

# Kinetic properties and tissular distribution of mammalian phosphomannomutase isozymes

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Human tissues contain two types of phosphomannomutase, PMM1 and PMM2. Mutations in the *PMM2* gene are responsible for the most common form of carbohydrate-deficient glycoprotein syndrome [Matthijs, Schollen, Pardon, Veiga-da-Cunha, Jaeken, Cassiman and Van Schaftingen (1997) *Nat. Genet.* **19**, 88–92]. The protein encoded by this gene has now been produced in *Escherichia coli* and purified to homogeneity, and its properties have been compared with those of recombinant human PMM1. PMM2 converts mannose 1-phosphate into mannose 6-phosphate about 20 times more rapidly than glucose 1-phosphate to glucose 6-phosphate, whereas PMM1 displays identical  $V_{\max}$  values with both substrates. The  $K_m$  values for both mannose 1,6-bisphosphate and glucose 1,6-bisphosphate are significantly lower in the case of PMM2 than in the case of PMM1. Like PMM1, PMM2 forms a phosphoenzyme with the chemical

characteristics of an acyl-phosphate. PMM1 and PMM2 hydrolyse different hexose bisphosphates (glucose 1,6-bisphosphate, mannose 1,6-bisphosphate, fructose 1,6-bisphosphate) at maximal rates of  $\approx 3.5$  and 0.3% of their PMM activity, respectively. Fructose 1,6-bisphosphate does not activate PMM2 but causes a time-dependent stimulation of PMM1 due to the progressive formation of mannose 1,6-bisphosphate from fructose 1,6-bisphosphate and mannose 1-phosphate. Experiments with specific antibodies, kinetic studies and Northern blots indicated that PMM2 is the only detectable isozyme in most rat tissues except brain and lung, where PMM1 accounts for about 66 and 13% of the total activities, respectively.

**Key words:** glycoprotein synthesis, mannose 1-phosphate, phosphoenzyme.

## INTRODUCTION

Carbohydrate-deficient glycoprotein syndromes (CDGs) are a series of genetic multisystemic disorders characterized by defective N-glycosylation of serum and cellular proteins [1,2]. The type-IA syndrome, by far the most frequent type, is an autosomal recessive disorder, linked to chromosome 16p13 [3–5] and characterized by psychomotor retardation, severe cerebellar dysfunction, peripheral neuropathy, liver insufficiency and abnormal subcutaneous adipose tissue distribution [1,2]. In this disorder, there is no apparent change in the structure of the protein-bound oligosaccharides but a decrease in their number [6]. This results from abnormal incorporation of mannose into dolichol-pyrophosphate oligosaccharide [7] due to a deficiency in phosphomannomutase (PMM) [8,9], an enzyme involved in the synthesis of GDP-mannose.

A first *PMM* gene (*PMM1*), on human chromosome 22q13 [10–12], was identified and shown to encode a protein that has equal PMM and phosphoglucosyltransferase activities [10] and which becomes phosphorylated on a conserved aspartate residue when incubated with its cofactor mannose 1,6-bisphosphate [13,14]. The PMM present in rat liver as well as that found in several human cell types were found to have a distinctly higher affinity for mannose 1,6-bisphosphate than recombinant PMM1, and did not react with antibodies directed against this protein, indicating the existence of a second isozyme [13]. A second *PMM* gene was then identified, which was assigned to chromosome 16p13 and shown to be mutated in CDG IA [15,16].

The existence of two different isozymes of PMM raises the question of their respective roles. They could differ from each other by their kinetic properties and/or by their tissular distributions. The purpose of the present work was to express and characterize the enzyme encoded by the *PMM2* gene and compare its kinetic properties with those of human recombinant PMM1. It was also to evaluate both at the protein and at the mRNA levels the distribution of these two enzymes in rat tissues.

## MATERIALS AND METHODS

### Materials

Chemicals were from Sigma (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany). Radiochemicals, Thermosequenase, Hybond membranes, the enhanced chemiluminescence Western-blotting detection reagents and Protein A–Sepharose were from Amersham Pharmacia Biotech (Roosendaal, Netherlands). Phosphomannose isomerase was from Sigma; other auxiliary enzymes, the Random Primed DNA-labelling kit, *Pwo* DNA polymerase and NADP<sup>+</sup> were from Boehringer Mannheim (Mannheim, Germany). *E. coli* fructose 1-phosphate kinase was a kind gift of M. Veiga-da-Cunha (ICP, Brussels, Belgium). It has been over-expressed at 19 °C in BL21(DE3)pLysS cells using a pET3a expression vector [17] containing the open reading frame of the *E. coli FruK* gene [18]. The protein was purified to homogeneity by ammonium sulphate fractionation, gel filtration on Sephadex G-25 and DEAE-Sepharose chromatography (M. Veiga-da-

Abbreviations used: PMM, phosphomannomutase; CDG, carbohydrate-deficient glycoprotein syndrome; GPI, glycosylphosphatidylinositol.

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Cunha, unpublished results). Recombinant human PMM1 was prepared and purified to homogeneity as previously described [10]. Mannose 6- $^{32}\text{P}$ ]phosphate and mannose 1,6-bisphosphate were prepared as previously described [13]. Mannose 1,6- $^{32}\text{P}$ ]bisphosphate was prepared by incubating mannose 6- $^{32}\text{P}$ ]phosphate ( $10^8$  c.p.m., 2.5 nmol) [13] with 25  $\mu\text{M}$  mannose 1,6-bisphosphate/5 mM  $\text{MgCl}_2$ /25 mM Hepes, pH 7.4/0.5 units of PMM2 for 10 min at 20 °C in a volume of 5 ml. The reaction was stopped by heating for 5 min at 80 °C and mannose 1,6-bisphosphate was purified by chromatography on a 1 ml column of Dowex AG1-X8 ( $\text{Cl}^-$  form; 200–400 mesh) using a stepwise NaCl gradient to elute the phosphate esters.

### Expression and purification of recombinant PMM2

The open reading frame of *PMM2* was amplified by PCR with *Pwo* DNA polymerase, using a clone isolated from a human lymphoblast cDNA library [15] as template. The primers used in this reaction (5'-GACGCATATGGCAGCGCCTGGCCCA-GC-3' and 5'-TAGGATCCTTAGGAGAACAGCAGTTCA-CA-3') were 5'-phosphorylated and contained an *Nde*I and a *Bam*HI restriction site (underlined), respectively. The  $\approx 750$  bp amplification product was isolated by electrophoresis in a 1% agarose gel, purified with the QiaQuick gel extraction kit (Qiagen, Germany) and ligated into the *Eco*RV site of the pBlueScript KS<sup>+</sup> vector, to generate pBS-PMM2. The insert of this plasmid was sequenced [19] to rule out any PCR errors. An *Nde*I–*Bam*HI restriction fragment was then excised and inserted into the expression vector pET3a [17] to generate pET-PMM2. The recombinant protein was expressed in *E. coli* BL21(DE3)pLysS [17] as in [10].

Cell extracts were prepared as in [20]. For the purification, a cell pellet derived from a 1 litre culture was resuspended in 50 ml of lysing buffer, and the fraction of protein precipitating between 6 and 24% poly(ethylene glycol) was resuspended in 20 ml of a buffer (Buffer A) containing 25 mM Hepes, pH 7.1, 5 mM  $\text{MgCl}_2$ , 2  $\mu\text{g}/\text{ml}$  antipain, 2  $\mu\text{g}/\text{ml}$  leupeptin and 1 mM dithiothreitol. This preparation was applied to a DEAE-Sepharose column (1.5  $\times$  16 cm), which was washed with 40 ml of Buffer A and developed with a NaCl gradient (0–500 mM in 2  $\times$  150 ml of Buffer A). The flow-through fractions, which contained PMM2, were pooled and concentrated 10-fold by ultrafiltration in an Amicon (Beverly, MA, U.S.A.) pressure cell equipped with a YM-10 membrane and applied to a Q-Sepharose column (1.2  $\times$  17 cm). The column was washed with 40 ml of Buffer B (25 mM Tris/HCl, pH 8.5/5 mM  $\text{MgCl}_2$ /2  $\mu\text{g}/\text{ml}$  leupeptin/2  $\mu\text{g}/\text{ml}$  antipain/1 mM dithiothreitol) and developed with a NaCl gradient (0–500 mM in 2  $\times$  150 ml of Buffer B). The active fractions were pooled and concentrated 20-fold by ultrafiltration. The enzyme was stored at  $-80$  °C in the presence of 25 mM Hepes, pH 7.1/2 mg/ml BSA/1 mM dithiothreitol/20% glycerol.

### Enzyme and protein assays

All enzymic activities were assayed spectrophotometrically at 340 nm by the reduction of NADP<sup>+</sup> to NADPH in a reaction mixture incubated at 30 °C and containing, unless otherwise stated, 50 mM Hepes, pH 7.1, 5 mM  $\text{MgCl}_2$ , 0.25 mM NADP<sup>+</sup> and 10  $\mu\text{g}/\text{ml}$  yeast glucose 6-phosphate dehydrogenase. Phosphoglucomutase activity was measured in the presence of 500  $\mu\text{M}$  glucose 1-phosphate and 10  $\mu\text{M}$  glucose 1,6-bisphosphate, and PMM activity was measured in the presence of 10  $\mu\text{M}$  mannose 1,6-bisphosphate, 100  $\mu\text{M}$  mannose 1-phosphate, 10  $\mu\text{g}/\text{ml}$  phosphoglucose isomerase and 3.5  $\mu\text{g}/\text{ml}$  phosphomannose iso-

merase. Hexose bisphosphatase activities were measured in the same reaction mixture with the indicated concentrations of glucose 1,6-bisphosphate, mannose 1,6-bisphosphate or fructose 1,6-bisphosphate, and the appropriate auxiliary enzymes.

In some experiments, the reaction was performed in the absence of NADP<sup>+</sup> and auxiliary enzymes and was arrested by mixing 0.2 ml of the mixture with 0.4 ml of 10% perchloric acid. The extracts were neutralized with 3 M  $\text{KHCO}_3$ , centrifuged and the resulting supernatants used to measure metabolites. Fructose 6-phosphate, mannose 6-phosphate and mannose 1-phosphate were measured spectrophotometrically with end-point assays performed in the same reaction mixture as above, adding successively glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, phosphomannose isomerase and 10  $\mu\text{g}/\text{ml}$  PMM2. Fructose 1,6-bisphosphate [21] and fructose 1-phosphate [22] were measured as indicated and mannose 1,6-bisphosphate through the stimulation of the PMM activity of PMM2, using mannose 1,6-bisphosphate as a standard.

Protein was assayed using the method of Bradford [23] with bovine  $\gamma$ -globulin as a standard.

### Labelling of PMM with mannose 1,6- $^{32}\text{P}$ ]bisphosphate

PMM (typically  $\approx 5$   $\mu\text{g}$  of PMM1 or 3.2  $\mu\text{g}$  PMM2) was incubated at 0 °C with various concentrations of mannose 1,6-bisphosphate/100 000 c.p.m. mannose 1,6- $^{32}\text{P}$ ]bisphosphate/5 mM  $\text{MgCl}_2$ /25 mM Hepes, pH 7.1/0.1 mg of BSA in a final volume of 0.1 ml. The reaction was arrested after 10 s (for PMM1) or 30 s (for PMM2) by addition of 0.25 ml of 5% trichloroacetic acid; the precipitate was collected by filtration and counted for radioactivity [13].

### Tissular distribution of PMM1 and PMM2

Rat tissue extracts were prepared by homogenizing fresh tissue samples in 4 vols. of a buffer containing 25 mM Hepes, pH 7.1, 5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 5  $\mu\text{g}/\text{ml}$  leupeptin and 5  $\mu\text{g}/\text{ml}$  antipain. The homogenates were centrifuged for 10 min at 10 000 g and stored frozen at  $-80$  °C.

For the preparation of antiserum, New Zealand rabbits were injected subcutaneously with 100  $\mu\text{g}$  of homogeneous recombinant PMM2 in Freund's complete adjuvant. The injections were repeated four times at 2-week intervals. Western blots were performed as described in [24] using the enhanced chemiluminescence detection system [25,26].

For the immunotitration experiments, 50  $\mu\text{l}$  of Protein A-Sepharose was incubated for 30 min at room temperature with the indicated volume of antiserum (0–100 ml) in the presence of PBS (8 mM  $\text{Na}_2\text{HPO}_4$ /1.5 mM  $\text{KH}_2\text{PO}_4$ /150 mM NaCl/2.5 mM KCl) in a final volume of 300  $\mu\text{l}$ . The mixture was centrifuged for 30 s at 1000 g and the supernatant removed. The pellet was washed 5 times with 300  $\mu\text{l}$  of PBS and incubated for 1 h at room temperature in the presence of 100  $\mu\text{l}$  of tissue extract in a final volume of 300  $\mu\text{l}$ . The tubes were centrifuged for 2 min at 1000 g and the PMM activity was measured in the supernatant. Protein A-Sepharose preincubated without serum was used as a control.

### Northern blots

Total RNA was isolated from rat tissues by the guanidinium isothiocyanate/CsCl procedure [27,28]. Poly(A)<sup>+</sup> RNA, prepared with the QuickPrep micro mRNA purification kit of Amersham Pharmacia Biotech, was subjected to electrophoresis in 1.5%

agarose/formaldehyde gels and transferred by capillarity on to Hybond membranes [29]. The probes used were a *NotI*-*EcoRI* restriction fragment of  $\pm 1000$  bp from a mouse *PMM1* cDNA, comprising the whole coding region, and a  $\approx 400$  bp *BglII*-*SalI* restriction fragment corresponding to the 3' end of the coding sequence of mouse *PMM2* (G. Matthijs and E. Schollen, unpublished work). Both were labelled with [ $\alpha$ - $^{32}$ P]dCTP by random priming [29]. The filters were prehybridized for 4 h and hybridized for 14 h at 60 °C in a solution containing 6  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 5  $\times$  Denhardt's solution, 0.5% SDS and 20  $\mu$ g/ml denatured herring sperm DNA. The filters were washed at 60 °C (2  $\times$  SSC, 2  $\times$  15 min; 2  $\times$  SSC/0.1% SDS, 2  $\times$  20 min; 2  $\times$  SSC, 15 min), dried and exposed to a Kodak XAR-5 film at -80 °C for 36 h.

## RESULTS

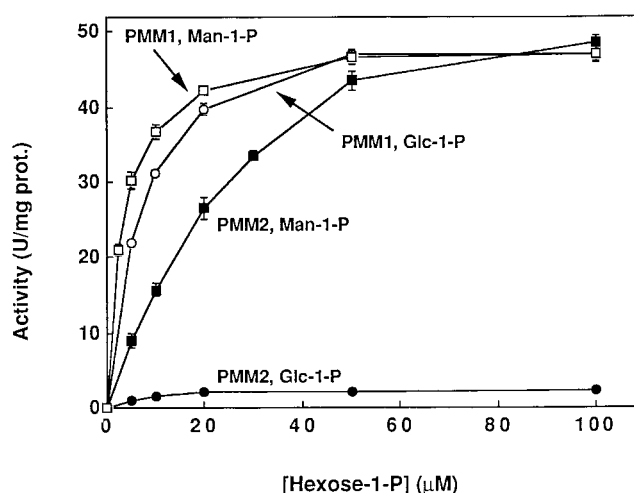
### Expression and purification of PMM2

Extracts of cells prepared 5 h after the addition of isopropylthiogalactoside displayed a PMM activity of 0.42  $\mu$ mol/mg of protein per min, as compared with 4 nmol/mg of protein per min in extracts of cells harbouring a pET3a plasmid devoid of insert. The recombinant enzyme was purified to near-homogeneity by a combination of poly(ethylene glycol) precipitation and chromatography on DEAE-Sepharose and Q-Sepharose. In contrast to PMM1 [13], PMM2 was not retained when chromatographed on DEAE-Sepharose at pH 7.1. It was completely retained on Q-Sepharose at pH 8.8 and could then be eluted at a salt concentration of 200 mM. At this stage, the enzyme displayed a specific activity of 49  $\mu$ mol/mg of protein per min. SDS/PAGE showed that the most purified fractions contained only one major band of 28000 Da, which made up about 90% of the protein present in the preparation (results not shown). As shown in Table 1, the overall purification was 115-fold, with a 45% yield.

### Kinetic properties in the phosphomutase reaction

Like other phosphomutases, PMM2 was found to be entirely dependent on the presence of a hexose bisphosphate for its activity, glucose 1,6-bisphosphate being as potent as mannose 1,6-bisphosphate. In the presence of 10 and 100  $\mu$ M mannose 1-phosphate, half-maximal stimulation of PMM2 was observed at 0.5 and 1  $\mu$ M mannose 1,6-bisphosphate, respectively. The corresponding values for PMM1 were significantly higher (1.3 and 5  $\mu$ M). PMM1 was also stimulated by high concentrations of fructose 1,6-bisphosphate through an indirect mechanism (see below).

In the presence of a saturating concentration of hexose 1,6-bisphosphate, PMM2 converted mannose 1-phosphate into



**Figure 1** Saturation curves of recombinant PMM1 and PMM2 with mannose 1-phosphate or glucose 1-phosphate

The enzymic activities (units/mg of protein) were assayed with 10  $\mu$ M mannose 1,6-bisphosphate and the indicated concentrations of mannose 1-phosphate or glucose 1-phosphate. The results shown are the means  $\pm$  S.E.M. of 3 determinations obtained with two different enzyme preparations.

mannose 6-phosphate at approximately the same rate as did PMM1 ( $\approx 46$   $\mu$ mol/mg of protein per min [10]), but it was about 20 times less active in the conversion of glucose 1-phosphate into glucose 6-phosphate (Figure 1). The  $K_m$  values of PMM2 for mannose 1-phosphate and glucose 1-phosphate were 18 and 12  $\mu$ M, respectively.

The pH-activity profile of human recombinant PMM2 was identical to that of PMM1 [13], with an optimum at pH 6.5 and about 80% of the maximal activity at pH 7.1 (results not shown). The sensitivity to vanadate was also the same as that described previously for PMM1, 50% inhibition being reached at about 50  $\mu$ M vanadate.

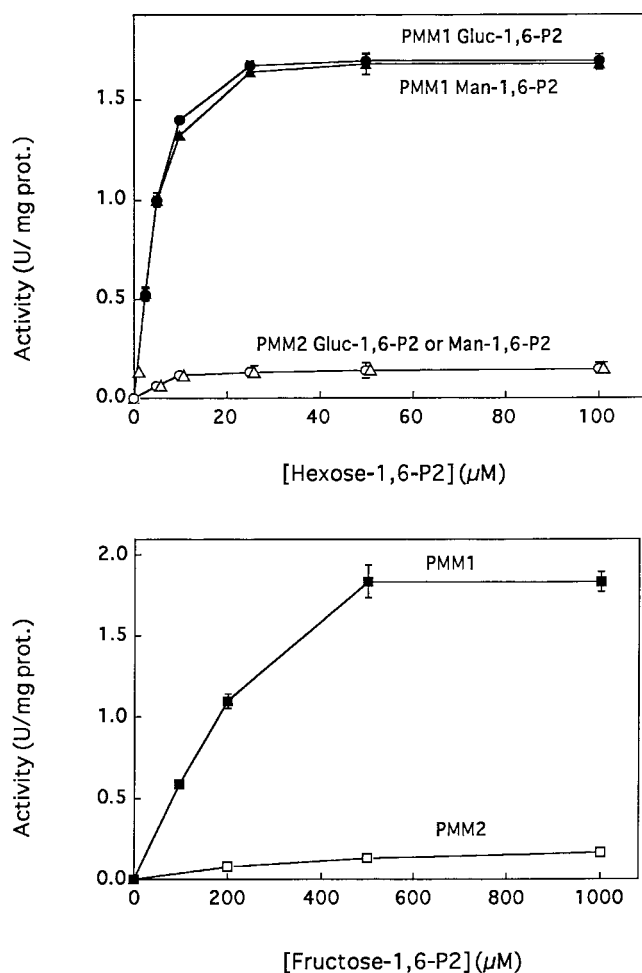
### Hydrolysis of hexose bisphosphates

As shown in Figure 2, PMM1 catalysed the formation of hexose 6-phosphate from either glucose 1,6-bisphosphate or mannose 1,6-bisphosphate and also, but at about 30-fold higher concentration of substrate, from fructose 1,6-bisphosphate. The maximal rates of these hexose bisphosphatase activities amounted

**Table 1** Purification of recombinant PMM2

The purification was performed starting from a 1 litre culture. The PMM activity of the recombinant protein was assayed with 5  $\mu$ M mannose 1,6-bisphosphate and 100  $\mu$ M mannose 1-phosphate. The results shown are representative of 4 independent experiments.

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (-fold)	Yield (%)
Bacterial extract	50	1175	500	0.42	1	100
6–24% Poly(ethylene glycol) fraction	10	255	430	1.68	4	86
DEAE-Sepharose	50	22	333	15	36	66
Q-Sepharose + Amicon YM10	3.5	4.6	225	49	115	45



**Figure 2** Hexose bisphosphatase activities of PMM1 and PMM2

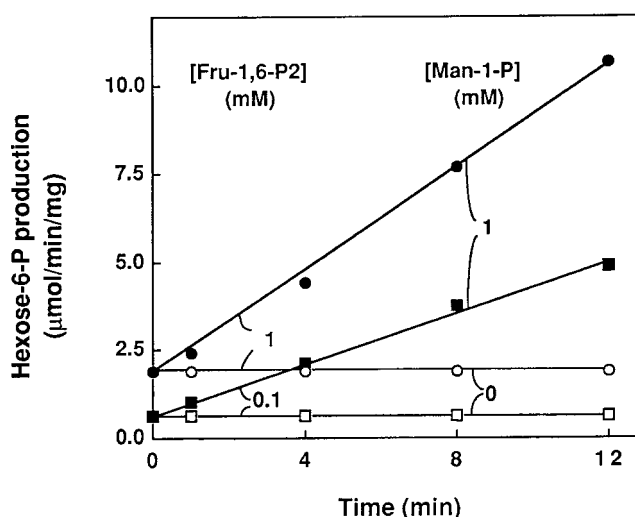
The enzymic activities were measured through the production of the corresponding hexose 6-phosphates with the indicated concentrations of glucose 1,6-bisphosphate (●,○), mannose 1,6-bisphosphate (▲,△) or fructose 1,6-bisphosphate (■,□). The results shown are the means  $\pm$  S.E.M. for  $n = 3$ .

to 1.7–1.9  $\mu\text{mol}/\text{mg}$  of protein per min, i.e. 3.5–4% of the PMM activity. PMM2 was about 10 times less active than PMM1 in this respect. Since both PMM1 and PMM2 have phosphotransferase activities far in excess of their phosphatase activities, it was not possible to determine the proportion of aldohexose 1-phosphate and 6-phosphate that they formed during these reactions. The fate of fructose 1,6-bisphosphate is discussed in the next paragraph.

#### The action of PMM1 on fructose 1,6-bisphosphate

The formation of fructose 6-phosphate from fructose 1,6-bisphosphate by PMM1 was 40% inhibited by 50  $\mu\text{M}$  vanadate, an inhibitor of PMMs, but was unaffected by AMP (1 mM), fructose 2,6-bisphosphate (50  $\mu\text{M}$ ) or EDTA (0.1 mM), known effectors of fructose 1,6-bisphosphatase [30].

The fact that fructose 1,6-bisphosphate was hydrolysed by PMM1 indicated that it phosphorylates the catalytic site of the enzyme and hence can participate in the various phosphoryl-exchange reactions catalysed by PMM1, including the formation of mannose 1,6-bisphosphate from mannose 1-phosphate. Fruc-



**Figure 3** Effect of fructose 1,6-bisphosphate on the activity of PMM1

PMM1 (2  $\mu\text{g}$ ) was incubated in the presence of the indicated concentrations of mannose 1-phosphate and fructose 1,6-bisphosphate in a final volume of 1 ml and the formation of hexose 6-phosphates (mannose and fructose 6-phosphates) was monitored continuously. The graph shows the instantaneous rate at the indicated times. The results are the means of three experiments. S.E.M. values (not shown) amounted to  $\leq 8\%$  of the corresponding means.

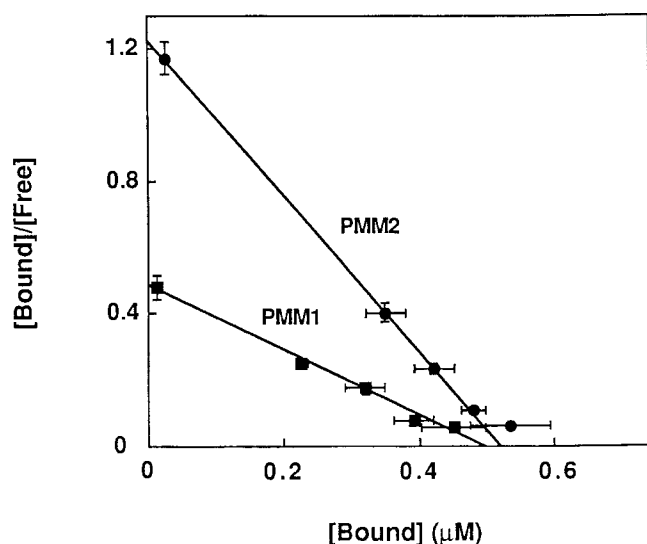
tose 1,6-bisphosphate was therefore expected to stimulate the PMM activity of PMM1. Figure 3 shows an experiment in which PMM1 was incubated with fructose 1,6-bisphosphate in the absence or presence of mannose 1-phosphate. It appears that the rate of hexose 6-phosphate production, which at zero time corresponded to the formation of fructose 6-phosphate from fructose 1,6-bisphosphate, increased progressively with time in the presence of mannose 1-phosphate. In contrast, the reaction was linear when glucose 1,6-bisphosphate or mannose 1,6-bisphosphate was used as a cofactor (results not shown). Unlike PMM1, PMM2 was not stimulated by fructose 1,6-bisphosphate (results not shown).

Other experiments showed that, when incubated with 1 mM mannose 1-phosphate and 1 mM fructose 1,6-bisphosphate, PMM1 formed mannose 1,6-bisphosphate at a rate corresponding to about 20% of the rate of fructose 1,6-bisphosphate disappearance. The latter was converted into a mixture of fructose 1-phosphate and fructose 6-phosphate in a 1:4 ratio (results not shown).

#### Formation of a phosphoenzyme

The phosphorylation of recombinant PMMs was studied using mannose 1,6- $^{32}\text{P}$  bisphosphate as substrate. Preliminary experiments with 0.1  $\mu\text{M}$  mannose 1,6- $^{32}\text{P}$  bisphosphate showed that maximal incorporation was observed after 10 s at 0  $^{\circ}\text{C}$  with PMM1 and after 30 s with PMM2. These times were used to determine the kinetic parameters of these incorporations. As shown in Figure 4, Scatchard plots were linear for both enzymes, allowing us to calculate  $K_{\text{diss}}$  values of 1.03 and 0.46  $\mu\text{M}$  and phosphorylation stoichiometries of 0.48 and 0.30 mol/mol of subunit for PMM1 and PMM2, respectively.

Incorporation of radioactivity was also observed when PMMs were incubated with mannose 6- $^{32}\text{P}$  phosphate and unlabelled mannose 1,6-bisphosphate [13], presumably because mannose 1,6- $^{32}\text{P}$  bisphosphate was formed under these conditions (results



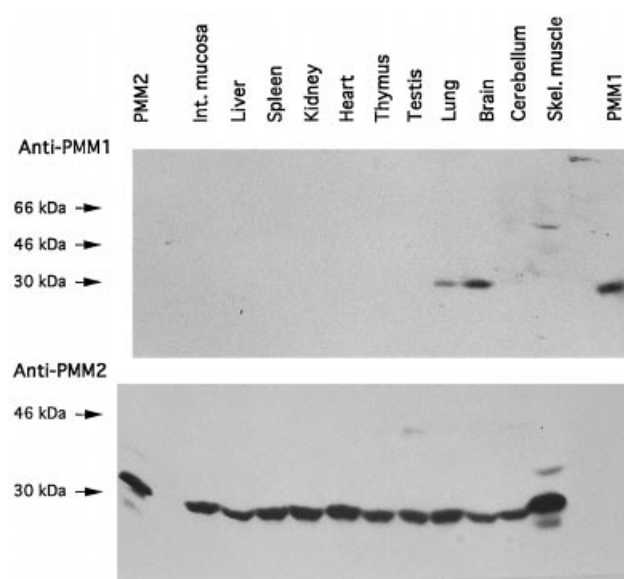
**Figure 4** Scatchard plot of the incorporation of phosphate from mannose 1,6- $^{32}\text{P}$ bisphosphate into PMM1 and PMM2

Recombinant PMM1 (50  $\mu\text{g/ml}$ ) and PMM2 (33  $\mu\text{g/ml}$ ) were incubated at 0  $^{\circ}\text{C}$  for 10 s and 30 s, respectively, with mannose 1,6- $^{32}\text{P}$ bisphosphate and different concentrations of unlabelled mannose 1,6-bisphosphate. Results shown are means of triplicates  $\pm$  S.E.M. The regression lines are shown ( $r = 0.958$  and  $0.957$ , respectively, for  $n = 13$  degrees of freedom).

not shown). The phosphoenzyme displayed the same sensitivity to acid, base and  $\text{NH}_2\text{OH}$  as PMM1 [13] (results not shown).

#### Distribution of PMM1 and PMM2 in rat tissues

Table 2 lists the PMM activity in various rat tissues. The most active one was intestinal mucosa, particularly when the result was expressed per gram of protein, and the tissue with the lowest activity was skeletal muscle. The  $K_a$  for mannose 1,6-bisphosphate (measured with 100  $\mu\text{M}$  mannose 1-phosphate) amounted to about 1  $\mu\text{M}$  in all cases, except for brain, in which it was significantly higher (4.2  $\mu\text{M}$ ). Tissues were also analysed by Western blotting with antibodies directed against PMM1 or



**Figure 5** Analysis of the distribution of PMM1 (top) and PMM2 (bottom) in rat tissues by Western blotting

Recombinant PMM1 or PMM2 (3 m-units) or samples from the indicated rat tissues and containing 3 m-units of PMM were submitted to SDS/PAGE. Protein was transferred to nitrocellulose membranes, which were tested with antibodies raised against human recombinant PMM1 or PMM2. Similar results were obtained in 3 other experiments. Int., intestinal; Skel., skeletal.

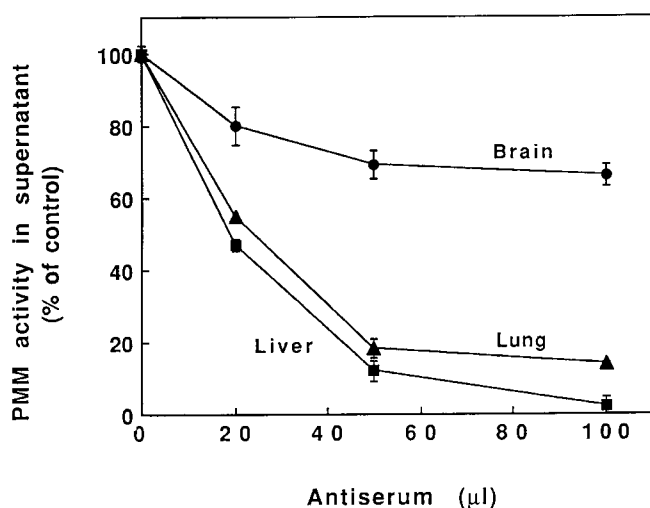
PMM2. PMM2 was found in all investigated tissues, whereas PMM1 was only present in brain and in lung (Figure 5). To quantify more precisely the amounts of the two isozymes present in different tissues, extracts were incubated with a Protein A-Sepharose-anti-PMM2 antibody complex; the mixture was centrifuged and PMM activity was measured in the supernatant. Such experiments, illustrated in Figure 6, indicated that PMM2 accounted for essentially all of the PMM activity in the investigated tissues except for brain and lung where about 66% and 13% of the activity remained in the supernatant (Table 2).

The presence of two distinct isozymes in brain was confirmed in an experiment in which a poly(ethylene glycol) fraction prepared from brain was chromatographed on DEAE-Sepharose.

**Table 2** Activity of PMM in rat tissues and reactivity to antibodies against PMM1 or PMM2

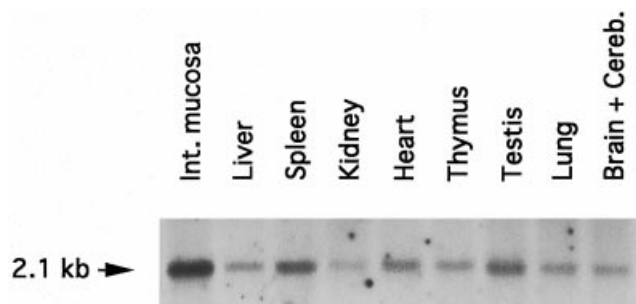
Man-1,6-P<sub>2</sub>, mannose 1,6-bisphosphate.

Tissue	Activity ( $n = 6$ )		$K_a$ for Man-1,6-P <sub>2</sub> ( $n = 2$ ) ( $\mu\text{M}$ )	Reactivity in Western blot ( $n = 4$ )		PMM activity not precipitated with anti-PMM2 Ab ( $n = 3$ ) (% of PMM activity)
	(units/g of tissue)	(units/g of protein)		Anti-PMM1	Anti-PMM2	
Intestinal mucosa	$1.83 \pm 0.11$	$22 \pm 1.8$	1.1, 1.1	—	+	< 5
Liver	$1.45 \pm 0.06$	$5.1 \pm 0.3$	1.2, 1.1	—	+	< 5
Kidney	$1.41 \pm 0.16$	$7.2 \pm 1.4$	1.1, 1.1	—	+	< 5
Spleen	$1.02 \pm 0.08$	$7.0 \pm 0.8$	1.1, 1.0	—	+	< 5
Heart	$0.90 \pm 0.05$	$4.8 \pm 0.5$	1.2, 1.1	—	+	< 5
Thymus	$0.77 \pm 0.04$	$7.2 \pm 0.1$	1.1, 1.1	—	+	< 5
Testis	$0.53 \pm 0.07$	$3.9 \pm 0.8$	1.2, 1.1	—	+	< 5
Lung	$0.42 \pm 0.01$	$4.5 \pm 0.6$	1.2, 1.2	weak	+	$13 \pm 0.3$
Brain	$0.25 \pm 0.02$	$2.6 \pm 0.2$	4.2, 4.3	+	±	$66 \pm 4.2$
Cerebellum	$0.24 \pm 0.05$	$4.1 \pm 0.1$	1.2, 1.1	—	+	< 5
Skeletal muscle	$0.12 \pm 0.01$	$1.4 \pm 0.1$	1.1, 1.0	—	+	< 5



**Figure 6** Immunotitration of PMM2 in rat tissue extracts

Extracts from the indicated tissues were incubated with anti-PMM2 antibodies bound to Protein A–Sepharose. PMM activity was measured in the supernatant and is expressed as percentage of the activity found with control Protein A–Sepharose. The results shown are the means  $\pm$  S.E.M. for  $n = 3$



**Figure 7** Distribution of the PMM2 mRNA in rat tissues

Poly(A)<sup>+</sup> RNA (5 µg) from the indicated tissues was analysed by Northern blotting. A 2.1 kb PMM2 mRNA was also observed in skeletal muscle (results not shown). Results shown are representative of 2 experiments. Int., intestinal; Cereb., cerebellum.

Two peaks of PMM activity were separated, which displayed a  $K_m$  for mannose 1,6-bisphosphate of 1 and 5 µM, respectively, and could therefore be identified as PMM2 and PMM1, respectively. One single peak, corresponding to PMM2, was observed when poly(ethylene glycol) fractions derived from intestinal mucosa, kidney and heart were chromatographed (results not shown).

Northern-blot analysis of poly(A)<sup>+</sup> RNA showed the presence of a 1.5 kb PMM1 mRNA in brain and lung, but not in liver, testis, heart, intestinal mucosa, spleen, thymus or kidney (results not shown). A 2.1 kb PMM2 mRNA was found in all investigated tissues, being most abundant in intestinal mucosa and testis (Figure 7).

## DISCUSSION

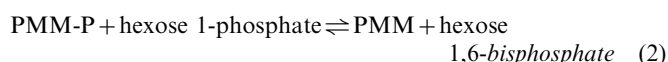
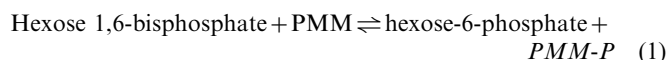
### Properties of PMM1 and PMM2

We have successfully produced the protein encoded by the human *PMM2* gene and shown that it has properties similar

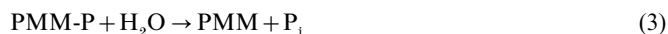
although not identical to those of PMM1. Both proteins, which share about 65% sequence identity, have a similar pH curve and form a phosphoenzyme with the characteristic sensitivity of acyl-phosphate bonds to alkali and NH<sub>2</sub>OH. In PMM1 the phosphorylated residue has been shown to be the first aspartate (Asp-19) in a LFDVDGTL sequence [14]. Since this sequence is completely conserved in PMM2 as well as in other eukaryotic PMMs, we may conclude that the phosphorylated residue is Asp-12 in this isozyme.

The kinetic studies that we have performed indicate that PMM2 is essentially a PMM, whereas PMM1 acts on glucose phosphates and mannose phosphates at equal rates. Furthermore, PMM1, but not PMM2, has a significant phosphatase activity on the hexose 1,6-bisphosphates as well as the ability to form mannose 1,6-bisphosphate, and presumably also glucose 1,6-bisphosphate using fructose 1,6-bisphosphate as a phosphoryl-group donor.

These activities can be explained by the combination of reactions involving either the reversible formation of the phosphoenzyme from a hexose 1,6-bisphosphate:



or the hydrolysis of the phosphoenzyme:



The PMM and the phosphoglucomutase activities are the sum of eqns. (1) and (2). The hexose bisphosphatase activity is the result of eqn. (1) or the reversal of eqn. (2) plus eqn. (3). The formation of mannose 1,6-bisphosphate from fructose 1,6-bisphosphate involves eqns. (1) and (2) acting on both fructose 1,6-bisphosphate and mannose 1-phosphate. We have shown that eqn. (3) occurs to a significant extent only in the case of PMM1.

PMM2 is the first enzyme to be characterized as a specific PMM. Yeast PMM [31,32], which is homologous to PMM1 and PMM2, and the enzymes of *Xanthomonas campestris* [33] and *Pseudomonas aeruginosa* [34], which are not homologous to eukaryotic PMMs, display phosphoglucomutase activities that are about 20, 50 and 150% their PMM activities, respectively.

### Tissular distribution of PMM1 and PMM2

The kinetic properties and the reactivity to specific antibodies indicate that PMM2 is widely distributed in tissues, whereas the presence of PMM1 is restricted to brain and lung. However, the sensitivity of our techniques does not allow us to exclude the possibility that PMM1 contributes to a small proportion (< 5%) of the activity in other tissues. Furthermore, we may not formally rule out the existence of a third isozyme that would be closely related to PMM2 and would therefore be very similar to this enzyme in its kinetic properties and in its reactivity to antibodies. However, all human or mouse expressed sequence tags identified by a BLAST [35] search are identical to either PMM1 or PMM2. Furthermore, an attempt to clone a third isozyme by screening  $\approx 10^6$  clones of a human kidney library with a PMM2 probe under low-stringency conditions resulted in the isolation of 33 PMM2 clones and 2 PMM1 clones, but no other PMM-like clone (E. Schollen and G. Matthijs, unpublished work). These results argue against the existence of a third isozyme.

The results obtained with Northern blots in rat tissues are in agreement with the tissular distribution of the two proteins, the PMM1 mRNA being essentially present in brain and lung. This

finding contrasts with the Northern blots performed with human RNAs, which indicated a widespread distribution of the PMM1 mRNA [10,15]. The observation that PMM activity is < 5% of the normal activity in liver, skeletal muscle and leucocytes of patients with mutations in the *PMM2* gene [8] (E. Van Schaftingen, unpublished work) indicates that PMM1 contributes little to the activity in these cell types. Therefore we hypothesize that the PMM1 mRNA is inefficiently transcribed in some human tissues.

The existence of two different isozymes in rat brain was confirmed by their separation on DEAE-Sepharose. Interestingly, Guha and Rose [36] also separated two peaks of PMM activity when they chromatographed (rabbit) brain extracts on an anion-exchanger. It would be most interesting to know if the two isozymes are present in different cell types of this organ. This would also help to understand the function of PMM1. One interesting possibility is that PMM1 participates in the biosynthesis and the biodegradation of mannose 1,6-bisphosphate and glucose 1,6-bisphosphate; however, other enzymes have been reported to play a role in this process [37,38].

### Consequences of the PMM2 deficiency

One of the striking features of CDG type IA is its multi-organ involvement. Patients with this disease show indeed signs of dysfunction of the central nervous system (olivopontocerebellar hypoplasia accompanied by ataxia, abnormal movements of the eyes, head and limbs, marked psychomotor retardation), liver (liver insufficiency; abnormal secretion of liver proteins, including blood clotting factors), heart (pericardial effusion, cardiomyopathy) and kidney (tubular proteinuria, nephrotic syndrome, renal cysts) as well as dysmorphic features (nipple retraction, large ears, abnormal deposition of fat tissue) [1,2]. This multifaceted symptomatology is best explained by the wide distribution of PMM2, which appears to be practically the sole PMM present in most tissues.

Since PMM2 is quite specific in catalysing the interconversion of mannose 1-phosphate and mannose 6-phosphate, the enzyme defect in CDG IA must be entirely due to the lack of formation of mannose 1-phosphate and hence of the mannosyl donor GDP-mannose, which is involved not only in N-glycosylation of protein but also in the formation of the glycosylphosphatidylinositol (GPI) anchor of membrane proteins [39]. Of interest is the fact that there is no sign of haemolysis in patients with CDG IA, which indicates that PMM deficiency does not grossly perturb GPI-anchor synthesis. This is in contrast with paroxysmal nocturnal haemoglobinuria, which is due to a defect in the biosynthesis of GPI anchor [40].

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