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Affinity Capture and Elution/Electrospray Ionization Mass Spectrometry Assay of Phosphomannomutase and Phosphomannose Isomerase for the Multiplex Analysis of Congenital Disorders of Glycosylation Types Ia and Ib

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We report a new application of affinity capture-elution electrospray mass spectrometry (ACESI-MS) to assay the enzymes phosphomannomutase (PMM) and phosphomannose isomerase (PMI), which when deficient cause congenital disorders of glycosylation CDG-type Ia and type Ib, respectively. The novel feature of this mass-spectrometry-based assay is that it allows one to distinguish and quantify enzymatic products that are isomeric with their substrates that are present simultaneously in complex mixtures, such as cultured human cell homogenates. This is achieved by coupled assays in which the PMM and PMI primary products are in vitro subjected to another enzymatic reaction with yeast transketolase that changes the mass of the products to be detected by mass spectrometry. The affinity purification procedure is fully automated, and the mass spectrometric analysis is multiplexed in a fashion that is suitable for high-throughput applications.

Congenital disorders of glycosylation (CDG, also known as Carbohydrate-Deficient Glycoprotein Syndromes) are inherited defects in the biosynthesis of N-linked oligosaccharide chains.[1−3] Patients show multisystem deficiencies that usually include variable mental and psychomotor retardation, hypotonia, liver deficiencies, and feeding problems. The most common form, called CDG-type Ia (CDG-Ia), is caused by a deficiency in phosphomannomutase isozyme 2 (PMM2), which catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate.[1−3] This disorder is greatly underdiagnosed because there are few laboratories available for testing, and physician awareness of the disorders is limited. When used in conjunction with analysis of serum transferrin glycosylation, PMM2 assays on fibroblasts or leukocytes provide reliable diagnosis of CDG-Ia. Another disorder, CDG-type Ib (CDG-Ib), is caused by insufficient phosphomannose isomerase (PMI), which catalyzes the conversion of fructose-6-phosphate to mannose-6-phosphate.[1−3] Prompt diagnosis of this potentially lethal condition is important because dietary mannose supplements provide a highly effective treatment.[1−3] Simple, high-throughput assays for both PMM2 and PMI activities would encourage more CDG testing for the identification of treatable patients. In response to this need, we have developed a rapid mass spectrometry-based multiplex assay to identify both PMM2- and PMI-deficient patients.

Mass spectrometry in combination with separation methods has been used previously in clinical analysis of errors of metabolism, as reviewed in ref 4. In particular, mass spectrometry offers high sensitivity and specificity in detecting metabolites in body fluids.[5,6] However, since mass spectrometry distinguishes and detects compounds by ion mass-to-charge ratios, analysis of PMM2 and PMI enzymatic products, which are isomeric with the substrates, represents a serious challenge. Phosphorylated monosaccharides, for example, mannose-1-phosphate, glucose-1-phosphate, and galactose-1-phosphate, show only modest differences in the MS/MS spectra of anions produced by electrospray ionization.[7,8] Hence, distinction and quantitation of these isomers in complex mixtures such as cell homogenates would be very difficult. Clearly, a new strategy is needed to tackle this analytical problem.

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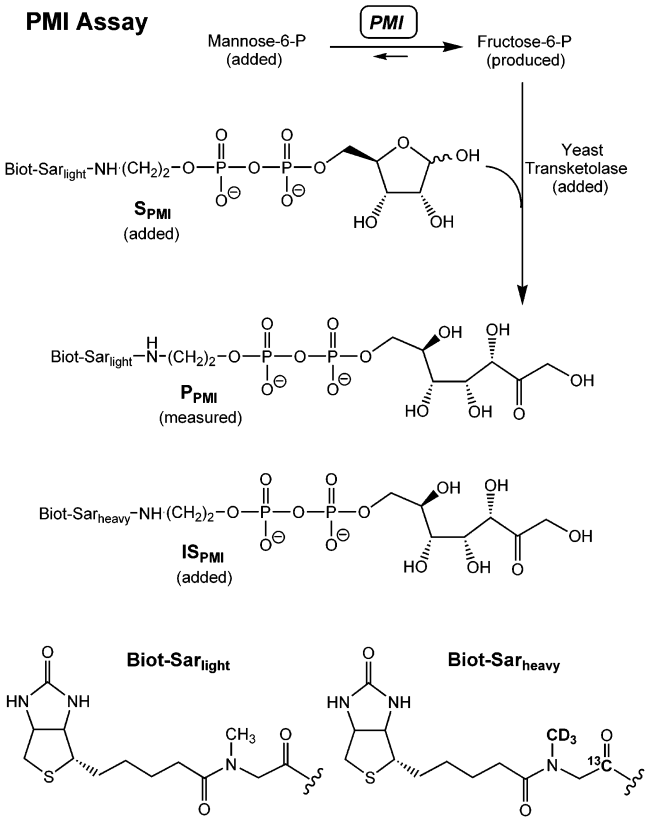
Over the past few years we have developed a new procedure for the quantitative analysis of enzymatic reaction velocities using mass spectrometry.[9] The assay relies on a method that we have termed "affinity capture and elution/electrospray ionization mass spectrometry" (ACESI-MS). The method uses a synthetic enzyme substrate that is covalently conjugated to biotin via a linker. This enables the biotinylated product to be easily purified prior to quantification by mass spectrometry with the aid of an internal standard, which is the product conjugate bearing heavy isotope substitution. Multiple enzymes can be analyzed in a single mass spectrometric run (multiplex analysis), as long as each product conjugate has a unique mass and does not interfere strongly with electrospray ionization. ACESI-MS assays have been developed for the analysis of β-galactosidase,[9] Sanfilippo syndromes types A−D,[10,11] and acid sphingomyelinase and galactocerebroside β-galactosidase,[12] as reviewed.[13] These assays can typically be carried out with a few micrograms or less of cell protein and, thus, are suitable for biomedical diagnosis using limited quantities of human tissue. ACESI-MS has also been automated for high-throughput clinical applications.[14]

In principle, ACESI-MS should be applicable to the analysis of virtually any enzyme, thus providing the advantage that a large array of clinical assays can be carried out using mass spectrometry as a single analytical platform. One potential limitation of ACESI-MS is that it cannot be used to directly assay an enzyme that does not cause a change in mass of the substrate upon conversion to product (e.g., isomerases). This is relevant to PMM2 and PMI. In the present study, we have developed ACESI-MS assays for PMM2 and PMI by coupling the products of these enzymatic reactions to an additional enzymatic pathway that leads to a change in mass of the original PMM2 and PMI substrate conjugates. Furthermore, the PMM2 and PMI reactions can be combined and analyzed in a single multiplex analysis, which can be automated. This study serves to illustrate that the ACESI-MS method can be generalized to include isomerases.

EXPERIMENTAL SECTION

Materials. Lyophilized yeast transketolase (TK), baker's yeast PMI (suspension in 3.2 M $(NH_4)_2SO_4$), mannose-6-phosphate, mannose-1-phosphate, and thiamine pyrophosphate (TPP) were purchased from Sigma. TK activity was measured according to the literature procedure.[15] The TK preparation at a concentration of 30 mU μL^{-1} in 50 mM *N*-(2-hydroxyethylpiperazine-*N*′-(2-ethanesulfonic acid) buffer (HEPES, Sigma) was aliquoted into Eppendorf tubes, lyophilized, and stored at −20 °C until used. The PMI suspension was centrifuged at 10000*g* for 10 min at 4 °C, the supernatant was dialyzed using a Slide-A-Lyzer MINI Dialysis Unit (Pierce, 10 kDa cutoff) in 50 mM HEPES (pH 7.5), and aliquots were stored at −20 °C. Mannose-6-phosphate, mannose-1-phosphate, and TPP were stored at −20 °C as 0.1 M aqueous stock solutions. TPP solutions older than one month were discarded. Human skin fibroblasts from healthy anonymous donors and CDG-Ia and CDG-Ib patients were cultured and harvested according to standard protocol[16,17] and stored at −80 °C. Mannose-1,6-diphosphate was made as described[24] and stored at −20 °C.

Scheme 1



Substrate Conjugates and Internal Standards. The substrate conjugates for PMI (S_{PMI}), PMM2 (S_{PMM}), and the corresponding internal standards IS_{PMI} and IS_{PMM} and product-conjugates P_{PMI} and P_{PMM} were synthesized as described in the Supporting Information. The chemical structures are shown in Scheme 1. The syntheses were optimized for the preparation of milligram quantities (5−50 mg) of conjugates. All compounds were characterized by ^{1}H NMR and ESI-MS. Stock solutions in water were quantified by integrating the appropriate proton signals in the ^{1}H NMR spectra using the signal of dimethyl sulfoxide (DMSO) as an internal standard.

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Procedures. *Preparation of the Cell Extract.* The cell extract was prepared according to the literature procedure[17] but with slight modification as follows: The cell pellet was suspended in a homogenization buffer that contained 20 mM HEPES, 25 mM KCl, 1 mM dithiothreitol, and 10 μg/mL each of leupeptin and antipain (pH 7.5). The suspension was frozen at −50 °C for 2 min, then thawed and centrifuged at 10000*g* for 3 min at 0 °C. The resulting supernatant was used as the cell extract for PMI and PMM2 assays. The amount of buffer was adjusted to give a protein concentration of 0.5−2 μg/mL when assayed using the Bradford reagent (Bio-Rad) calibrated with bovine serum albumin. The cell extract was kept on ice until used.

Incubation. The PMM2 and PMI assays were carried out in 10 μL of 25 mM HEPES, 3.0 mM $MgCl_2$, and 0.15 mM TPP (pH 7.5) in a 1.7-mL polypropylene microcentrifuge tube. For the PMI assay, the mixture also contained 0.25 mM mannose-6-phosphate, 0.10 mM (1.0 nmol) S_{PMI}, 24 mU TK, and 0.5 μg of cell extract protein. For the PMM2 assay, the mixture contained 0.13 mM mannose-1-phosphate, 1 μM mannose-1,6-diphosphate, 0.12 mM (1.2 nmol) S_{PMM}, 100 mU TK, 100 mU of PMI, and 1 μg of cell extract protein. The assay mixtures were incubated at 28 °C for 1 h and quenched by adding 3 μL of 50 mM ribose-5-phosphate and 50 mM fructose-6-phosphate. Internal standards, IS_{PMI} and IS_{PMM}, 0.2 nmol each, were added to the PMI and PMM2 assay mixtures, respectively, from 0.1 mM aqueous stock solutions. The PMI assay mixture was diluted by adding 50 μL of purified water (Milli Q, Millipore Corp.) and combined with the PMM2 assay mixture for affinity purification and duplex ESI-MS analysis.

To enhance the activity of TK and, thus, to reduce the amount of TK needed in the assay, the incubation conditions were modified from the previously reported procedures.[17,18] HEPES-buffered solution of pH 7.5 and an incubation temperature of 28 °C were used instead of pH 7.1 and 30 °C. We also found that the high salt content, mostly $(NH_4)_2SO_4$, in the commercial PMI suspension significantly reduces PMM2 activity and needs to be removed by dialysis. The dialyzed PMI in HEPES-buffered solution did not show any loss of activity after one month at −20 °C, even after frequent freezing and thawing.

Batch Procedure for Affinity Capture−Release. The combined PMI and PMM2 assay mixture (~70 μL) was transferred to a Microbiospin column (Bio-Rad) containing a slurry of immobilized streptavidin beads (Immunopure, Pierce, 2.2 nmol of biotin binding capacity, suspension supplied by the manufacturer). The columns were gently rocked, allowed to sit at room temperature for 5 min, and centrifuged (~1200*g* for 10 s). The beads were washed four times by adding 150 μL of Milli Q water followed by centrifugation. To release the conjugates, 25 μL of 1 mM aqueous biotin methyl ester solution was added, and the mixture was incubated at room temperature for at least 3 h after capping the column. An eluate containing product conjugates, internal standards, and unreacted substrates together with biotin methyl ester was obtained by centrifugation of the columns at 6000*g* for 5 s. The eluate was directly infused into the mass spectrometer for quantitative analysis.

ESI-MS Analysis. Mass spectra were obtained on a Bruker-Esquire (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer operating in negative ion mode. Samples were infused at 3 μL/min and ionized in a standard orthogonal Bruker

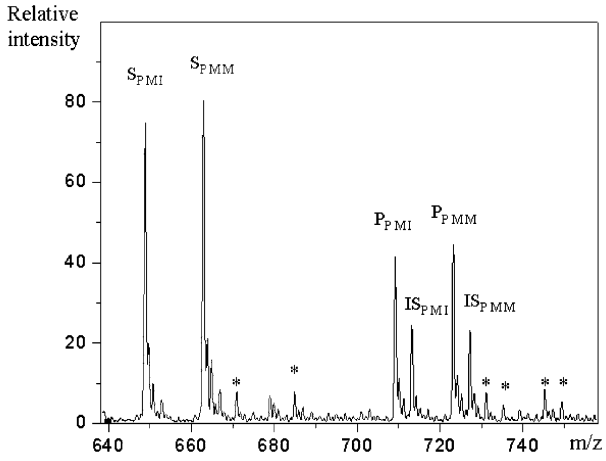


Figure 1. ESI mass spectrum of PMI− and PMM−substrate conjugates, product conjugates and internal standards. The peaks of deprotonated conjugates, $(M - H)^-$, are annotated as follows: S_{PMI}−PMI substrate conjugate, at *m*/*z* 649.3; S_{PMM}−PMM2 substrate conjugate, at *m*/*z* 663.3; P_{PMI}−PMI product conjugate, at *m*/*z* 709.3; IS_{PMI}−PMI internal standard, at *m*/*z* 713.3; P_{PMM}−PMM2 product conjugate, at *m*/*z* 723.3; IS_{PMM}−PMM2 internal standard, at *m*/*z* 727.3. The peaks labeled by asterisks correspond to sodiated ions, $(M - 2H + Na)^-$, that appear at *m*/*z* values 22 mass units higher than the corresponding $(M - H)^-$ ions.

ESI source. The mass spectrometric conditions were as follows: electrospray needle, −4000 V; end plate, −1500 V; nebulizer, 9.0 psi; drying gas flow rate, 5.0 L/min; drying gas temperature, 250 °C; skimmer 1, −30.0 V; skimmer 2, −6.0 V; capillary exit offset, −70.0 V; capillary exit, −100 V; octopole −2.0 V; trap drive, 55.0; lens I voltage, 5.0 V; lens II voltage, 60.0 V. Mass spectra were collected over a range of *m*/*z* 640−760 for ~1 min, and the ion intensities were averaged over several hundred spectra. The amount of product formation was calculated from the ratio of ion intensities corresponding to $(M - H)^-$ species for the products and internal standards. Peaks of the most abundant monoisotopic species were used for quantitation. For each assay, a blank sample was prepared that contained all components except for cell homogenate and, following affinity purification, was used to measure the background ion intensities at *m*/*z* values corresponding to the enzymatic products. The blank intensities, relative to those of the internal standards, were subtracted from the ion intensities measured in complete assays. The enzyme velocities were calculated in units of nanomoles of product per minute of incubation per milligram of cell lysate protein in the assay (mU mg^{-1}). The relevant regions of the mass spectra containing the substrate conjugates, product conjugates, and internal standards are shown in Figure 1 and discussed below.

Lab-on-Valve (LOV) Affinity Purification and ESI-MS Analysis. Affinity capture, purification, elution, and on-line MS analysis of assay samples were also carried out in a fully automated fashion using a bead injection[19] Lab-on-Valve-ESI-MS apparatus that has been described in detail previously.[14] The apparatus consists of a FIAlab-3000 system (FIAlab Instruments Inc.) that is controlled by the FIAlab 5.0 software. The apparatus performs sequential infusion of solutions and bead suspension through six radial ports by reversible action of a syringe pump, which is combined with valve rotation that distributes the flow through the ports. A 3-way isolation solenoid valve (Nresearch Inc.) is used to direct the eluate flow either to the ESI-MS or to waste.

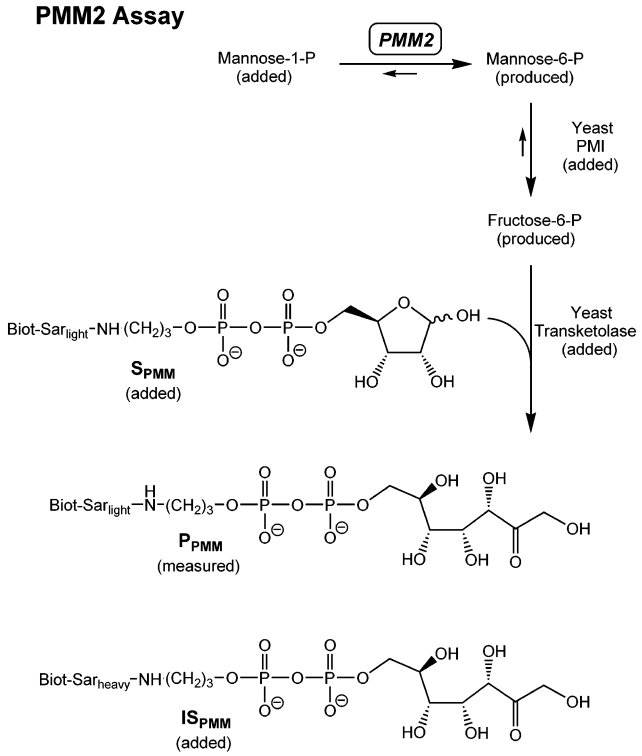
The following procedure is representative. Enzyme reaction mixture was diluted to 400 μL with Milli Q water and stored at −20 °C until further analyzed. Immediately before analysis, 4 μL of 20% acetic acid aqueous solution was added to the sample, and the reaction tube was attached to port 6 of the LOV apparatus.[14] Acetic acid was found to increase the capture efficiency for the biotin-containing conjugates. Immobilized streptavidin bead slurry (10 μL) was drawn from the bead reservoir and delivered to the bead-holding compartment, where the beads were packed above a short piece of a 1/16-in.-o.d. PEEK rod that was loosely inserted into the LOV channel. The space between the PEEK rod and the channel (∼30 μm) allows the solution to pass through the channel, while the ∼100 μm o.d. beads are retained in the compartment.[14] Reaction mixture (100 μL) was then drawn and passed through the bead bed at a flow rate of 5 μL/s, and the beads were washed with 350 μL of Milli Q water at 20 μL/s to remove nonspecifically bound components. Following this purification, the biotin-containing conjugates were eluted with 90 μL of 45 μM biotin methyl ester in methanol/water (1:1 by volume) and infused to the ESI-MS at a flow rate of 1 μL/s. The mass spectrometric conditions were as in the batch mode measurements with the following changes: end-plate, −500 V; skimmer 1, −50.0 V; capillary exit offset, −60.0 V. After mass spectrometric analysis, the beads were removed from the compartment by reversing the solvent flow, and the line to the ESI-MS was flushed with carrier solution before the next analysis. This full sequence of operations, including the MS analysis, takes 4.7 min.

RESULTS AND DISCUSSION

The strategy for the PMI assay is outlined in Scheme 1. PMI catalyzes the reversible reaction of fructose-6-phosphate to mannose-6-phosphate. The flux direction through this step can be controlled by the substrate concentrations. In the PMI assay, the enzyme in the cell homogenate is exposed to an excess of exogeneously added mannose-6-phosphate, which reverses the natural enzymatic pathway and results in the production of fructose-6-phosphate. The latter reacts with the PMI substrate conjugate (S_{PMI}) via catalysis by exogeneously added yeast transketolase (TK) to produce a sedoheptulose-7-phosphate conjugate containing the biotin handle (P_{PMI}). TK was chosen to be the coupling enzyme in part because of its commercial availability and affordable price.[15,20] The rate of formation of the TK product-conjugate (P_{PMI}) is controlled by the concentration of fructose-6-phosphate, which in turn is controlled by the PMI activity.

In the PMM2 assay (Scheme 2), the enzyme in the cell homogenate is exposed to an excess of exogeneously added mannose-1-phosphate to produce mannose-6-phosphate. The latter is converted by exogeneously added yeast PMI to fructose-6-phosphate, which is reacted with the PMM2 substrate conjugate (S_{PMM}) under yeast TK catalysis to form the PMM2 product conjugate (P_{PMM}). The rate of P_{PMM} formation is controlled by the concentration of fructose-6-phosphate, which in turn is controlled by the PMM2 activity. Because of the coupled-enzyme nature of the assays, PMI and PMM2 cannot be assayed simultaneously in the same cell sample. However, separate PMI and PMM2 assays can be run in parallel and combined, purified, and analyzed by ESI-MS in a single run because the product conjugates are distinguished in mass by the different number of methylene groups in the linker between the biotinyl-sarcosinyl group and the pyrophosphate bridge (Schemes 1 and 2). One additional note is that the potentially interfering isoform-1 of PMM is restricted to brain and lung and is thought to contribute <5% of the total PMM activity measured in other tissues.[21]

Scheme 2



Substrate conjugates S_{PMI} and S_{PMM} (Schemes 1 and 2) are composed of three functional substructures: biotin with an *N*-methylglycine (sarcosine) linker, amino alkyl phosphate, and ribose-5-phosphate. Biotin works as an affinity handle to be captured by immobilized streptavidin beads. Sarcosine was conjugated to biotin to form an *N*-methyl biotinamide linkage, which resists cleavage by the enzyme biotinidase present in biological samples.[22] The amino alkyl phosphate provides sufficient distance between the affinity handle and the enzyme substrate so that the conjugate capture and release by affinity chromatography is independent of the substrate structure.[22] At the same time, it provides a simple synthetic way to conjugate the affinity handle and the enzyme substrate, ribose-5-phosphate, by amide and pyrophosphate bonds, respectively.[23,24] Although TK has wide substrate specificity, ribose-5-phosphate, a natural substrate of TK, was chosen to be incorporated into the substrate conjugates because of its low K_m (Michaelis−Menten constant) and stability.

The chemical change induced by TK results in the transfer of a $C_2H_4O_2$ fragment from fructose-6-phosphate to the ribose moiety of S_{PMI} and S_{PMM}, resulting in a 60-Da mass increase in the sedoheptulose product conjugates P_{PMI} and P_{PMM}, which are readily resolved in the ESI-mass spectra. Moreover, the structures of S_{PMI} and S_{PMM} differ by a CH_2 unit in the linker, which causes a 14-Da mass difference in the products, and the same increment distinguishes internal standards IS_{PMI} and IS_{PMM} to achieve mass spectrometric differentiation. IS_{PMI} and IS_{PMM} are further distinguished from their respective products by the presence of four heavy isotopes ($^{13}C_1$ and $^2H_3$), such that all species present in the

duplex assay are unambiguously distinguished by mass and can be quantified simultaneously.

The activities of PMM2 and PMI toward the synthetic substrate conjugates were measured using cultured skin fibroblasts from anonymous donors. Figure 1 shows a representative result obtained from a duplex PMI and PMM2 analysis of cells from a healthy donor, as measured by ESI-MS in the negative ion detection mode. The peaks at m/z 709.3−711.3 (annotated P_{PMI}) and 723.3−725.3 (annotated P_{PMM}) correspond to products and their naturally occurring isotope satellites formed by PMI and PMM2, respectively, whereas peaks at m/z 713.3−715.3 and 727.3−729.3 correspond to the heavy isotope-labeled internal standards, IS_{PMI} and IS_{PMM}, respectively. The unreacted substrate conjugates are seen as peaks at m/z 649.3−651.3 and 663.3−665.3 for PMI and PMM2, respectively. Small peaks corresponding to sodiated ions, $(M - 2H + Na)^-$, also appear in the mass spectra (annotated with asterisks) but do not interfere with the measurements. The enzyme activities were quantified in $mU \cdot mg^{-1}$ by measuring the intensity ratios for the most abundant product and internal standard peaks. The ESI-MS spectra demonstrate excellent signal-to-noise ratios and clear product speciation by m/z values. Also noteworthy is the absence of interfering species in the affinity-purified samples.

The assay conditions were optimized by measuring the product formation using skin fibroblasts from a healthy individual, in which the amount of added TK, concentrations of substrate conjugates, incubation time, and amount of cell lysate protein were varied. The effect of the TK amount is shown in Figure 2a. The rate of product formation levels off at TK concentrations higher than 12 mU and 60 mU for PMI and PMM2, respectively. Hence, to ensure that the observed velocities are rate-limited by PMI and PMM2, the TK concentrations were kept at 24 mU and 100 mU in all PMI and PMM2 assays, respectively. The effect of substrate conjugate concentrations is shown in Figure 2b. The rate of product formation maximizes at S_{PMI} and S_{PMM} concentrations of 0.15 mM and 0.25 mM, respectively. To conserve substrate conjugates, S_{PMI} and S_{PMM} concentrations of 0.10 mM and 0.12 mM for PMI and PMM2, respectively, were used.

The kinetic data for P_{PMI} and P_{PMM} formation were fitted to the Michaelis−Menten formula and plotted in Figure 2b. The K_m values were 0.096 and 0.13 mM for PMI and PMM2, respectively, and the respective V_{max} values were 4.5 and 3.25 mU mg^{-1}. Note that the K_m for PMI is for binding mannose-6-phosphate, which is the product of the natural enzyme action (Scheme 1). Likewise, the K_m for PMM2 is for binding mannose-1-phosphate, which is the product of the natural biochemical pathway (Scheme 2).

The effects of the incubation time and amount of cell extract protein are summarized in Figure 3a,b. Both PMI and PMM2 assays showed linear response in product formation as a function of incubation time and amount of protein, as shown by the correlation coefficients for linear regression (r^2), which are 0.991 (PMI) and 0.997 (PMM2) in Figure 3a and 0.988 (PMI) and 0.999 (PMM2) in Figure 3b.

To demonstrate the utility of the ACESI-MS assays in clinically relevant cases, we measured PMI and PMM2 activities in cultured skin fibroblasts from six healthy individuals and four patients afflicted with CDG-Ia or CDG-Ib. The results obtained by batch assays are summarized in Table 1. Triplicate measurements of

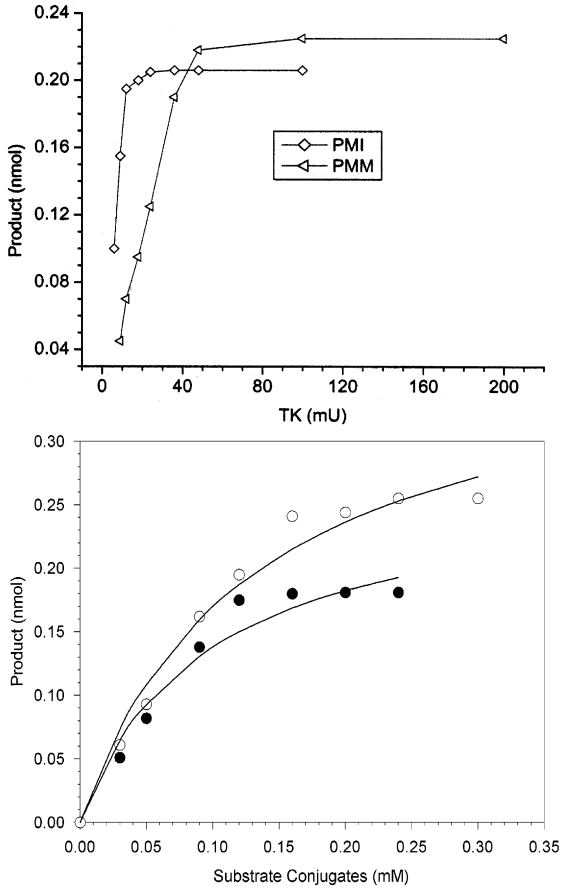


Figure 2. (a) Effect of TK amount on the product conjugate formation in PMI and PMM2 assays. The cell lysate was from an anonymous healthy donor. Amount of cell lysate protein used: PMI assay, 1 μg; PMM2 assay, 2 μg. Incubation time: 1 h. (b) Effect of the substrate conjugate concentration on the product conjugate formation in PMI and PMM2 assays. The cell lysate was from an anonymous healthy donor. The amount of cell lysate protein and incubation time was as in (a). The curves are from least-squares Michaelis−Menten fits for K_m and V_{max} (see text).

assays from each cell culture showed very good reproducibility with <5% one standard deviation (SD). The mean PMM2 activity of healthy individuals (1.7 ± 0.4 mU mg^{-1}) is comparable to the value reported by Van Schaftingen et al. (1.32 ± 0.37 mU mg^{-1})[17] and by Charlwood et al. (1.0−2.1 mU mg^{-1}).[18] The mean PMI activity of healthy individuals (3.5 ± 0.9 mU mg^{-1}) is lower than the value reported by Van Schaftingen et al. (14.6 ± 4.1 mU mg^{-1}) and Charlwood et al. (14−23 mU mg^{-1}). The measurements showed ~19 and ~25% variability in the PMM2 and PMI activities, respectively, among healthy individuals. In contrast, the affected patients showed only ~10% residual activities compared to healthy individuals, as shown in Figure 4B and C. It is also evident from the results that the activities of PMM2 and PMI are not cross-correlated (Table 1 and Figure 4). The PMM2 activity in the cell extracts from two CDG-Ia-affected patients remained within 1 SD of the mean activities measured for healthy individuals. Likewise, the PMI activity in the cell extracts from two CDG- Ib-affected patients remained within 1 SD of the mean activities of healthy individuals. These results are consistent with previous studies that demonstrated the lack of cross-correlation in enzyme activity between PMI and PMM2.[17,18]

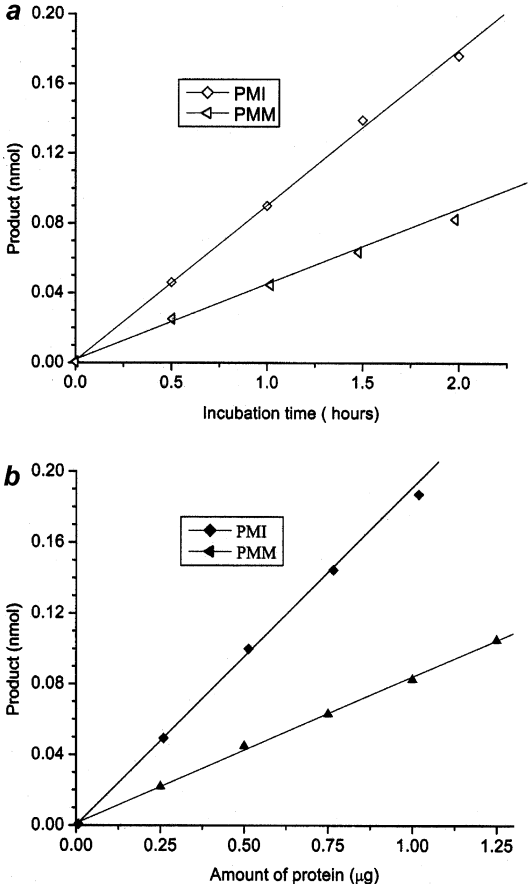


Figure 3. (a) Effect of the incubation time on the formation of the PMI and PMM2 product conjugates. The assay conditions are the same as that described in the Experimental section, except for the incubation time which was varied. (b) Effect of the amount of cell lysate protein on the formation of the PMI and PMM2 product conjugates. The assay conditions are the same as that described in the Experimental section, except for the amount of cell lysate which was varied.

Efforts were also made to reduce the substrate consumption per assay and to shorten the time per analysis. To achieve the first objective, PMI and PMM2 activities were measured in a cell extract from a healthy individual, in which the amount of cell lysate protein and all other assay solutions were reduced by one-half. The mass spectrum showed ion intensities for PMI and PMM2 product formation that were 10 times higher than the background peaks in a blank sample that was assayed in the absence of a cell extract. Thus, reducing the amount of substrate to 0.4 μg/assay and cell extract to one-half provided a signal-to-noise ratio that was sufficient for accurate diagnosis. At this reduced scale, 1 mg of a substrate conjugate is sufficient for carrying out 2500 assays. Experiments also showed that the PMI and PMM2 activities in the cell extract were surprisingly stable, as only ~10% of activity was lost after storage at 4 °C for 10 days.

The analysis time per assay was substantially shortened by using the LOV-ESI-MS apparatus, which allows automated affinity capture and release of biotin-containing conjugates on immobilized streptavidin and on-line analysis of the released conjugates by ESI-MS.[14] With this system, the entire purification and analysis cycle was completed in 4.7 min. This is compared with a batch analysis in which the elution process took ~3 h with additional manual pipetting time. A representative elution profile from the LOV

Table 1. PMI and PMM2 Activities Measured with Respect to Cell Lysate Protein

	enzyme activity[a]	
cell source	PMI	PMM2
normal 1	3.3 ± 0.1[b]	1.2 ± 0.1[b]
normal 2	4.1 ± 0.2	1.8 ± 0.0
normal 3	3.5 ± 0.1	2.2 ± 0.1
normal 4	2.8 ± 0.0	1.9 ± 0.1
normal 5	5.1 ± 0.2	1.6 ± 0.1
normal 6	2.4 ± 0.1	1.4 ± 0.2
mean normal activity	3.5 ± 0.9[c]	1.7 ± 0.4[c]
normal 7.1[d]	2.7 ± 0.05[b]	2.1 ± 0.12
normal 7.2[d]	2.9 ± 0.16	1.8 ± 0.18
normal 7.3[d]	2.8 ± 0.16	2.1 ± 0.07
CDG-Ia ED[e]	3.2 ± 0.1[b]	0.15 ± 0.1[b]
CDG-Ia JD[e]	2.9 ± 0.2	0.10 ± 0.0
CDG-Ib MK[e]	0.3 ± 0.1	2.0 ± 0.1
CDG-Ib BJ[e]	0.4 ± 0.0	1.5 ± 0.1

[a] In units of mU mg^{-1}. 1U corresponds to the amount of enzyme that forms 1 μmol of product per minute. [b] Standard deviations of triplicate batch measurements. [c] Standard deviations of the mean activities from six normal donors. [d] Measured using LOV-ESI-MS for three different incubations of cell lysate from normal 7 donor. [e] Anonymous affected patients.

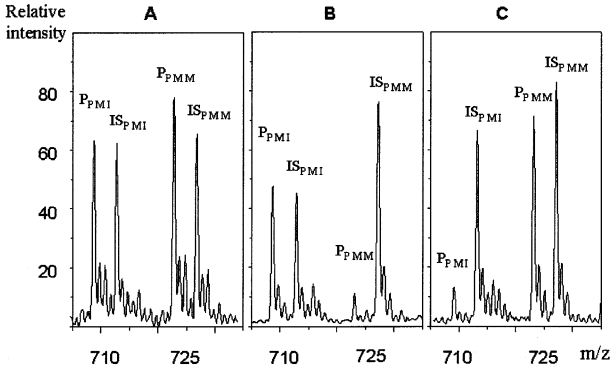


Figure 4. ESI mass spectra of the PMI and PMM2 product conjugates and internal standards from assays of skin fibroblasts: panel A, a healthy control; panel B, a CDG-Ia-afflicted patient; panel C, a CDG-Ib-afflicted patient.

apparatus is shown in Supporting Information (Figure S1) as a mass-selected ion chromatogram of unreacted substrate S_{PMI} that was monitored at m/z 649.3. The elution of the captured conjugate is practically complete in 3 min. The elution profiles of S_{PMM}, product conjugates, and internal standards were virtually identical to that of S_{PMI}. For an even faster analysis, the elution was terminated at 1.4 min, and the ion intensities of the products and internal standards were obtained from the spectra that were averaged over 20 s at elution times corresponding to the maximum intensity (1.00 to 1.35 min, Figure S1). The enzyme velocities in cell homogenates from a healthy donor (normal 7), measured by using the fast LOV-ESI-MS protocol, are summarized in Table 1. Three enzyme reaction batches were prepared using the same cell extract, and triplicate analyses were performed with each assay sample. The mass spectra of duplex assay samples show excellent signal-to-noise ratios and good reproducibility. In addition, the enzyme velocities measured by the LOV-ESI-MS (Table 1) show good agreement with the population means of normal PMI and PMM2 activities measured by the batch method, with differences being within 0.75 SD.

CONCLUSIONS

One of our major goals with ACESI-MS is to illustrate its general utility for the analysis of virtually any enzymatic reaction of clinical or biochemical significance by use of mass spectrometry as a common analytical platform. Currently there is no single enzyme assay methodology that has been demonstrated to be applicable to most enzymes. As shown in this study, ACESI-MS can be readily applied to enzymes that do not cause a change in mass for the substrate-to-product conversion. The remaining factor that needs to be addressed is to develop ACESI-MS assays of enzymes that cannot utilize a substrate conjugate. Current studies are underway to use coupled enzymatic assays with ACESI-MS to fully establish the generalization of the approach. We can envision that this will at least provide a technology that can be used in a high-throughput fashion for widespread analysis of genetic diseases, although routine screening for genetic diseases remains a controversial topic. More and more clinical laboratories are adopting mass spectrometry as a diagnostic tool. Although instrumentation is expensive, the overall costs are probably less when one considers that a single mass spectrometer may replace a collection of instruments (i.e., spectrofluorimeter, spectrophotometer, scintillation counter, etc.) that are currently needed to carry out a wide variety of enzyme assays for the biochemical confirmation of diagnoses of genetic diseases. The ACESI-MS substrate conjugates are often more expensive per gram than substrates used in conventional enzyme assays, but the cost is more than offset by the relatively small amounts of reagents needed for ACESI-MS analysis (typically 10 μL reaction volumes). In this context, it may be noted that 50 mg quantities of S_{PMM} and S_{PMI} and 20 mg quantities of IS_{PMM} and IS_{PMI} are currently available upon request from our laboratory. This is sufficient for ~70 000 ACESI-MS assays at a reagent cost of $0.05 per assay. When one considers the cost of all reagents needed (coupling enzymes, cofactors, buffers), the cost of the ACESI-MS assay of PMM2 and PMI is comparable to that of the conventional assay.[17,18] Other advantages of ACESI-MS are that the method does not require radioactive substances, it achieves product speciation with a low background, and allows multiple enzyme assays to be carried out simultaneously. It is also fully automatable, as shown by the results of the present study.

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SUPPORTING INFORMATION AVAILABLE

Synthetic procedures with references (6 pp) and Figure S1 are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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