Effect of mutations found in carbohydrate-deficient glycoprotein syndrome type IA on the activity of phosphomannomutase 2

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Abstract **Seven mutant forms of human phosphomannomutase 2 were produced in *Escherichia coli* and purified. These mutants had a V_{max} of 0.2–50% of the wild enzyme and were unstable. The least active protein (R141H) bears a very frequent mutation, which has never been found in the homozygous state whereas the second least active protein (D188G) corresponds to a mutation associated with a particularly severe phenotype. We conclude that total lack of phosphomannomutase 2 is incompatible with life. Another conclusion is that the elevated residual phosphomannomutase activity found in fibroblasts of some patients is contributed by their mutated phosphomannomutase 2.**

Key words: Protein glycosylation; Phosphomannomutase; Mannose; Oligosaccharide

1. Introduction

Carbohydrate-deficient glycoprotein (CDG) syndromes are a series of genetic disorders characterized by a defective *N*-glycosylation of serum and cellular proteins [1,2]. The type IA syndrome (CDG IA), by far the most frequent type, is due to a deficiency in phosphomannomutase (PMM) [3,4], an enzyme involved in the synthesis of GDP-mannose. This defect results in abnormal incorporation of mannose into dolichol-pyrophosphate oligosaccharide [5] and in a decrease in the number of oligosaccharides per protein [6].

CDG IA patients bear mutations in the PMM2 gene [7], which is present on chromosome 16, in a region (p13) previously identified as the CDG IA locus [8,9]. A total of 23 different missense mutations and one single base pair insertion have been found [7,10]. The most frequent mutation (R141H), found in 43 of 53 patients, was never observed in the homozygous state, suggesting that homozygosity for this mutation either is incompatible with life or may lead to a different, possibly normal phenotype.

To explain these and other findings, including the rather high residual PMM activity found in the fibroblasts of some patients [4,10], we have now analyzed the effect of several mutations on the activity and the stability of human recombinant PMM2.

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Abbreviations: CDG, carbohydrate-deficient glycoprotein; CDG IA, carbohydrate-deficient glycoprotein syndrome type IA; PMM, phosphomannomutase

2. Materials and methods

2.1. Materials

Chemicals were from Sigma or Merck. Phosphomannose isomerase was from Sigma; other auxiliary enzymes and NADP were from Boehringer. Mannose 1,6-bisphosphate, prepared as in [3], was purified as in [11]. Extracts of fibroblasts were prepared as in [3].

2.2. Site-directed mutagenesis

All mutants were constructed as in [12]. Site-directed mutagenesis was performed by using *Pwo* DNA polymerase, 'back-to-back' mutated primers and the plasmid pBS-PMM2 [13]. To facilitate the selection of the mutated sequences, an additional silent mutation that introduces or removes a restriction site was systematically added. The PCR-amplified plasmids were isolated by electrophoresis in a 1% agarose gel, purified with the QiaQuick gel extraction kit and recircularized. The inserts of these mutants were checked by sequencing [14] to rule out any PCR errors. *Nde*I-*Bam*HI restriction fragments were excised and inserted into the expression vector pET3a [15].

2.3. Expression of the recombinant proteins

E. coli BL21(DE3)pLys harboring the expression plasmids were grown in 0.3 l of M9 medium at 37°C until A_{600} reached 0.5–0.6. The culture was maintained at 4°C for 20 min before addition of isopropylthiogalactoside to a final concentration of 0.4 mM. The incubation was pursued at 18°C until A_{600} reached $\approx$1.7 (i.e. for about 20 h). Bacterial extracts were prepared and the recombinant enzyme purified by a combination of poly(ethylene glycol) precipitation and chromatography on a DEAE-Sepharose column (1.2×18 cm) [13]. The flow-through fractions containing wild-type or mutant PMM2 were pooled and concentrated 10-fold by ultrafiltration in an Amicon pressure cell equipped with a YM-10 membrane. The enzymes were stored at −80°C in the presence of 20 mM HEPES pH 7.1, 2 mg/ml bovine serum albumin and 1 mM dithiothreitol.

2.4. Enzyme and protein assays

Unless otherwise indicated, PMM was assayed as described [13] except that the concentration of mannose 1-phosphate was 200 μM and mannose 1,6-bisphosphate, 10 μM. Protein was measured as in [16] with bovine gamma-globulin as a standard. PMM assay in fibroblasts was performed as previously described [4].

2.5. Assay of thermal stability

The mutant and the wild-type PMM2s were incubated at the indicated temperatures and for the indicated times at a concentration of 75 μg/ml in the presence of 25 mM HEPES pH 7.1, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol and 1 mg/ml bovine serum albumin. The PMM activity was then measured and compared to the initial activity.

3. Results

3.1. Expression and purification of PMM2 mutants

Preliminary experiments indicated that some of the mutant proteins were insoluble or unstable when expressed at 37°C. Expression was therefore carried out at 18°C for all proteins including the wild-type. Extracts of cells prepared 20 h after

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addition of isopropylthiogalactoside displayed PMM activities ranging between 0.76 nmol/min/mg protein for the R141H mutant to 938 nmol/min/mg protein for the wild-type enzyme (Table 1).

The recombinant PMM2 proteins were purified by poly-(ethylene glycol) precipitation followed by chromatography on DEAE-Sepharose at pH 7.1. This procedure allowed us to separate PMM2, which is not retained by the column [13], from bacterial PMM, which is only eluted when the salt gradient is applied [17]. As previously reported for wild-type PMM2, the protein present in the flow-through fractions is purified to near-homogeneity, as assessed by SDS-PAGE (not shown). Similar results were obtained for all mutants. Purification factors ranged from 40- to 180-fold and the yields were always higher than 85% indicating that all proteins were stable at low temperature.

3.2. Effect of the mutations on the kinetic properties

Table 1 shows the kinetic properties of the mutants. Five substitutions decreased the V_{max} of PMM2 by 2- to 5-fold. In one of them (F119L) this change was accompanied by a marked decrease in the affinity for the substrate and for the activator mannose 1,6-bisphosphate, whereas in other mutants, these effects were either absent (V129M, V231M) or modest (R162W); the D65Y showed a 1.7-fold increase in the affinity for its substrate. The two other substitutions had a marked effect on the V_{max}, which decreased to about 2 and 0.4% of the control value for the D188G and R141H mutant, respectively. In the case of the D188G mutant, this effect was partially compensated for by a 3.5-fold increase in the affinity for the substrate, whereas for the R141H mutant, the decrease in V_{max} was accompanied by an about 10-fold decrease in the affinity for the substrate and for the activator mannose 1,6-bisphosphate.

Similar observations were made when the mutations were introduced into mouse PMM1, the R141H giving rise to the least active recombinant protein (0.5% of the wild-type value in crude extracts).

3.3. Thermal stability of PMM2 and mutants

As shown in Fig. 1A, all mutant proteins were significantly less stable than the wild-type enzyme. The most unstable one was the V231M mutant which lost about 50% of its activity in 10 min; at the other extreme were the D188G and R162W mutant, whose half-lives at 40°C were about 30 min. All other mutants showed intermediate behaviors with half-lives of about 15 min.

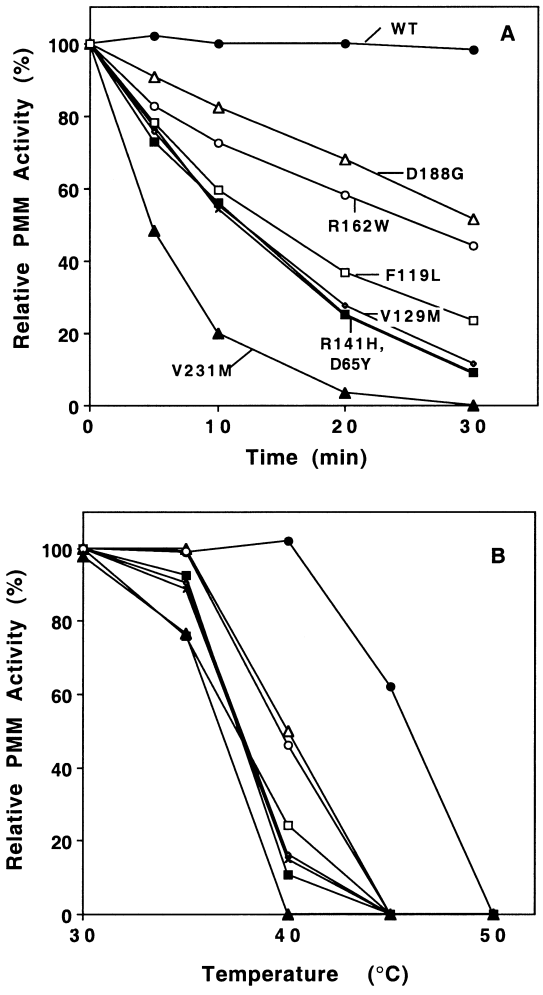

Fig. 1. Thermal stability of wild-type and mutant forms of PMM2. The enzymes were incubated for the indicated times at 40°C (A) or for 30 min at the indicated temperatures (B). The significance of the symbols is indicated in (A), except for (X): D65Y; (■), R141H. Each point represents the mean of three experiments. S.E.M. values have been omitted for the sake of clarity; they represented $<10\%$ of the value to which they refer.

The thermal stability was also analyzed by testing the effect

Table 1
Kinetic properties of wild-type and mutant forms of PMM2

Mutation	Specific activity[a]			K_m for mannose 1-P (μM)	K_a for mannose 1,6-P_2 (μM)
	extract (nmol/min/mg prot.)	purified protein			
		(μmol/min/mg prot.)	(% of control)		
Wild-type	938	35.1	100	18	1.1
D65Y	286	17.7	50.6	11	1.0
F119L	81	8.6	24.5	45	4.9
V129M	355	17.5	50	21	1.1
R141H	0.76	0.14	0.4	165	10.6
R162W	131	6.51	18.6	18	2.1
D188G	9.5	0.66	1.88	5	1.0
V231M	244	13.4	38.5	18	1.1

[a]The enzymic activity was assayed with 200 μM mannose 1-phosphate and 10 μM mannose 1,6-bisphosphate, except for the F119L mutant (200 μM mannose 1-phosphate, 50 μM mannose 1,6-bisphosphate) and R141H mutant (500 μM mannose 1-phosphate, 100 μM mannose 1,6-bisphosphate). Values shown are the means of three values; S.E.M. were less than 5% of the value to which they refer.

Table 2
Phosphomannomutase activity and severity of the affection according to the genotype in patients with CDG IA

Genotype	PMM activity in fibroblasts (mU/mg protein)		No. of deaths in infancy/no. of patients with indicated genotype
	Measured	Theoretical	
WT/WT	3.77 ± 0.30 (8)	3.77	n.a.
D65Y/D65Y	0.64	1.91	0/1
D65Y/R141H	0.82	0.95	1/1
F119L/F119L	0.13	0.92	0/1
F119L/R141H	0.19 ± 0.04 (11)*,**	0.46	2/19
V129M/R141H	0.44	0.94	0/4
R162W/R141H	0.43	0.35	0/1
D188G/R141H	0.10 ± 0.03 (4)*,**	0.04	4/5***
V231M/R141H	0.49 ± 0.10 (6)*	0.72	3/9

Results are individual values or means ± S.E.M. for the indicated number of subjects; they are taken from [4,10]. Theoretical values are calculated based on the activity of the control fibroblasts and the loss of V_{max} found wiht the different mutations (column 4 of Table 1).
Abbreviations: WT, wild-type; n.a., not applicable.
*Significantly different from control ($P < 0.0001$).
**Significantly different from V231M/R141H ($P < 0.05$) by Student's *t*-test.

of 30 min incubations at different temperatures (Fig. 1B). The results of such experiments were consistent with those mentioned above, the wild-type enzyme being by far the most stable protein and the V231M mutant, the most unstable one, with an about 10°C difference between these two extremes. All other proteins showed intermediate values with the same ranking as shown in Fig. 1A.

3.4. PMM activities in fibroblasts of CDG IA patients

Table 2 shows the PMM activities measured in extracts of fibroblasts from patients homozygous or heterozygous for mutations studied in the present work. As previously stressed [10,18], most of the patients are compound heterozygotes with the R141H mutation. PMM activities are compared to 'theoretical' values integrating the effect of the mutations on the V_{max} of the enzyme. Since the R141H mutant is almost inactive, the activity found in extracts of fibroblasts is virtually entirely contributed by the other alleles in subjects heterozygous for this mutation.

The less decreased PMM activities were observed in extracts from patients with the D65Y mutation either in the homozygous state or in the compound heterozygous state with R141H. Intermediary values were observed with the patients heterozygous for the V129M, R162W and V231M mutations, whereas the F119L and most particularly the D188G mutations were associated with the lowest PMM activities. Remarkably, fibroblast extracts from patients with the D65Y, F119L, V129M and V231M mutations had lower PMM values than expected, whereas this did not appear to be the case for the R162W and the D188G mutations. The discrepancy between the calculated and observed value was even more pronounced in extracts of liver or leukocytes though not from lymphocytes.

Table 2 also shows that the D188G/R141H phenotype appears to be associated with a particularly poor vital prognosis [10].

4. Discussion

We show in this work that all seven CDG IA mutations that we have studied cause a decrease in the activity and in the stability of PMM. This entirely confirms the view that mutations in the *PMM2* gene are responsible for the disease. The mutation that most affects the V_{max} of PMM and its affinity for mannose 1-phosphate and mannose 1,6-bisphosphate is R141H. Since, in addition, this mutation destabilizes the enzyme, we conclude that it must lead to a virtually inactive protein in vivo. The fact that this very frequent mutation was never observed in the homozygous state [10,18], whereas patients homozygous for other mutations (D65Y, F119L) have been described [10,19] indicates that a total lack of PMM2 activity is incompatible with life. This is in keeping with the fact that the PMM2 isozyme appears to contribute essentially all of the PMM activity in most tissues [13] and in fibroblasts [11].

Remarkably, D188G, the second most severe mutation after R141H appears to be particularly detrimental in vivo, four of the five cases with the D188G/R141H genotype having died in infancy.

Enzymatic assays have shown variable residual PMM activity in fibroblasts of CDG IA patients [4,10]. It appears now that this residual activity depends on the genotype, being particularly elevated in the case of patients bearing mutations that do not affect greatly the activity of the recombinant protein whereas it is much lower in patients heterozygous for the severe mutation D188G. In some cases (fibroblasts with mutations D65Y, F119L, V129M, R141H, V231M) but not in others (D188G, R162W), the activity observed in extracts is lower than expected from the activity of the recombinant protein. This is easily explained by the fact that the D188G and R162W mutants are more stable than the other mutant proteins.

The fact that some of the mutant proteins still display substantial activity in fibroblast extracts suggests that analysis of this material should be made with caution. It is indeed possible that some mutations are overlooked because they cause only a modest decrease in the PMM activity of fibroblasts.

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