early stage of the forward reaction and plotted against t to obtain a linear curve. According to the result shown in Fig. 3B, the maximum P/t was approximated to be 14.3 μM/min at 0 min. With the final enzyme concentration of 117 ng/mL used for this experiment, $V^f_{\max}$ was calculated to be 122.5 μmol/(min mg). With these parameters known, $K_{M(Glc6P)}$ was calculated to be 0.28 mM according to Eq. (6). This value correlates well with those obtained using traditional methods, which approximate 0.3 mM [39,43,44].

3.5. K_M and V_{max} measurements for both substrates of PMI

Due to the lack of a suitable assay, the K_M and $V_{\max}$ values for Fru6P as substrate of PMI have not been reported in the literature. Therefore, we decided to study the kinetics of PMI in both directions using our methodology to gain this information. Since the K_{eq} for this reaction is approximately 1, which indicates that both directions are thermodynamically equal, two individual saturation plots were generated with each isomer as starting substrate. Time curves were first recorded to determine suitable quenching time for each substrate concentration to ensure initial velocity conditions. The saturation plot and the corresponding Lineweaver–Burk plot for Man6P are shown in Fig. 4A. Using the same methodology as for PGI, K_M for Man6P was calculated to be 1.21 ± 0.10 mM, which agrees well with those values reported previously, i.e., approximately 1.3 mM [1,45,46]. The $V_{\max}$ in the Man6P to Fru6P direction was calculated to be 55.6 μM/min or 69.5 μmol/(min mg), which agrees with the value of 65.4 μmol/(min mg) obtained by UV-coupling assay. When the substrate concentration was varied between 0.05 and 2 mM for Fru6P, substrate inhibition effect was observed as the substrate concentration exceeded 0.5 mM (data not shown). When the data corresponding to 0.05–0.4 mM Fru6P were fitted according to Eq. (5) as shown in Fig. 4B, the values of K_M and $V_{\max}$ were calculated to be 0.15 ± 0.10 mM, and 7.7 ± 0.5 μmol/(min mg), respectively. There are no reported K_M and $V_{\max}$ values of Fru6P in the literature for comparison, however, the accuracy of these kinetic parameters could be verified using the Haldane relationship. As shown below, the equilibrium constant for this reaction can be calculated either by dividing the equilibrium concen-

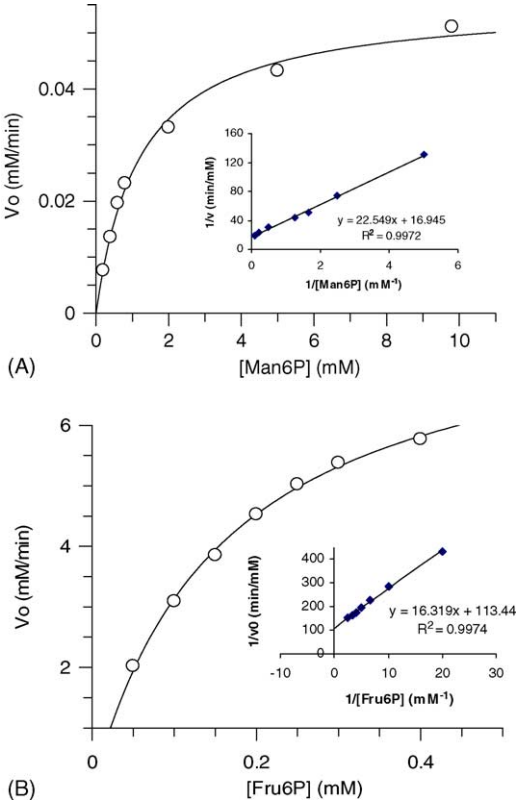

Fig. 4. Kinetic measurement of PMI. (A) Saturation plot of Man6P with Lineweaver–Burk plot shown in the inset, Man6P concentration was varied between 0.5 to 10 mM, final PMI concentration was 200 ng/mL; (B) saturation plot of Man6P with Lineweaver–Burk plot shown in the inset, Fru6P concentration was varied between 0.05 and 0.4 mM, final PMI concentration was 400 ng/mL.

tration of product by that of substrate or by the Haldane relationship.

$$K_{eq} = \frac{C_{eq(Fru6P)}}{C_{eq(Man6P)}} = \frac{V^{f}_{max} K_{M(Fru6P)}}{V^{r}_{max} K_{M(Man6P)}}$$

By inputting the four kinetic parameters of PMI obtained through saturation plots to the Haldane relationship, K_{eq} was calculated to be 1.12, which agrees quite well with the value of 1.10 measured as the equilibrium concentration ratio between Fru6P and Man6P as described above.

4. Conclusion

A novel mass spectrometry based assay was developed to quantify phosphorylated aldose–ketose isomers and was successfully applied to the kinetic measurements of phosphoglucose isomerase and phosphomannse isomerase. Using tandem mass spectrometry combined with a multicomponent quantification method, the isomeric substrate–product pair between Glc6P and Fru6P or between Man6P and Fru6P, can be directly quantified. Kinetic parameters K_M and V_{max} for both directions of the two isomerases can be easily determined, and the equilibrium constant for these reversible reactions can also be calculated from a single assay. All the kinetic parameters obtained agree well with those values measured using other traditional methods, except for those of Fru6P as substrate for phosphomannose isomerase, which are reported for the first time. These values help to more comprehensively characterize PMI and will also aid further mechanistic studies and inhibitor design toward this enzyme. The sensitivity (as low as 5 μM substrate with 3% conversion to product) is comparable to that obtained using chromatographic methods, but the assay time is dramatically shorter and therefore can be considered as an efficient kinetic assay. It is also a general assay method that can be easily adapted to other aldose–ketose isomerase systems providing tandem mass spectrometry can generate distinguishable spectra for the isomeric substrate–product pair. The tandem mass spectrometry strategy for PGI and PMI and the ion/molecule reaction strategy developed previously for PGM, have extended the application of mass spectrometry in enzyme kinetic analysis to isomerases and mutases whose substrates and products have the same mass.

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