Journal of Enzyme Inhibition and Medicinal Chemistry, April 2006; 21(2): 187–192

Taylor & Francis
Taylor & Francis Group

Preliminary studies on the inhibition of D-sorbitol-6-phosphate 2–dehydrogenase from *Escherichia coli* with substrate analogues

CÉLINE ROUX, LAURENT SALMON*, & CATHERINE VERCHÈRE-BÉAUR†

Laboratoire de Chimie Bioorganique et Bioinorganique, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8124, Institut de Chimie Moléculaire et des Matériaux d'Orsay, Bâtiment 420, Université Paris-Sud XI, 15 rue Georges Clemenceau, 91405 Orsay Cedex, France

(Received 23 September 2005; in final form 23 November 2005)

Abstract
D-Sorbitol-6-phosphate 2-dehydrogenase catalyzes the NADH-dependent conversion of D-fructose 6-phosphate to D-sorbitol 6-phosphate and improved production and purification of the enzyme from *Escherichia coli* is reported. Preliminary inhibition studies of the enzyme revealed 5-phospho-D-arabinonohydroxamic acid and 5-phospho-D-arabinonate as new substrate analogue inhibitors of the F6P catalyzed reduction with IC_{50} values of (40 ± 1) μM and (48 ± 3) μM and corresponding K_m/IC_{50} ratio values of 14 and 12, respectively. Furthermore, we report here the phosphomannose isomerase substrate D-mannose 6-phosphate as the best inhibitor of *E. coli* D-sorbitol-6-phosphate 2-dehydrogenase yet reported with an $IC_{50} = 7.5 \pm 0.4$ μM and corresponding K_m/IC_{50} ratio = about 76.

Keywords: *Sorbitol-6-phosphate dehydrogenase, phosphomannose isomerase, phosphoglucose isomerase, inhibition, phosphate sugars*

Abbreviations: *5PAA, 5-phospho-d-arabinonate; 5PAH, 5-phospho-d-arabinonohydroxamic acid; Ec, Escherichia coli; F6P, d-fructose 6-phosphate; G6P, d-glucose 6-phosphate; G6PDH, d-glucose-6-phosphate dehydrogenase; M6P, d-mannose 6-phosphate; NAD, nicotinamide adenine dinucleotide; PMI, phosphomannose isomerase; S6P, d-sorbitol 6-phosphate; S6PDH, d-sorbitol-6-phosphate 2-dehydrogenase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis*

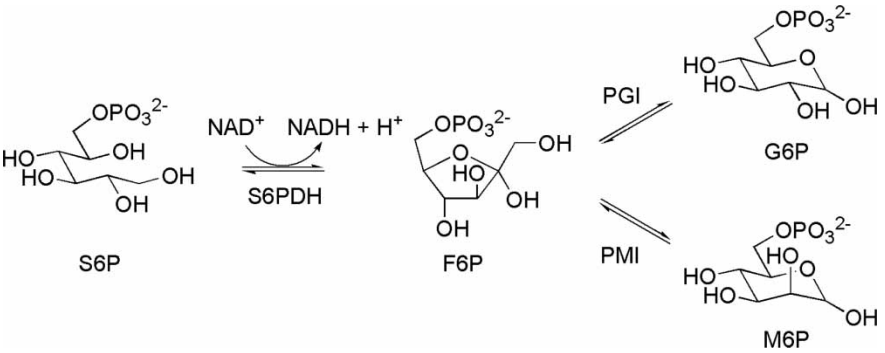
Introduction

There are three main hexitol transport systems in *Escherichia coli*, specific for D-galacticol, D-mannitol and D-sorbitol (also called D-glucitol). This last sugar alcohol catabolic enzyme system is encoded within the *gut* operon on the *E. coli* chromosome, having the gene order: *gutC-gutA-gutB-gutD*, where *gutC* is a regulatory gene, and *gutA*, *gutB*, and *gutD* encode the sorbitol-specific enzyme II, the sorbitol-specific enzyme III and D-sorbitol 6-phosphate 2-dehydrogenase (S6PDH, E.C. 1.1.1.140), respectively [1,2]. This transport system proceeds *via* the phosphoenolpyruvate:sugar phosphotransferase system [3], a group translocation process utilizing phosphoenolpyruvate as a substrate in phosphoryl transfer involving two non-specific cytoplasmic proteins: enzyme I and Heat stable Protein. The membrane-bound II^{gut}- III^{gut} complex of the phosphotransferase system transports and phosphorylates external d-sorbitol [4–6]. The resulting cytoplasmic D-sorbitol 6-phosphate (S6P, Scheme 1) is then reversibly converted by the NAD^+ dependent S6DPH to the glycolytic intermediate D-fructose 6-phosphate (F6P, Scheme 1).

As part of our general interest in the study of enzymes involved in the metabolism of 6-carbons phosphorylated monosaccharides, like phosphoglucose isomerases (PGI) [7] and phosphomannose isomerases (PMI) [8], we were first interested in the design and kinetic evaluation of substrate analogue

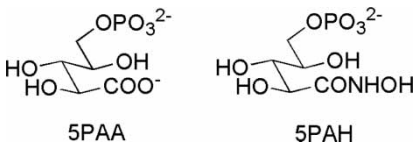
Correspondence address: L. Salmon. Tel: 33 1 69 15 63 11. Fax: 33 1 69 15 72 81. E-mail: lasalmon@icmo.u-psud.fr
†Tel: 33 1 69 15 74 40. Fax: 33 1 69 15 72 81. E-mail: cvercher@icmo.u-psud.fr

ISSN 1475-6366 print/ISSN 1475-6374 online © 2006 Taylor & Francis
DOI: 10.1080/14756360500535260



Scheme 1. Reversible transformations catalyzed by d-sorbitol-6-phosphate 2-dehydrogenase (S6PDH), phosphoglucose isomerase (PGI), and phosphomannose isomerase (PMI) between, respectively, d-sorbitol 6-phosphate (S6P) and D-fructose 6-phosphate (F6P), F6P and D-glucose 6-phosphate (G6P), and F6P and D-mannose 6-phosphate (M6P).

inhibitors of S6PDH. PGI and PMI are the aldose-ketose isomerases which reversibly isomerize, respectively, F6P to D-glucose 6-phosphate (G6P), and F6P to D-mannose 6-phosphate (M6P) as depicted in Scheme 1. Because S6PDH, a quite poorly studied enzyme, shares F6P as a common substrate with PGI and PMI, we proposed to evaluate known PGI and PMI inhibitors and substrates as potential substrate analogue inhibitors of S6PDH. To our knowledge, such inhibitors have never been reported in the literature for this enzyme. They would provide the first tools available to the scientific community for future structural and kinetic studies of the S6PDH mechanism of action. In the meantime, our further interest in S6PDH was closely related to our recent work published on phospho-mannose isomerases [8]: we hypothesized that a PMI/S6PDH coupled enzyme system could constitute a new and useful enzymatic assay for PMI activity measurement in the M6P to F6P direction (Scheme 1). Such a PMI assay would be an alternative method to the known thiobarbituric acid colorimetric assay and PMI/PGI/glucose-6-phosphate dehydrogenase (G6PDH) coupled enzyme assay [8]. Indeed, the former method proved tedious for K_i determinations, while the latter cannot be practically used in the particular case of a weak PMI competitive inhibitor which would also be a strong PGI competitive inhibitor, as we reported for 5-phospho-D-arabinonate (5PAA, Scheme 2) [8]. Hence, a PMI/S6PDH enzymatic assay would have the advantages of being simple to carry out for K_i determinations while avoiding the use of PGI. For that, we needed to check whether or not PMI substrate M6P and known PMI inhibitors [8], like 5PAA and 5-phospho-D-arabinonohydroxamic acid (5PAH, Scheme 2), would inhibit the F6P reduction catalyzed by S6PDH from *E. coli*. Because M6P, 5PAA and 5PAH can also be considered as good structural analogues of the S6PDH substrates F6P and S6P (Schemes 1 and 2), their kinetic evaluation on S6PDH would either allow the design of a new PMI assay or the finding of the first substrate analogue inhibitors for the S6PDH catalyzed reaction.



Scheme 2. 5-Phospho-D-arabinonate (5PAA) and 5-phospho-D-arabinonohydroxamic acid (5PAH) inhibitors evaluated on *E. coli* S6PDH-catalyzed reduction of F6P.

In this communication, we first report an improved and detailed procedure derived from the literature for production and purification of S6PDH from *E. coli*. We also report that both PMI (and PGI) inhibitors 5PAA and 5PAH are good inhibitors of S6PDH from *E. coli*, as well as the PMI substrate M6P which appears as the best EcS6PDH inhibitor ever evaluated, while G6P behaves neither as a substrate nor inhibitor. Although the postulated PMI/EcS6PDH coupled enzyme assay, which would avoid PGI, is therefore very unlikely to be used for PMI activity measurements, our study reports the first substrate analogue inhibitors ever evaluated on a S6PDH.

Materials and methods

Materials

The trisodium salt of 5PAA and the disodium salt of 5PAH were synthesized according to the reported procedures [9–11]. M6P was purchased as the barium salt and converted to the sodium salt by ion-exchange on a Dowex® 50X4–400 resin. 50 mM TRIS-HCl pH 7.5 buffer (T buffer) and 50 mM HEPES pH 7.1 buffer were used after filtration through a 0.22 μm filter. All other commercial chemicals, biochemicals and enzymes were of reagent grade from Sigma-Aldrich® and used without further purification. All

solutions were stored at − 20°C, except NADH which was freshly prepared.

Instruments

UV absorbance measurements were made with a SAFAS® 190 DES spectrophotometer equipped with a Julabo® thermostat regulation device, using 1 mL Brand® polystyrene disposable cuvettes of 1 cm optical path.

Preparation of E. coli D-sorbitol-6-phosphate 2-dehydrogenase

E. coli BL21 pLysS carrying plasmid pET3a-*s6pdh* [12] was grown in 1 L of LB medium with 100 μg/mL ampicillin at 37°C until the culture reached an $OD_{600} = 0.6$. Then, overexpression of the *Ecs6pdh* gene was induced by addition of 0.4 mM isopropylthiogalactoside and the temperature kept overnight at 19°C. Ampicillin [13] was regularly added to a final concentration of 100 μg/mL. At the same time, the pH was maintained at 7.2 ± 0.2 by the controlled addition of 100 mM NaOH, to prevent acidification of the culture due to degradation of the antibiotic by β-lactamase. After growth, the cells were harvested by centrifugation (5000 rpm, 15 min, 4°C), suspended in 30 mL of homogenization buffer (T buffer with 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride) and passed once through a French pressure cell. The cell lysis was completed by addition of DNase (125 U) and RNase (20 μg) in the presence of 10 mM magnesium sulfate. The lysate was centrifuged at 10000 rpm for 1 h at 4°C. The clear supernatant was fractionated by solid ammonium sulfate precipitation: the protein was precipitated at 50% saturation. The extract was placed 72 h at 4°C. After a further centrifugation at 10000 rpm for 1 h at 4°C, the proteins were dialyzed twice against T buffer and chromatographed on a High Load 16/60 Superdex® 200 pg column equilibrated with T buffer (sample volume < 1 mL, flow rate = 1 mL/min). Fractions containing S6PDH activity were pooled, concentrated by precipitation at 50% saturation of ammonium sulfate, and dialyzed against T buffer, ready to use in kinetic studies. For long time storage at 4°C, the proteins were precipitated at 70% saturation of ammonium sulfate. Proteins concentrations were determined by the Bradford method, and the ratio of protein purification was estimated by densitometry with the Alphadigidoc software.

S6PDH assays

In the course of its purification, EcS6PDH was assayed at 25°C through the change in OD_{340}, in 1 mL assay mixture containing T buffer, 3 mM F6P, and 170 μM NADH. Specific activities were measured under the same conditions using substrate concentrations of at least 5 times their corresponding K_m value. Kinetic parameters were determined in the presence of 100–800 μM F6P and 170 μM (> $5\,K_m$) of NADH, or 3 mM (~ $5\,K_m$) of F6P and 5–50 μM NADH. Inhibitors were evaluated for their inhibition ability at the active site of the enzyme compared to F6P. IC_{50} (inhibitor concentration that gives an initial rate equal to 50% of the rate in the absence of inhibitor) of each inhibitor was evaluated in 1 mL assay mixture containing T buffer, 500 μM F6P, and 170 μM NADH. Inhibitors were included at final concentrations of 0–400 μM for 5PAA, 0–300 μM for 5PAH and 0–50 μM for M6P (100 mM aqueous solutions appropriately diluted 10 or 100 times). Duplicate kinetic data were analyzed by double reciprocal plots of the initial reaction velocity *versus* F6P (or NADH) concentration. IC_{50} determinations were based on ten points and were not duplicated. Following preincubation in the spectrophotometer compartment to control absorbance stability, the reaction was initiated by addition of 0.01 U of EcS6PDH.

Results

Purification of the S6PDH from E. coli

S6PDH from *E. coli* was produced and purified according to previously described procedures

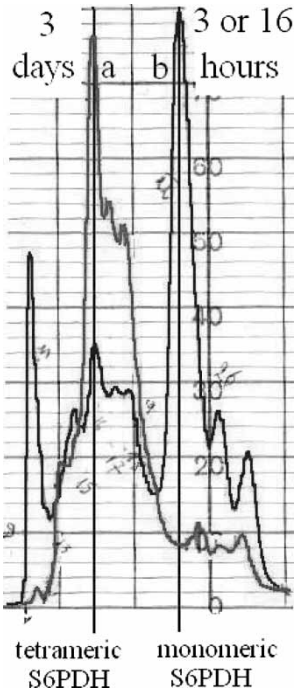


Figure 1. Variable electrophoretic profiles for gel filtration chromatography depending on ammonium sulfate precipitation time: (a) 3 days (grey line), (b) 3 or 16 h (black line).

[12,14]. However, the ammonium sulfate precipitation step, roughly described in both publications, clearly appears as a crucial step. Indeed, depending on the time of proteins precipitation, 72 h (Figure 1a) or 16 h (Figure 1b), electrophoretic profiles of the gel filtration chromatography are significantly different. In the mean time, dehydrogenase activity for the main peak of each profile is quite different and much more important after 72 h than 16 h.

Previous standardization of the column indicates that the main peak in Figure 1a corresponds to proteins with molecular weights of about 130 kDa and in Figure 1b to proteins with molecular weights of about 29 kDa. Moreover, SDS-PAGE analysis revealed that both protein extracts present a 29 kDa major band (Figure 2). Based on these results and kinetic measurements, we assumed that the main peak in Figure 1a corresponds to the tetrameric and active form of S6PDH, and in Figure 1b to the monomeric and inactive form of S6PDH. So, renaturation of this protein is likely to depend on the precipitation time. Hence, a 72 h precipitation time would allow dithiotreitol to be naturally oxidized by air, and consequently, intra- and intermolecular disulfide bonds formation.

Finally, about 12000 U of more than 90% homogeneous EcS6PDH (Figure 2) could be obtained in this way, which was therefore used without further purification for kinetic analyses.

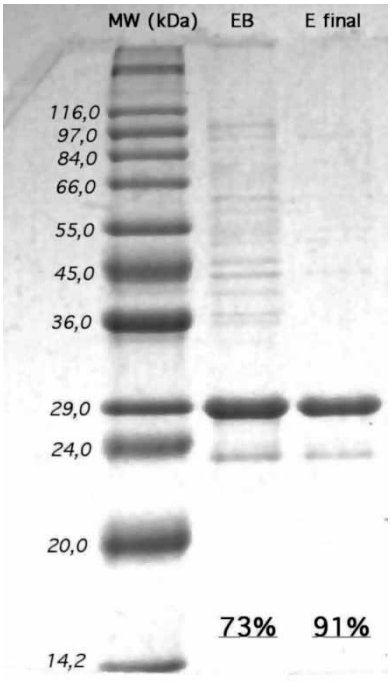


Figure 2. SDS-PAGE of EcS6PDH purification. Lane 1: molecular weight (MW, in kDa); lane 2: crude extract (EB); lane 3: final extract (E final).

Preliminary studies on the inhibition of EcS6PDH by 5PAA, 5PAH, M6P and G6P

First of all, kinetic parameters of F6P and NADH on EcS6PDH had to be determined under our analytical conditions. K_m values of (568 ± 66) μM for F6P and (25 ± 3) μM for NADH were determined in T buffer at 25°C (Figure 3). These values are in the range of those previously reported in 50 mM pH 7.1 HEPES buffer at 30°C (380 μM and 28 μM, respectively) [12].

Preliminary inhibition studies of the F6P reduction catalyzed by EcS6PDH, performed at a substrate concentration equal to K_m, allowed the determination of IC_{50} values of (48 ± 3) μM for 5PAA (Figure 4a) and (40 ± 1) μM for 5PAH (Figure 4b) from the fitted representation of V_i/V_0 (%) against inhibitor concentration using the equation below:

$$\frac{V_i}{V_0} = \frac{100}{1 + \frac{[I]}{IC_{50}}}$$

Although IC_{50} values might be dependent on substrate concentration (as an example for competitive inhibition), 5PAA and 5PAH appear to behave as relatively good substrate analogue inhibitors of EcS6PDH with corresponding K_m/IC_{50} ratio values of 12 and 14, respectively. Thereafter, the PMI substrate M6P was first evaluated as a potential substrate of EcS6PDH in the presence of NADH. No OD_{340} change could be detected. In addition, no PMI activity could be measured with the PMI/S6PDH coupled enzyme system. Indeed, evaluation of M6P as a potential inhibitor of the EcS6PDH-catalyzed reduction of F6P gave us an IC_{50} value of (7.5 ± 0.4) μM (Figure 4c), corresponding to a K_m/IC_{50} ratio value of 76, the highest value ever obtained for an inhibitor of the enzyme. Preliminary studies of G6P on EcS6PDH indicated that the initial rate of the F6P to S6P catalyzed reduction, observed in the presence of as much as 200 μM in G6P, was still 90% of the rate measured in the absence of any inhibitor. This result shows that G6P behaves neither as a substrate nor an inhibitor of EcS6PDH.

Discussion

Among other possible molecules, 5PAA, 5PAH, M6P and G6P were designed as potentially good structural analogues of the 6-carbon phosphorylated monosaccharides S6P and F6P, the two substrates of S6PDH. Their kinetic evaluation on the F6P reduction catalyzed by S6PDH from *E. coli* allowed us to provide for the first time original and valuable tools for future structural and kinetic analyses of the enzyme mechanism of action. Indeed, only irreversible inhibitors, not designed as substrate or reaction intermediate analogues, like diethyl carbonate and

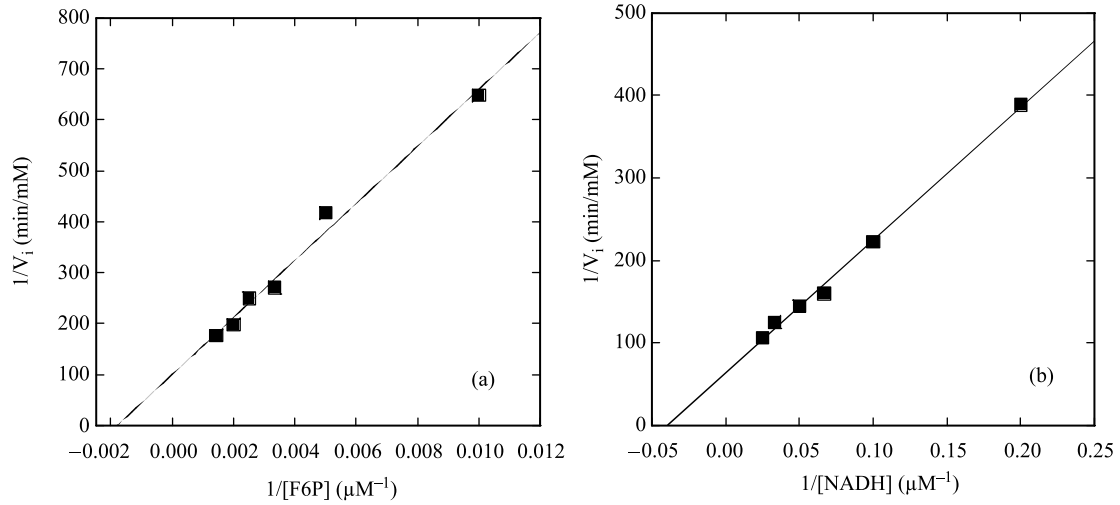


Figure 3. Determination of K_m values of (a) F6P and (b) NADH for the S6PDH-catalyzed reaction.

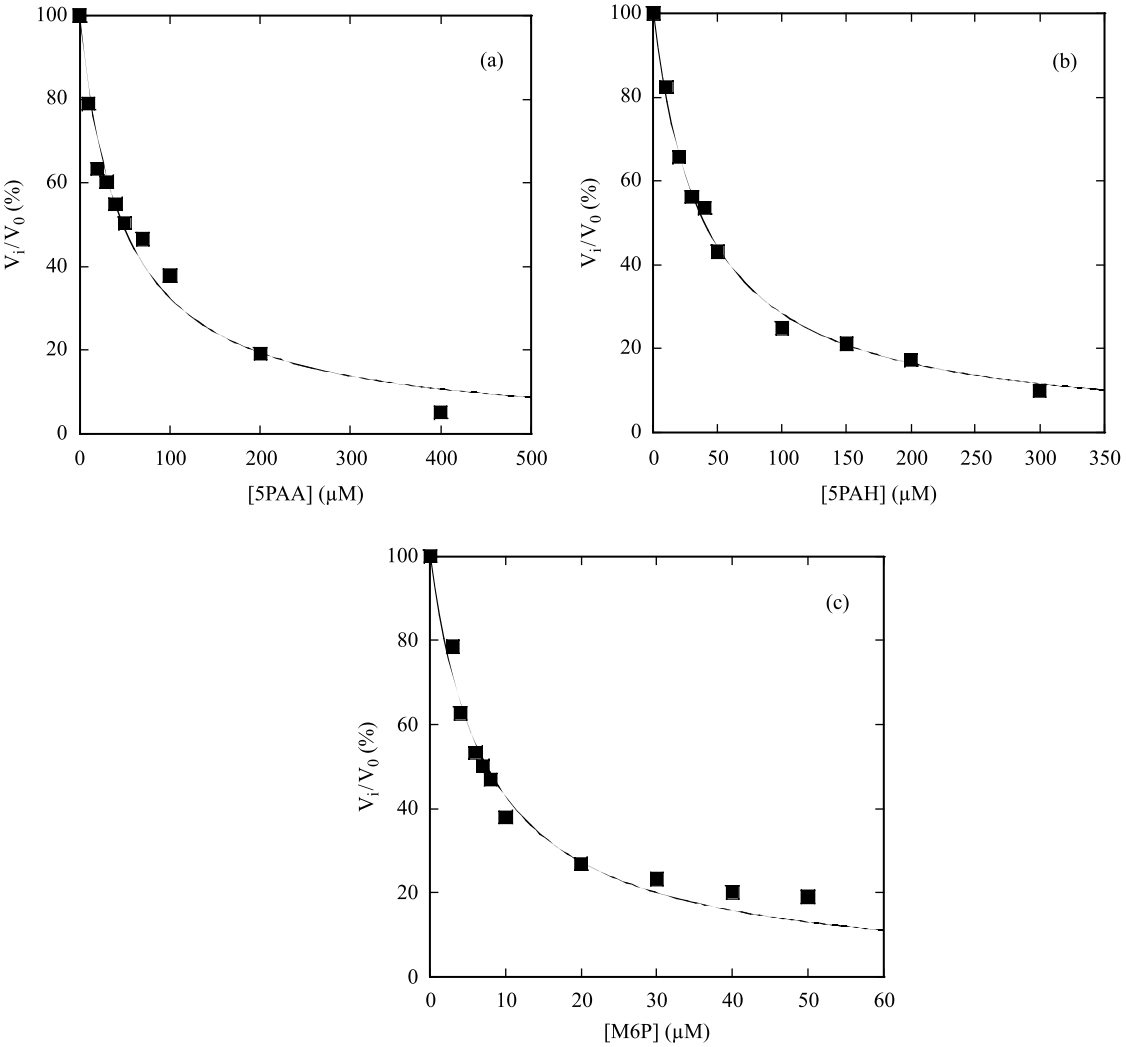


Figure 4. Determination of IC_{50} values of (a) 5PAA, (b) 5PAH, and (c) M6P *versus* EcS6PDH at [F6P] = 500 μM. Values of the ratio of initial reaction velocity in presence (V_i) or absence (V_0) of inhibitor were plotted against inhibitor concentrations, which gives inhibitor concentration = IC_{50} for $V_i/V_0 = 50\%$.

N-ethylmaleimide have been reported in the literature [14]. 5PAA and 5PAH, the two PMI and PGI inhibitors originally designed as mimics of the postulated 1,2-*cis*-enediolate reaction intermediate of the corresponding enzyme-catalyzed isomerization reactions, behave as good substrate analogue inhibitors of EcS6PDH, but not as strong reaction intermediate analogue inhibitors as observed for 5PAA with PGI [15], and 5PAH for PGI [16] and PMI [8]. Indeed, as known for all NAD^+-dependent dehydrogenases, S6PDH most likely involves a hydride transfer from NADH to the keto group of F6P, without formation of an 1,2-*cis*-enediolate high energy intermediate.

Kinetic evaluations of PMI substrate M6P and its C2 epimer, PGI substrate G6P, are quite interesting. While the latter behaves neither as a substrate nor an inhibitor of the EcS6PDH catalyzed reduction of F6P, the former behaves as the strongest structural substrate analogue inhibitor of EcS6PDH yet reported in the literature. Because M6P, the PMI substrate, binds 76 times more tightly S6PDH than its own substrate F6P, a new PMI assay that would use S6PDH as the coupled enzyme is unrealistic. In the case of G6P, however, a new PGI/S6PDH coupled enzyme assay monitoring F6P formation from the G6P to F6P reversible isomerization reaction catalyzed by PGI [17] (Scheme 1) is likely to be efficient and will soon be under investigation. Such an assay will allow us to evaluate PGI inhibitors targeted against pathogens that rely entirely on glycolysis for their development like the parasite *Trypanosoma brucei* involved in African trypanosomiasis.

In conclusion, this paper reports the first S6PDH inhibitors designed as substrate analogues. M6P and G6P kinetic properties determined on *E. coli* S6PDH-catalyzed reduction of F6P are not in accord with the development of a PMI/S6PDH coupled enzyme assay for measuring PMI activity on F6P. However, a PGI/S6PDH coupled enzyme assay for measuring PGI activity on G6P is likely to be developed. Complete kinetic analyses of the bisubstrate mechanism of S6PDH are currently under investigation and will be reported later, as well as elucidation of the inhibitors binding mode through K_i determinations.

Acknowledgements

Pr. Emile Van Schaftingen (Laboratoire de Chimie Physiologique, Christian de Duve Institute of Cellular Pathology and Université Catholique de Louvain, B-1200 Brussels, Belgium) is greatly acknowledged for providing us with the *Escherichia coli* BL21 pLysS bacteria carrying the pET3a-*s6pdh* plasmid and for helpful discussions during protein purification.

References

[1] Lengeler J, Steinberger H. Analysis of regulatory mechanisms controlling the activity of the hexitol transport systems in *Escherichia coli* K12. Mol Gen Genet 1978;167:75–82.

[2] Sarno MV, Tenn LG, Desai A, Chin AM, Grenier FC, Saier Jr., MH. Genetic evidence for glucitol-specific enzyme III, an essential phosphocarrier protein of the *Salmonella typhimurium* glucitol phosphotransferase system. J Bacteriol 1984;157:953–955.

[3] Saier Jr., MH, Grenier FC, Lee CA, Waygood EB. Evidence for the evolutionary relatedness of the proteins of the bacterial phosphoenolpyruvate: Sugar phosphotransferase system. J Cell Biochem 1985;27:43–56.

[4] Dills SS, Apperson A, Schmidt MR, Saier Jr., MH. Carbohydrate transport in bacteria. Microbiol Rev 1980;44: 385–418.

[5] Lengeler J. Mutations affecting transport of the hexitols d-mannitol, d-glucitol, and galactitol in *Escherichia coli* K-12: isolation and mapping. J Bacteriol 1975;124:26–38.

[6] Lengeler J. Nature and properties of hexitol transport systems in *Escherichia coli*. J Bacteriol 1975;124:39–47.

[7] Hardré R, Bonnette C, Salmon L, Gaudemer A. Synthesis and evaluation of a new inhibitor of phosphoglucose isomerase: the enediolate analogue 5-phospho-d-arabinohydroxamate. Bioorg Med Chem Lett 1998;8:3435–3438.

[8] Roux C, Lee JH, Jeffery CJ, Salmon L. Inhibition of Type I and Type II phosphomannose isomerases by the reaction intermediate analogue 5-phospho-d-arabinonohydroxamic acid supports a catalytic role for the metal cofactor. Biochemistry 2004;43:2926–2934.

[9] Chirgwin JM, Noltmann EA. The enediolate analogue 5-phosphoarabinonate as a mechanistic probe for phosphoglucose isomerase. J Biol Chem 1975;250:7272–7276.

[10] Hardré R, Salmon L. Competitive inhibitors of yeast phosphoglucose isomerase: synthesis and evaluation of new types of phosphorylated sugars from the synthon d-arabinonolactone-5-phosphate. Carbohydr Res 1999;318:110–115.

[11] Salmon L, Prost E, Merienne C, Hardré R, Morgant G. A convenient synthesis of aldonohydroxamic acids in water and crystal structure of L-erythronohydroxamic acid. Carbohydr Res 2001;335:195–204.

[12] Moukil MA, Van Schaftingen E. Analysis of the cooperativity of human beta-cell glucokinase through the stimulatory effect of glucose on fructose phosphorylation. J Biol Chem 2001;276:3872–3878.

[13] Hoffman BJ, Broadwater JA, Johnson P, Harper J, Fox BG, Kenealy WR. Lactose fed-batch overexpression of recombinant metalloproteins in *Escherichia coli* BL21 (DE3): Process control yielding high levels of metal-incorporated, soluble protein. Protein Expr Purif 1995;6:646–654.

[14] Novotny MJ, Reizer J, Esch F, Saier Jr., MH. Purification and properties of d-mannitol-1-phosphate dehydrogenase and d-glucitol-6-phosphate dehydrogenase from *Escherichia coli*. J Bacteriol 1984;159:986–990.

[15] Jeffery CJ, Hardré R, Salmon L. Crystal structure of rabbit phosphoglucose isomerase complexed with 5-phospho-d-arabinonate identifies the role of Glu357 in catalysis. Biochemistry 2001;40:1560–1566.

[16] Arsenieva D, Hardré R, Salmon L, Jeffery CJ. The crystal structure of rabbit phosphoglucose isomerase complexed with 5-phospho-d-arabinonohydroxamic acid. Proc Natl Acad Sci USA 2002;99:5872–5877.

[17] Ruijter GJG, Visser J. Characterization of *Aspergillus niger* phosphoglucose isomerase. Use for quantitative determination of erythrose 4-phosphate. Biochimie 1999;81:267–272.