Comparison of PMM1 with the phosphomannomutases expressed in rat liver and in human cells

Michel Pirard[a], Jean-François Collet[a], Gert Matthijs[b], Emile Van Schaftingen[a,*]

[a] *Laboratory of Physiological Chemistry, International Institute of Cellular and Molecular Pathology and University of Louvain, Avenue Hippocrate 75, B-1200 Brussels, Belgium*
[b] *Center for Human Genetics, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium*

Received 22 April 1997

Abstract Carbohydrate-deficient glycoprotein syndrome type I (CDGI) is most often due to phosphomannomutase deficiency; paradoxically, the human phosphomannomutase gene *PMM1* is located on chromosome 22, whereas the CDGI locus is on chromosome 16. We show that phosphomannomutases present in rat or human liver share with homogeneous recombinant PMM1 several kinetic properties and the ability to form an alkali- and NH_2OH-sensitive phosphoenzyme with a subunit mass of $\approx 30\,000$ M_r. However, they have a higher affinity for the activator mannose-1,6-bisphosphate than PMM1 and are not recognized by anti-PMM1 antibodies, indicating that they represent a related but different isozyme. Phosphomannomutases belong to a novel mutase family in which the active residue is a phosphoaspartyl or a phosphoglutamyl.

1. Introduction

Carbohydrate-deficient glycoprotein syndromes (CDG) are multisystemic disorders characterized by a deficiency in the N-glycosylation of serum and cellular proteins [1,2]. The type I syndrome, by far the most frequent type, is an autosomal recessive disorder. Linkage studies have assigned the corresponding locus to the short arm of chromosome 16 [3–5]. Van Schaftingen and Jaeken reported in 1995 that phosphomannomutase activity was deficient in liver, leucocytes and/or fibroblasts of six patients with CDG type I [6]. This deficiency has now been confirmed in cells from 45 out of 52 patients [7]. The seven other cases are presumably due to a different enzyme deficiency.

Two different types of phosphomannomutases have been described. In prokaryotes, the enzyme is a $\approx 50\,000$ M_r polypeptide that has the consensus phosphorylation site (TASHNP) of phosphoglucomutases from prokaryotes and eukaryotes [8–11]. In contrast, phosphomannomutases of *Saccharomyces cerevisiae* and *Candida albicans* are dimers of $\approx 30\,000$ M_r subunits, which do not show sequence similarity with phosphoglucomutases. A human cDNA encoding a phosphomannomutase homologous to the fungal enzyme has recently been cloned [12] from a liver library and the corresponding gene (*PMM1*) shown to be present on chromosome 22. The chromosomal localization of *PMM1* excludes that mutations in this gene are responsible for CDG syndrome type I.

The purpose of the present work is to compare the properties of the PMM1 enzyme with those of phosphomannomutases present in rat liver and in human cells, to investigate the possibility of the existence of different isozymes.

2. Materials and methods

2.1. Materials

Chemicals were from Sigma or Merck. [γ-^{32}P]ATP was from Amersham. Phosphomannose isomerase was from Sigma; other auxiliary enzymes and NADP were from Boehringer. Mannose-1,6-bisphosphate, prepared as in [6], was purified by chromatography on Dowex AG 1-X8 (200–400 mesh; formate form) equilibrated with 50 mM ammonium formate, pH 5.0; elution was performed with a stepwise gradient of NaCl. The purified product was titrated by measuring the mannose-6-phosphate liberated after a 60 min incubation at 95°C in 0.1 M HCl. Recombinant human phosphomannomutase was prepared and purified to homogeneity as previously described [12]. Mannose-6-[^{32}P]phosphate was prepared from mannose and [γ-^{32}P]ATP, and purified as previously described for glucose-6-[^{32}P]phosphate [13]. Extracts of human liver, lymphocytes and fibroblasts were prepared as in [6].

2.2. Partial purification of rat liver phosphomannomutase

The livers of eight rats were homogenized in 4 vols. of a buffer containing 25 mM HEPES, pH 7.1, 250 mM sucrose, 100 mM NaCl and 1 mM dithiothreitol; the homogenate was centrifuged for 15 min at $15\,000 \times g$ and a 6–22% poly(ethylene glycol) fraction was prepared from the supernatant [14]. This fraction was resuspended in 0.6 vol. (compared to the initial homogenate) of buffer A (25 mM HEPES, pH 7.1, containing 1 mM dithiothreitol), clarified by centrifugation and applied onto a DEAE-Sepharose (Pharmacia) column (2.5×40 cm). The column was washed with 400 ml of buffer A and developed with a NaCl gradient (0–500 mM in 2×250 ml of buffer A). The most active fractions were pooled, concentrated 10-fold by ultrafiltration in an Amicon pressure cell (YM-10) and applied onto a Sephacryl S-200 (Pharmacia) column equilibrated with buffer A containing 100 mM NaCl.

2.3. Preparation of polyclonal antibodies and Western blots

For preparation of antiserum, New Zealand rabbits were injected subcutaneously with 500 μg of homogeneous human PMM1 [12] in Freund's complete adjuvant. The injection was repeated twice at 3-week intervals and blood was collected 10 days after the last injection. Western blots were performed and developed as in [15].

2.4. Enzyme and protein assays

Unless otherwise indicated, phosphomannomutase was assayed spectrophotometrically at 30°C in a mixture containing 50 mM HEPES, pH 7.1, 5 mM $MgCl_2$, 0.25 mM NADP, 10 μg/ml yeast glucose-6-phosphate dehydrogenase, 10 μg/ml phosphoglucose isomerase, 3.5 μg/ml phosphomannose isomerase, 0.1 mM mannose-1-phosphate and 10 μM mannose-1,6-bisphosphate. Phosphoglucomutase was assayed as in [6]. One unit of enzyme is the amount catalysing the formation of 1 μmol of product per minute under the assay conditions described above. Protein was assayed according to Bradford [16] with bovine serum albumin as a standard.

2.5. Labelling of the enzyme with mannose-6-[^{32}P]phosphate

Phosphomannomutase (typically ≈ 10 μg of PMM1) was incubated at 0°C with 1 μM mannose-6-phosphate, 100 000 c.p.m. of mannose-6-[^{32}P]phosphate, the indicated concentrations of mannose-1,6-bi-

*Corresponding author. Fax: (32) 2-7647598.
E-mail: vanschaftingen@bchm.ucl.ac.be

PII S0014-5793(97)00704-7

sphosphate, 25 mM HEPES, pH 7.1, 5 mM $MgCl_2$ and 0.1 mg bovine serum albumin in a final volume of 100 μl. The reaction was arrested by addition of 250 μl of 5% ice-cold trichloroacetic acid and the resulting mixture was filtered on polyethersulfone filters (Supor® 200 from Gelman), which were washed with 5 ml of 5% trichloroacetic acid, dried and counted for radioactivity in the presence of 5 ml of HiSafe2 Packard scintillant. In some experiments, the acidified mixture was centrifuged for 10 min at $10\,000\times g$; the pellet was resuspended in 50 μl of a mixture containing 2.5% SDS, 12% sucrose, 12 mM dithiothreitol, 0.02% Brilliant Blue and 50 mM Tris-HCl, pH 6.8. These samples were loaded onto 10% SDS polyacrylamide gels, which were run for 5 h at 0°C and 20 V/cm. The gels were dried and radioactivity was localized with a PhosphorImager apparatus (Molecular Dynamics).

3. Results and discussion

3.1. Partial purification of rat liver phosphomannomutase

Phosphomannomutase was partially purified from rat liver by a procedure involving poly(ethylene glycol) precipitation and chromatography on DEAE-Sepharose and on Sephacryl S-200. As illustrated in Fig. 1A, the peak of phosphomannomutase activity was distinct from that of phosphoglucomutase

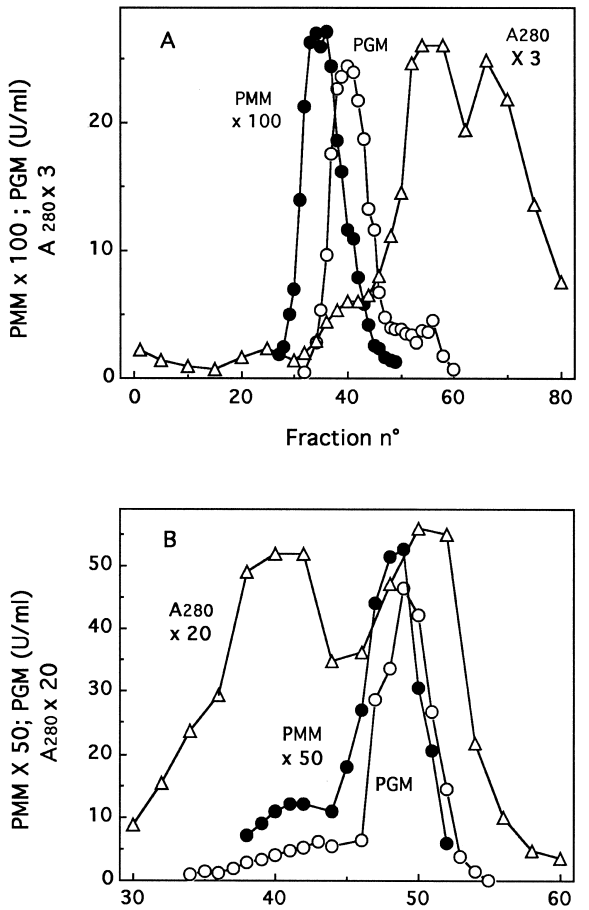

Fig. 1. Elution profile of rat liver phosphomannomutase (●) and phosphoglucomutase (○) from a DEAE-Sepharose column (A) and from a Sephacryl S-200 column (B). A poly(ethylene glycol) fraction obtained from 80 g of liver was applied onto a DEAE-Sepharose column (2.5×40 cm), which was developed with a NaCl gradient; 5 ml fractions were collected; fractions 31–39 were pooled and concentrated before further purification. In (B) a fraction purified by chromatography on DEAE-Sepharose and containing 6.8 U phosphomannomutase was applied onto a Sephacryl S-200 column. Fractions of 1 ml were collected. A_{280} was also measured (△).

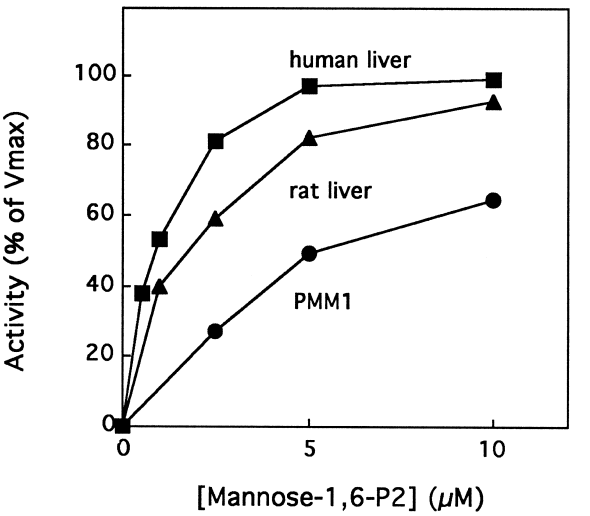

Fig. 2. Saturation curve of human PMM1 (●), rat liver phosphomannomutase (▲) and human liver phosphomannomutase (■) with mannose-1,6-bisphosphate. The enzymic activity was assayed with 100 μM mannose-1-phosphate and the indicated concentrations of mannose-1,6-bisphosphate. Results are expressed as percentage of the activities measured with 100 μM mannose-1,6-bisphosphate. These amounted to 46 (PMM1), 0.3 (rat liver) and 0.009 (human liver) μmol/min/mg protein. One representative experiment is shown.

activity in the DEAE-Sepharose effluent, confirming that the two enzymes are different proteins [17]. The peaks of the two mutases were almost coincident in the Sephacryl S-200 column (Fig. 1B), indicating that phosphomannomutase has a molecular mass close to that of phosphoglucomutase, a monomeric enzyme of about 60 000 M_r [18]. The specific activity of the purified enzyme was ≈300 mU/mg protein compared to ≈3 mU/mg protein in the extract; the yield of the purification was about 10%.

3.2. Kinetic properties of human PMM1 and of rat and human liver phosphomannomutases

Human recombinant PMM1 catalysed the conversion of both mannose-1-phosphate ($K_m = 3.2$ μM) and glucose-1-phosphate ($K_m = 6$ μM) to the corresponding hexose-6-phosphates with a V_{max} for both substrates of approximately 46 μmol/min/mg protein. The enzyme was dependent for its activity on the presence of a hexose-bisphosphate, with a K_a for mannose-1,6-bisphosphate (Fig. 2) and for glucose-1,6-bisphosphate (not shown) of 5 ± 0.2 μM (mean+SEM for $n = 3$). The lack of specificity of PMM1 contrasts with the narrow specificity of mammalian phosphoglucomutases, which are at least 500-fold more active with glucose-1-phosphate than with mannose-1-phosphate [18].

Due to the presence of contaminating phosphoglucomutase, the activity of rat liver phosphomannomutase could not be determined with glucose-1-phosphate. This enzyme displayed a K_m of 3 μM for mannose-1-phosphate (not shown) and K_as for mannose-1,6-bisphosphate (Fig. 2) and glucose-1,6-bisphosphate (not shown) that were significantly lower (1.2 ± 0.1 μM) than those of PMM1. A similar value for mannose-1-phosphate was observed for the enzyme present in crude human liver extracts, the K_a for mannose-1,6-bisphosphate (Fig. 2) and glucose-1,6-bisphosphate (not shown) being equal to 0.9 ± 0.1 μM.

Rat and human liver phosphomannomutases displayed the

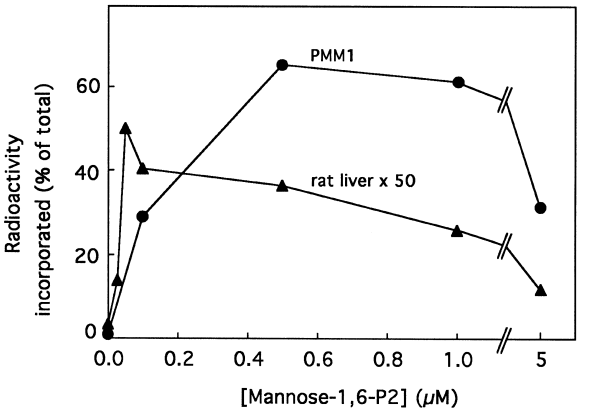

Fig. 3. Effect of the concentration of mannose-1,6-bisphosphate on the formation of a phosphoenzyme. Human recombinant PMM1 (0.45 U; ●) or partially purified rat liver phosphomannomutase (1.5 mU, ▲) was incubated at 0°C for 30 s in the presence of 1 μM mannose-6-phosphate and the indicated concentrations of mannose-1,6-bisphosphate. The results obtained with rat liver phosphomannomutase were multiplied by 50. Each point is the mean of at least three measurements ± SEM.

same pH dependency as PMM1 with an optimum at pH 6.5, about one unit more acidic than that of rabbit muscle or rat liver phosphoglucomutase (not shown). The three phosphomannomutases showed also the same sensitivity to vanadate ($K_i = 100$ μM; not shown), a compound known to inhibit phosphotranferases that form a phosphoenzyme intermediate [19]. This value was much higher than the K_i observed with the phosphoglucomutases mentioned above (1 μM). Furthermore, the three phosphomannomutases were insensitive to arsenate, whereas this compound, a known inhibitor of phosphoglucomutases [20], inhibited the rat and the human liver enzymes by about 80% at 5 mM. These results suggested that rat and human liver phosphomannomutases were most likely enzymes of the same family as PMM1, but with different kinetic properties. Furthermore, the lack of effect of arsenate on the conversion of mannose-1-phosphate to mannose-6-phosphate indicated that this assay specifically measures phosphomannomutase, without contribution of phosphoglucomutase.

3.3. Phosphoenzyme formation

The sensitivity to vanadate [19] and the dependence on bisphosphate esters suggested that the reaction mechanism of phosphomannomutase involved the formation of a phosphoenzyme intermediate. As shown in Fig. 3, incubation of PMM1 with mannose-6-[^{32}P]phosphate in the presence of mannose-1,6-bisphosphate resulted in the incorporation of phosphate into the enzyme. The labelling was dependent on the presence of mannose-1,6-bisphosphate (K_a of ≈ 0.5 μM) and an inhibition at high concentrations of the hexose-bisphosphate was observed. At 5 μM mannose-1,6-bisphosphate, half-saturation of the labelling was obtained with 18 μM mannose-6-phosphate and up to 0.3 mol of phosphate was incorporated per mole of enzyme subunit (not shown). The phosphoenzyme intermediate that was formed was labile, as indicated by the fact that it was completely destroyed after a 10 min incubation at 20°C in 1 M NaOH or in a 0.2 M hydroxylamine/0.1 M sodium acetate (pH 5.5) mixture. It was also half-maximally degraded after 10 min of incubation at 50°C in 5% trichloroacetic acid (not shown). These properties, particularly the sensitivity to hydroxylamine, suggest an acylphosphate bond, as in phosphoaspartate or in phosphoglutamate [21,22]. They allow to exclude a phosphohistidine, which is acid-labile but alkali-stable, and a phosphoserine, which is alkali-labile (though much less than in the present case) but stable to acid [23].

The lability of the phosphoenzyme prevented us from isolating a tryptic [^{32}P]phosphopeptide for identification of the phosphorylated residue. PMM1 appears therefore to belong to a third class of mutases, distinct from both phosphoglucomutases and from phosphoglycerate mutases. The reaction mechanism of these enzymes is indeed known to involve the formation of a phosphoserine and a phosphohistidine, respectively [24,25].

Incorporation of phosphate into protein was also observed upon incubation of the partially purified rat liver phosphomannomutase. In this case a half-maximal stimulation was observed with 0.1 μM mannose-1,6-bisphosphate (Fig. 3). In the experiment shown in Fig. 4, we incubated different fractions of the Sephacryl S-200 column (see Fig. 1B) with 1 μM mannose-6-[^{32}P]phosphate and 0.05 μM mannose-1,6-bisphosphate and submitted the samples to SDS-PAGE. Two different bands of about 30 000 and 60 000 M_r were labelled; the elution profile of these polypeptides corresponded to those of phosphomannomutase and phosphoglucomutase, respectively. Remarkably, the 30 000 M_r band migrated a little faster than PMM1. Phosphoglucomutase was labelled presumably because of partial conversion of mannose-6-phosphate to glucose-6-phosphate by contaminating phosphoglucose isomerase and phosphomannose isomerase. The 30 000 M_r band was no longer apparent if the denatured samples were incubated for 10 min with 1 M NaOH or 0.2 M NH_2OH at pH 5.5, whereas the labelling of the 60 000 M_r band remained unchanged after these treatments (not shown). These data confirm that rat liver phosphomannomutase forms a phosphoenzyme with properties similar to those of PMM1.

Incubation of human liver, lymphocyte or fibroblast extracts with mannose-6-[^{32}P]phosphate together with man-

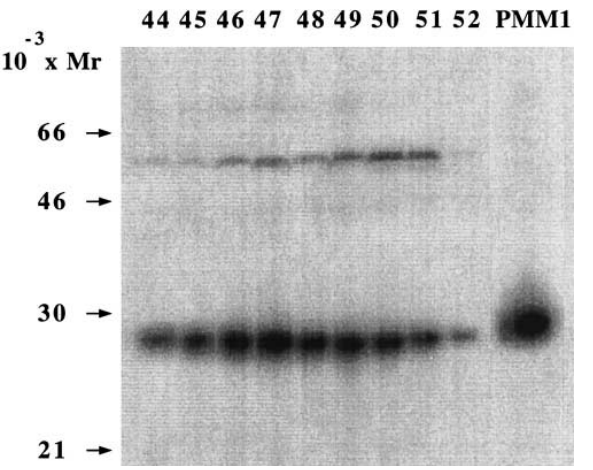

Fig. 4. SDS-PAGE analysis of the proteins labelled with mannose-6-[^{32}P]phosphate. Twenty microlitres of the indicated fractions of the Sephacryl S-200 column shown in Fig. 1B or 0.45 U of PMM1 were incubated with mannose-6-[^{32}P]phosphate and 0.05 μM (Sephacryl S-200 fractions) or 1 μM (PMM1) mannose-1,6-bisphosphate for 30 s at 0°C.

nose-1,6-bisphosphate resulted in a faint but detectable incorporation of phosphate in the 30 000 and the 60 000 M_r bands (not shown). Remarkably, the labelling of the 30 000 M_r band was not detectable in extracts of cells from patients with phosphomannomutase deficiency indicating that the 30 000 M_r polypeptide is either missing or inactive in CDG syndrome type I.

3.4. *Reactivity of phosphomannomutases with antibodies directed against PMM1*

Western blots were performed to check if the $\approx$30 000 M_r polypeptide present in human cell extracts was the same as PMM1. No polypeptide reacted with antibodies directed against PMM1 when samples from liver, lymphocytes and fibroblasts (containing between 0.25 and 0.7 mU phosphomannomutase) were tested (not shown). Controls run in parallel with PMM1 indicated that the technique allowed the detection of 0.01 mU of this enzyme. These data show that the principal phosphomannomutase present in human liver, lymphoblasts and fibroblasts is not PMM1. We may, however, not exclude that a small proportion ($\leq$10%) of the activity is contributed by PMM1. This enzyme could be responsible for the residual phosphomannomutase activity found in patients with CDG syndrome type I.

4. Conclusion

The phosphomannomutases present in human and rat livers have several properties in common with PMM1 including their sensitivity to pH and to vanadate, their ability to form a labile phosphoenzyme intermediate and their subunit molecular mass of about 30 kDa. However, they differ from this enzyme in their higher sensitivity to hexose-bisphosphates and in their lack of reactivity with antibodies directed against PMM1. Taken together these data indicate that they represent a different isozyme of the same protein family. This second phosphomannomutase isozyme is most likely mutated in carbohydrate-deficient glycoprotein syndrome type I. We have preliminary data in favour of this hypothesis (Matthijs, Cassiman and Van Schaftingen, unpublished results).

Acknowledgements: The authors are indebted to G. Berghenouse for technical help. This work was supported by the Actions de Recherche Concertées, by the Belgian Federal Service for Scientific, Technical and Cultural affairs and by the National Funds for Scientific Research. MP is a fellow of the Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture.

References

[1] Jaeken, J. and Carchon, H. (1993) J. Inhert. Metab. Dis. 16, 813–820.
[2] Jaeken, J., Matthijs, G., Barone, R. and Carchon, H. (1996) J. Med. Genet. 34, 73–76.
[3] Martinsson, T., Bjursell, C., Stibler, H., Kristiansson, B., Skovby, F., Jaeken, J., Blennow, G., Strömme, P., Hanefeld, F. and Wahlström, J. (1994) Hum. Mol. Genet. 3, 2037–2042.
[4] Matthijs, G., Legius, E., Schollen, E., Vandenberk, P., Jaeken, J., Barone, R., Fiurama, A., Visser, G., Lambert, M. and Cassiman, J.J. (1996) Genomics 35, 597–599.
[5] Bjursell, C., Stibler, H., Wahlström, J., Kristiansson, B., Skovby, F., Strömme, P., Blennow, G. and Martinsson, T. (1997) Genomics 39, 247–253.
[6] Van Schaftingen, E. and Jaeken, J. (1995) FEBS Lett. 377, 318–320.
[7] Jaeken, J., Artigas, J., Barone, R., Fiumar, A., De Koning, T.J., Poll-The, B.T., De Rijk-van Andel, J.F., Hoffman, G., Mayatepek, E., Pineda, M., Vilaseca, M.A., Saudubray, J.M., Schlüter, B., Wevers, R. and Van Schaftingen, E. (1997) J. Inherit. Metab. Dis., in press.
[8] Zielinski, N.A., Chakrabarty, A.M. and Berry, A. (1991) J. Biol. Chem. 266, 9754–9763.
[9] Stevenson, G., Lee, S.J., Romana, L.K. and Reeves, P.R. (1991) Mol. Gen. Genet. 227, 173–180.
[10] Köplin, R., Arnold, W., Hötte, B., Simon, R., Wang, G. and Pühler, A. (1992) J. Bacteriol. 174, 191–199.
[11] Boles, E., Liebetrau, W., Hofmann, M. and Zimmermann, F.K. (1994) Eur. J. Biochem. 220, 83–96.
[12] Matthijs, G., Schollen, E., Pirard, M., Budarf, M.L., Van Schaftingen, E. and Cassiman, J.J. (1997) Genomics 40, 41–47.
[13] Vandercammen, A., Detheux, M. and Van Schaftingen, E. (1992) Biochem. J. 286, 253–256.
[14] Vandercammen, A. and Van Schaftingen, E. (1990) Eur J. Biochem. 191, 483–489.
[15] Vandercammen, A. and Van Schaftingen, E. (1993) Biochem. J. 294, 551–556.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Guha, S.K. and Rose, Z.B. (1985) Arch. Biochem. Biophys. 243, 168–173.
[18] Ray, W.J., Hermodson, M.A., Puvathingal, J.M. and Mahoney, W.C. (1983) J. Biol. Chem. 258, 9166–9174.
[19] Climent, F., Bartrons, R., Pons, G. and Carreras, J. (1981) Biochem. Biophys. Res. Commun. 101, 570–576.
[20] Belocopitow, E. and Marechal, L.R. (1973) Anal. Biochem. 53, 108–114.
[21] Lipmann, F. and Tuttle, L.C. (1945) J. Biol. Chem. 159, 21–28.
[22] Hokin, L.E., Sastry, P.S., Galsworthy, P.R. and Yoda, A. (1965) Proc. Natl. Acad. Sci. USA 54, 177–184.
[23] Weller, M. (1979) in: Protein Phosphorylation, pp. 452–453, Pion, London.
[24] Ray, W.J. and Peck, E.J. (1972) in: P. Boyer (Ed.), The Enzymes, Vol. 6, pp. 407–477.
[25] Rose, Z.B. (1970) Arch. Biochem. Biophys. 140, 508–513.