First investigations for the characterization of glucosamine-6- 1 phosphate synthase by capillary electrophoresis

Miriam Beneito-Cambra, Pierre Gareil, Bernard Badet, Marie-Ange Badet-Denisot, Nathalie Delaunay

► To cite this version:

Miriam Beneito-Cambra, Pierre Gareil, Bernard Badet, Marie-Ange Badet-Denisot, Nathalie Delaunay. First investigations for the characterization of glucosamine-6- 1 phosphate synthase by capillary electrophoresis. *Journal of Chromatography B Biomedical Sciences and Applications*, 2018, 1072, pp.130-135. 10.1016/j.jchromb.2017.11.015 . hal-02329349

HAL Id: hal-02329349

<https://hal.science/hal-02329349>

Submitted on 23 Oct 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

First investigations for the characterization of glucosamine-6-phosphate synthase by capillary electrophoresis

Miriam Beneito-Cambrá^a, Pierre Gareil^b, Bernard Badet^c, Marie-Ange Badet-Denisot^c,
Nathalie Delaunay^{a,d,*}

^aLaboratory of Analytical and Bioanalytical Sciences and Miniaturization, UMR CBI 8231, ESPCI Paris, PSL Research University, 10 rue Vauquelin, 75005 Paris, France

^bChimie ParisTech, Laboratory of Physicochemistry of Electrolytes, Colloids and Analytical Sciences (PECSA), 75005 Paris, France

^cCentre de recherche de Gif, Institut de Chimie des Substances Naturelles, CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France

^dCNRS, UMR 8231, Paris, France

Corresponding author: Nathalie Delaunay, ESPCI Paris, Laboratory of Analytical and Bioanalytical Sciences and Miniaturization, UMR CBI 8231, 10 rue Vauquelin, 75231 Paris cedex 05, France. Tel: +33 140794651. Fax: +33 140794776.

e-mail adress: nathalie.delaunay@espci.fr

ABSTRACT

The enzyme glucosamine-6-phosphate synthase (GlmS) is an important point of metabolic control in biosynthesis of amino sugar-containing macromolecules and is therefore a potential target in order to design antibacterial and antifungal drugs. It has two oligomerization states, which are the active dimer and the inactive hexamer. For the first time, the potential of CE to separate and quantify the two forms was studied. After incubating GlmS with the D-glucosamine 6-phosphate (GlcN6P) inhibitor, an electrolyte based on sodium phosphate at pH 7.2 and an ionic strength of 100 mM plus GlcN6P (either 2 or 20 mM) allowed the hexamer-dimer separation. However, the displacement of the dimer/hexamer equilibrium during the analysis time prevented any improvement of the resolution when varying the effective separation length or the temperature of the analysis. Therefore, the use of a short-end CE method allowed the decrease in the analysis time to about 1 min. Some parameters such as the temperature and the time of incubation and the ratio of the inhibitor and enzyme concentrations were studied. Then, it was also possible to test, very rapidly and with a very small amount, some molecules having an inhibition potential for the GlmS enzyme (arabinose-5-phosphate oxime, 2-amino-2-deoxy-D-glucitol 6-phosphate, and glucose-6-phosphate).

Keywords: capillary electrophoresis; enzyme; glucosamine-6-phosphate synthase; inhibitor screening

Abbreviations

ADGP: 2-amino-2-deoxy-D-glucitol 6-phosphate, A5PO: arabinose-5-phosphate oxime, BGE: background electrolyte, BTP: Bis Tris Propane, DTT: dithiothreitol, EDTA: ethylenediaminetetraacetic acid, Glc6P: glucose-6-phosphate, GlcN6P: D-glucosamine 6-phosphate, GlmS: glucosamine-6-phosphate synthase, PTSA: p-toluenesulfonic acid.

1. Introduction

The enzyme glucosamine-6-phosphate synthase (GlmS) is an important point of metabolic control in the biosynthesis of amino sugar-containing macromolecules [1-2]. Indeed, it catalyzes the first step of the hexosamine metabolism since it irreversibly converts D-fructose-6-phosphate into D-glucosamine 6-phosphate (GlcN6P) using L-glutamine as a nitrogen source. The end product, uridine diphosphate N-acetylglucosamine-6-phosphate, is an essential building block of bacterial and fungal cell walls. Therefore, GlmS is a potential target in the design of antibacterial and antifungal drugs [1-3], which is a major issue because invasive fungal infections have increased dramatically over the past two decades and are associated with an increase in morbidity and mortality [4]. Moreover, as GlmS leads to compounds belonging to glycosylation pathways potentially implicated in the regulation of glucose, it is also a therapeutic target to prevent from complications in type-2 diabetes [1, 2].

The active form of GlmS from *Escherichia coli* is a dimeric assembly of two identical monomers, each consisting of a 27 kDa glutaminase domain and a 40 kDa synthase domain [1, 2]. A study using size exclusion chromatography, dynamic and quasi-elastic light scattering, native polyacrylamide gel electrophoresis, and ultracentrifugation demonstrated that the dimer is in equilibrium with a hexameric form, *in vitro* as well as *in cellulo* [5]. Moreover, the enzyme crystal structure studies allowed the identification of two enzyme conformations, which are the dimer and the hexamer. It was also observed that increasing the protein concentration results in a decrease in enzyme specific activity and that the presence of GlcN6P favours the hexameric form. Both effects strongly supported GlmS inhibition through hexamer stabilization, which would reflect a physiologically relevant allosteric regulation of *E. coli* enzyme. In addition to glucose 6-phosphate bound at the active site, the crystallographic hexameric organization of *E. coli* GlmS enables the binding of another linear sugar. Keeping this binding site occupied should then force the GlmS to remain in the inactive hexamer state, which should be useful for the development of specific inhibitors.

The product GlcN6P with weak inhibitory properties ($K_i = 0.38$ mM) was the first studied GlmS inhibitor [1]. Other reported inhibitors, such as arabinose-5-phosphate oxime (A5PO) ($K_i = 14$ μ M) and 2-amino-2-deoxy-D-glucitol 6-phosphate (ADGP) ($K_i = 25$ μ M), which exhibit better inhibition properties are good candidates for the

hexamer stabilization [3]. The potential use of GlmS as a therapeutic target is likely to explain the sustained interest in the synthesis of new GlmS inhibitors [6,7].

Different methods were described in the literature to determine the GlmS activity, such as the Morgan-Elson colorimetric assay, the continuous coupled assay or the radiometric assay employing [^{14}C]Fructose-6-phosphate and involving an electrophoretic separation of formed [^{14}C]GlcN6P [1]. They all involve several laborious steps. More recently, new alternatives were proposed such as isothermal titration calorimetry [8] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [9]. Separation techniques, such as high performance liquid chromatography (HPLC), could also be a valuable alternative. Indeed, HPLC allowed the study of the enzyme activity or the screening of inhibitors thanks to the separation of the substrates or the products followed by spectrophotometry [10, 11] or mass spectrometry detection [12].

In the last two decades, capillary electrophoresis (CE) has become a powerful technique for the study of enzymes [13-17]. Indeed, CE is a powerful miniaturized separation technique that combines a short analysis time, high resolutions, a low cost, and a low sample and reagent consumption. In this study, for the first time, the potential of CE is evaluated for the measurement of the GlmS activity via the simultaneous determination of its active dimer and inactive hexamer forms after a pre-incubation with an inhibitor. The commercially available GlcN6P was used to develop the CE separation, as it was previously observed that the addition of 20 mM of GlcN6P to a 15 μM GlmS solution (about 1 mg mL^{-1}) led to an equilibrium between the dimer and the hexamer [5]. GlcN6P was added to the GlmS sample before injection in the CE capillary, but also in the background electrolyte (BGE) to maintain the equilibrium between both forms. The CE method was also used to investigate the effect of the incubation time, the incubation temperature, and the concentration ratio of inhibitor and GlmS on the dimer/hexamer ratio. Finally, the method was evaluated for inhibitor screening with A5PO, ADGP, and glucose-6-phosphate (Glc6P).

2. Materials and methods

2.1 Chemical and reagents

1 M NaOH solution was from VWR (Fontenay-sous-Bois, France). Acetone (99.9%), HEPES (99.5%), GlcN6P monobasic (99%), sodium dihydrogen phosphate (98%), sodium hydrogen phosphate (99.0%), NaCl, Bis Tris Propane (BTP), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), D-arabinose-5-phosphate disodium, hydroxylamine, p-toluenesulfonic acid (PTSA), and Glc6P were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Ultrapure (UP) water was delivered by a Direct-Q3 system (Millipore, Molsheim, France).

GlmS was purified from *E. coli* cells at 4°C with an anion exchange column (Q Sepharose) with a linear gradient from 0 to 1 M NaCl in a 20 mM BTP, 1 mM EDTA, and 1 mM DTT buffer at pH 7.2 [18]. The fractions containing GlmS were pooled, concentrated by ultrafiltration (30 kDa, Amicon-Ultra Centrifugal Filter Unit, Millipore) and purified with a Superdex 200 gel filtration column (Sigma-Aldrich) and a 50 mM HEPES buffer at pH 7.2 containing 1 mM of EDTA. The collected fractions were concentrated by ultrafiltration (30 kDa Amicon-Ultra). The purity of the isolated GlmS was checked by SDS-PAGE with a protocol already described [18]. A dialysis was carried out in order to reduce the concentrations of HEPES and NaCl. Finally, a stock solution of GlmS at 25.5 g L⁻¹ (0.38 mM) in 50 mM HEPES and 50 mM NaCl buffer at pH 7.2 was obtained and kept at -20°C until use. A5PO was obtained from the reaction of the commercially available arabinose-5-phosphate with hydroxylamine [19]. ADGP was obtained by sodium borohydride reduction of GlcN6P [20].

2.2 Instrumentation

The CE experiments were carried out with an Agilent Technologies 7100 CE system (Massy, France) equipped with a photodiode array absorbance detector. The UV detector was set at 270 nm for the neutral marker (acetone) and between 192 and 200 nm for the GlmS enzyme. The data acquisition and the instrument control were carried out with the Chemstation software. The separations were performed with bare fused-silica capillaries (Photonlines, Marly-le-Roi, France) of 50 µm internal diameter and a total length of 35 or 58.5 cm (effective length 8.5, 26.5 or 50 cm, respectively).

2.3 Electrophoretic conditions

New capillaries were activated by successive flushes under *ca.* 1 bar with 1 M NaOH, 0.1 M NaOH, and water (10 min each, about 300 capillary volumes). Before each run, the capillary was rinsed with 0.1 M NaOH, UP water (5 min each one), and the appropriate BGE for 10 min. The appropriate amounts of GlcN6P, sodium dihydrogen phosphate, and sodium hydrogen phosphate were used to obtain the composition, pH, and ionic strength of the different tested BGEs. The GlcN6P pK_a values of 6.2 and 8.2 were used to make the calculations. The solutions of GlmS at concentration of 0.05 mg/mL (0.76 μ M), 0.13 g L⁻¹ (1.9 μ M), 0.25 g L⁻¹ (3.8 μ M) and 0.51 g L⁻¹ (7.6 μ M) were prepared by dilution of the GlmS stock solution in the appropriate BGE. A dilution of acetone in the BGE (4%, v/v) was used as neutral marker. The solutions of GlmS and neutral marker were separately hydrodynamically injected, in two successive plugs but analyzed in the same run, to avoid any enzyme denaturation or other interference phenomenon.

3. Results and discussion

3.1 Development of the CE method

A 100 mM phosphate buffer at pH 7.2 was selected as BGE in order to study the activity of the GlmS enzyme with a physiological environment. At pH 7.2, GlmS is expected to be negatively charged, as its isoelectric point value is 5.6 [21]. First, the GlmS electrophoretic profile was obtained with this phosphate buffer-based BGE, without adding the GlcN6P inhibitor in the sample and in the BGE. As shown in Figure 1a, 4 peaks appeared in the electropherogram. The migration time of the neutral marker (see the dotted electropherogram) is about 4 min. Consequently, the main peak with a migration time of 5.9 min corresponds to an anion, as expected for GlmS at pH 7.2. Since the enzyme is initially present in the dimer form [1, 2], the main peak was assigned to the GlmS dimer. The first two peaks with migration times of about 4 min, corresponding to the migration time of the neutral marker, may correspond to neutral impurities present in the enzyme solution. The peak with a migration time of 4.8 min was assigned to HEPES, which is present in the GlmS stock solution. This hypothesis was confirmed by the CE analysis of an aliquot of 0.3 mM HEPES diluted in the phosphate-based BGE (data not shown).

Figure 1

Figure 1: Electrophoretic profiles of the GlmS enzyme (7.6 μ M) (a) without or (b) with the addition of the GlcN6P inhibitor in the sample (incubation at room temperature) and in the BGE. In (a), the dotted line corresponds to the injection of the neutral marker (acetone). In (b), the concentration of the GlcN6P inhibitor was 20 mM and the incubation time was (b1) 10 min, (b2) 50 min, and (b3) 100 min. Electrophoretic conditions: bare-fused silica capillary, 50 μ m $\times$ 35 cm (26.5 cm to the detection). BGE: (a) sodium phosphate buffer at pH 7.2 (100 mM ionic strength) and (b) sodium phosphate buffer at pH 7.2 plus 20 mM GlcN6P (100 mM ionic strength). Separation voltage: 10 kV (operating current: 50 μ A). Analysis temperature: 20°C. Hydrodynamic injection: 30 mbar $\times$ 4 s. UV detection at 200 $\pm$ 10 nm for GlmS and 270 $\pm$ 10 nm for the neutral marker.

In order to obtain the GlmS hexamer form, the enzyme was incubated for a given time in a vial containing the phosphate-based BGE plus 20 mM of GlcN6P and next injected in the CE system, using the incubation media as BGE to maintain the hexamer forms during the analysis [22]. As can be seen Figure 1b, the presence of GlcN6P led to the

appearance of an additional peak with a slightly higher migration time than that of the dimer peak. In addition, the intensity of this new peak increases with the incubation time, while the peak assigned to the dimer decreases. The UV spectrum of this new peak was recorded and it was similar to the spectrum obtained for the dimer (data not shown). Accordingly this new peak was assigned to the hexamer form.

A higher migration time for the hexamer GlmS form than for the dimer form is consistent with the expected migration order due to an increase in charge to hydrodynamic size ratio from the dimer to the hexamer form due to a higher compactness, leading to a slight increase in the electrophoretic mobility (absolute value) for the hexamer. In the counter-electroosmotic flow mode, this resulted in an increase in the migration time.

3.2 Optimization of the dimer-hexamer resolution

In order to improve the resolution between the dimer and the hexamer peaks, the influence of the BGE pH was studied (Figure 2a). This modification can affect both electrophoretic (conformation and charge of the enzyme) and electroosmotic mobilities. When the pH increased from 7.2 to 8.2, an increase in the electroosmotic mobility was observed as expected, which induced a decrease in the migration times of the dimer and hexamer forms, despite the increase of the negative charge of the enzyme that would contribute to an increase in the absolute value of the electrophoretic mobility. Unfortunately, a pH increase from 7.2 to 8.2 resulted in a gradual decrease in the dimer-hexamer resolution. When a BGE with a pH of 6.2 was tested, there was a marked decrease in the peak intensities of the enzyme (data not shown), may be due to a partial precipitation of the enzyme at a pH near its pI value. The BGE pH was therefore set at 7.2 for the other experiments of the study.

Figure 2

Figure 2: Electropherograms of GlmS (0.76 μ M) obtained with a BGE composed of (a) a sodium phosphate buffer plus 20 mM GlcN6P (100 mM ionic strength) at pH 7.2, 7.6, and 8.2 or (b) a sodium phosphate buffer plus 20 mM GlcN6P at pH 7.2 with a total ionic strength of 50, 100, or 150 mM. The GlmS aliquots were incubated for 120 min at room temperature within the BGE. Operating currents: between 22 and 65 μ A. For other conditions, see Figure 1.

The ionic strength of the BGE can also affect the dimer-hexamer separation, since it modifies the GlmS effective charge, its potential adsorption to the capillary wall, and the electroosmotic mobility. Figure 2b shows the obtained electrophoretic profiles using the BGEs with an ionic strength of 50, 100, and 150 mM. Table 1 presents the resulting electroosmotic and electrophoretic mobilities, the effective selectivity, the resolution, and the efficiency for the dimer and hexamer peaks. As expected, when the separation was performed with a BGE having a higher ionic strength, an increase in migration times was observed due to a significant decrease in the electroosmotic mobility. Considering the absolute value of the electrophoretic mobility of the dimer, a decrease

is observed when the ionic strength increases from 50 to 150 mM. This behavior may be explained by an increase in the condensation of cations on the surface of the enzyme, which results in a decrease in its effective charge [23]. Both led to an improved resolution between the GlmS dimer and hexamer peaks when the BGE ionic strength increased, to reach a maximum around 100 mM. This ionic strength value was selected for the rest of the study, since the effective selectivity and the efficiencies are the same at 100 or 150 mM, but a lower ionic strength contributes to reduce the Joule heating and 100 mM provides maximum resolution. It is worthwhile to notice that for each tested condition in this manuscript, the voltage value was set in order to stay in the area of a linear increase in current with voltage. In that case, the rise in temperature in the capillary is not detrimental and a maximum difference value of 5°C was calculated between the set value and the temperature existing in the center of the capillary [24].

Table 1. Electroosmotic mobility (μ_{EO}), electrophoretic mobility of the dimer ($\mu_{ep,dimer}$), effective selectivity (α), resolution (Rs), hexamer-dimer corrected area ratio, and efficiency (N) for the dimer and hexamer peaks obtained at different ionic strengths of the BGE at pH 7.2. The aliquots of 0.76 μ M GlmS were incubated at room temperature during 120 min. Other conditions, see Figure 1.

BGE ionic strength	50 mM	100 mM	150 mM
μ_{EO} ($\times 10^5 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$)	41.5	38.7	36.4
$\mu_{ep,dimer}$ ($\times 10^5 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$)	-14.7	-13.2	-12.8
$\alpha_{(dimer - hexamer)}^a$	-	1.1	1.1
$Rs_{(dimer - hexamer)}^b$	0	1.0	0.8
hexamer-dimer corrected area ratio	-	1.0	1.9
$N_{(dimer)}^c$	-	8000	9000
$N_{(hexamer)}^c$	-	11000	10000

$$^a \alpha = (t_{hexamer} - t_{EOF}) / (t_{dimer} - t_{EOF}) \quad (t_i = \text{migration time})$$

$$^b Rs = 1.177 \cdot (t_{hexamer} - t_{dimer}) / (\delta_{hexamer} + \delta_{dimer}) \quad (\delta = \text{width at half height peak})$$

$$^c N_i = 5.54 \times (t_i / \delta_i)^2$$

The separation temperature was reduced to 15°C, in an attempt to improve the oligomer resolution, but no change in resolution was obtained (data not shown). An increase in the effective capillary length from 26.5 to 50 cm was next tested, keeping constant the other parameters. These two lengths led to analysis time of 7 and 27 min, respectively. This significant increase in migration times results both from an increase in the effective length and a decrease in the electrical field, as the voltage was kept constant. As can be observed in Table 2, the efficiencies increased with the effective capillary length as it is expected by the theory. However, the resolution between the two peaks was not improved. Antagonistic effects between the separation of the dimer and the hexamer along the capillary thanks to their different electrophoretic mobilities and the displacement of the equilibrium between the two forms may explain this particular behavior.

Table 2. Effective selectivity (α), resolution (R_s), and efficiency (N) for the dimer and hexamer peaks obtained with two different effective capillary lengths. BGE: sodium phosphate buffer plus 20 mM GlcN6P at pH 7.2 (100 mM ionic strength). Hydrodynamic injection: 30 mbar $\times$ 4 s or 80 mbar $\times$ 5 s for effective length capillaries of 26.5 and 50 cm, respectively. Separation voltage, 10 kV. Analysis temperature, 15°C. The aliquots of 0.76 μ M GlmS were incubated in BGE at room temperature during 120 min.

Effective capillary length (l)	26.5 cm	50 cm
l/L^a	0.76	0.85
$\alpha_{(\text{dimer} - \text{hexamer})}^a$	1.1	1.1
$Rs_{(\text{dimer} - \text{hexamer})}^b$	1.1	1.0
$N_{(\text{dimer})}^c$	8000	9000
$N_{(\text{hexamer})}^c$	12000	17000

^aL: total capillary length

To study this hypothesis, the effect of the analysis time on the ratio of the corrected areas of the hexamer and dimer peaks was investigated. As can be seen in Figure 3, the proportion of the hexamer form increased with the incubation time and reached a plateau after several tens of minutes. However, the analysis time led to significant different values of the ratio of the two forms, whatever the pre-incubation time of the sample before the injection in the capillary: the longer the analysis time, the higher the

ratio of hexamer to dimer. This behavior is consistent with a displacement of the dimer-hexamer equilibrium during the electrophoretic separation, despite the presence of the inhibitor in the BGE since the same solution is used for the CE analysis and the incubation step. Considering the increase in the hexamer proportion with the incubation time and the analysis time, which can be considered as an extension of the incubation time as the inhibitor is also present in the BGE, we can suggest that the kinetics of the hexamer formation is faster than its dissociation into dimer. To reinforce this hypothesis, a sample incubated for 50 min at room temperature was also analyzed with the longer capillary (50 cm effective length) at a higher temperature of analysis (35°C), but not so different from the incubation temperature value (room temperature, therefore about 10°C) and during a limited time (17 vs 50 min). In that case, the analysis time was reduced to 17 min and the ratio of the hexamer/dimer corrected areas was reduced as expected, from 1.9 to 0.7.

Figure 3

Figure 3: Plot of the ratio of the corrected areas of the hexamer to dimer peaks vs the incubation time for two different analysis times. Diamonds, analysis time: 7 min, effective capillary length: 26.5 cm. Squares, analysis time: 27 min, effective capillary length: 50 cm. The aliquots of 0.76 μ M GlmS were incubated in the BGE at room temperature. Each condition (incubation + electrophoretic analysis) was tested 3 times. The meaning of the error bar is the highest and the lowest obtained values. For other conditions, see Table 2.

315

316 With this first CE method, other characterizations of the enzyme-inhibitor system were
 317 carried out. As an example, using a GlcN6P inhibitor concentration fixed at 20 mM, the
 318 concentration of the GlmS enzyme was varied from 0.76 to 3.8 μ M, which corresponds
 319 to an inhibitor-enzyme molar ratio between 26,300 and 5,300. Figure 4a shows the ratio
 320 of the corrected areas of hexamer to dimer peaks *vs* the incubation time for the different
 321 ratios of the inhibitor to enzyme concentrations. The proportion of the inactive hexamer
 322 form increases with the concentration of the dimer, which is consistent with the mass
 323 action law.

Figure 4

324

325 **Figure 4:** Plots of the ratio of the corrected areas of the hexamer to dimer peaks *vs* the
 326 incubation time for (a) different ratios of the inhibitor and enzyme concentrations and
 327 (b) two incubation temperatures. (a) [GlcN6P] = 20 mM, [GlmS] = 0.76, 1.9, and
 328 3.8 μ M, which correspond to ratios of 26,300 (diamonds), 10,500 (squares), and 5,300
 329 (triangles), respectively. Incubation at room temperature; total capillary length, 35 cm;
 330 for other separation conditions see Table 2. (b) incubation at 4°C (squares) or at room
 331 temperature (diamonds); [GlmS] = 0.76 μ M and [GlcN6P] = 20 mM; total capillary

length, 58.5 cm; for other separation conditions see Table 2. Each condition was tested 2 and 3 times (incubation + electrophoretic analysis) in a and b, respectively. The meaning of the error bar is the highest and the lowest obtained values.

The incubation temperature was also studied: two aliquots of the GlmS enzyme were incubated with GlcN6P at 4 and 25°C, and injected at different incubation times (Figure 4b). In both cases, if the hexamer/dimer ratio increases with the incubation time, the low incubation temperature seems to favor the displacement of the equilibrium to the inactive hexamer form. We can notice that the equilibrium between the dimer and hexamer forms is temperature-dependent and that some Joule heating during the separation occurs, but it may have a little impact on it since, as already said, a maximum difference value of 5°C was calculated between the set value and the temperature existing in the center of the capillary [24] and, in most cases, the value is even below 2°C.

3.3 Short-end CE method

Since the time scale of the dimer-hexamer rearrangement kinetics is close to the analysis times previously used (7 and 27 min), a reduction of the analysis time was searched using an injection by the short-capillary end to reduce the effective length to 8.5 cm. Using this CE configuration, the analysis time was reduced to less than 2.5 min (data not shown). The resolution between the dimer and hexamer peaks was slightly improved to a value of 1.2, which confirms the hypothesis of a displacement of the dimer-hexamer equilibrium during the CE separation. It is worthwhile to notice that the injected volume was reduced to 2 nL (30 mbar for 2 s) keeping a non-significant contribution of the injection step to the peak band broadening, since the injected plug length was about 1% of the effective separation length.

To speed up further the analysis, the temperature was increased from 15 to 25°C and the voltage was increased as much as possible while preserving the resolution to a value of 15 kV. These conditions allowed a decrease in the analysis time to 1.2 min (Figure 5). The UV spectra of the peaks attributed to the dimer and the hexamer had a maximum of absorption around 200 nm. The detection wavelength was next optimized and an improvement of the signal to noise ratio of about 15% was obtained using 192 nm instead of 200 nm.

Figure 5: Electropherogram of the GlmS enzyme (1.9 μ M) obtained with a short-end injection. Electrophoretic conditions: bare-fused silica capillary, 50 μ m $\times$ 35 cm (8.5 cm to the detection). BGE: sodium phosphate buffer at pH 7.2 plus 20 mM GlcN6P (100 mM ionic strength). GlmS was incubated in the BGE at 4°C for 30 min. Separation voltage: 15 kV. Analysis temperature: 25°C. Hydrodynamic injection: 30 mbar $\times$ 2 s. UV detection at 192 ± 4 nm.

Using the short-end approach, the influence of the concentration of the GlcN6P inhibitor on the ratio of the corrected areas of the hexamer to dimer peaks was studied with an incubation at 4°C (Figure 6). A decrease in the GlcN6P concentration from 20 to 2 mM led to a smaller conversion into the inactive hexamer form, i. e. a decrease in the inhibition effect, which agrees with the mass action law, since the GlcN6P is a product of the enzymatic reaction. At a given inhibitor concentration, as already previously observed in Figure 4b, a decrease in the incubation temperature induced an increase in the inhibition effect. Then, a smaller inhibitor effect has to be expected at physiological temperatures. It is important to note that here, even if the analysis temperature is different from the incubation temperature, it must not affect significantly the hexamer-dimer ratio as the analysis time is very short (1.2 min) compare to the incubation time (up to 300 min).

This method was finally used to test three inhibitors, ADGP, A5PO, and Glc6P, each at a concentration of 10 mM in the sample and in the phosphate-based BGE at pH 7.2 and with an incubation at 4°C, which correspond to values close to the values used in this study with GlcN6P. All electropherograms show only one peak. In order to make the most correct identification possible using the migration time and to take into account the small variations of the electroosmotic flow, an internal standard was used. PTSA was selected as it has a migration time of about 2 min, has a strong absorption in UV and does not affect the profile of the GlmS peaks whatever the tested inhibitor. Table 3

presents the average, the standard deviation, and the confidence interval values of the corrected electrophoretic mobilities of the dimer and the hexamer forms obtained with the introduction of 20 mM of GlcN6P in the sample and in the BGE. The corresponding values pertaining to the single peak obtained by replacing GlcN6P by another potential inhibitor are also given. If for ADGP, it is not possible to conclude which form was present without further experiments such as spiking, the other two inhibitors allow the formation of the inactive hexamer form. These preliminary results confirm the great potential of this approach for a rapid screening of potential inhibitors.

Figure 6: Plots of the ratio of the average corrected areas of the hexamer to the dimer peaks vs the incubation time for a concentration of the GlcN6P inhibitor of 2 mM or 20 mM with an incubation at room temperature or at 4°C (n=2). Diamonds, 2 mM GlcN6P and incubation at 4°C. Squares, 20 mM GlcN6P and incubation at 4°C. Triangles, 20 mM GlcN6P and incubation at room temperature. [GlmS] = 1.9 μ M. Analytical conditions, see Figure 5.

Table 3. Average, standard deviation (SD), and interval values of the corrected electrophoretic mobilities (μ_{ep}) of the dimer and the hexamer, obtained with 20 mM of GlcN6P in the sample and in the BGE, or of the single peak obtained with 10 mM of ADGP, A5PO or Glc6P in the sample and in the BGE. [GlmS] = 1.9 μ M. Incubation between 60 and 180 min, at 4°C. PTSA was used as internal standard at a concentration of 0.1 mM. Other conditions: see Figure 6.

	$\mu_{ep}(\text{dimer})/\mu_{ep}(\text{PTSA})$ (n ^b =12)	$\mu_{ep}(\text{hexamer})/\mu_{ep}(\text{PTSA})$ (n=12)	$\mu_{ep}(\text{peak obtained with ADGP})/\mu_{ep}(\text{PTSA})$ (n=4)	$\mu_{ep}(\text{peak obtained with A5PO})/\mu_{ep}(\text{PTSA})$ (n=5)	$\mu_{ep}(\text{peak obtained with Glc6P})/\mu_{ep}(\text{PTSA})$ (n=8)
average	0.508	0.547	0.524	0.541	0.539
SD	0.011	0.008	0.009	0.005	0.002
confidence interval^a	0.487 - 0.529	0.531 - 0.563	0.506 - 0.542	0.532 - 0.549	0.534 - 0.544

^avalues calculated for a 95% probability

^bnumber of repetition of the electrophoretic analysis

4. Conclusion

The GlmS enzyme is characterized by an allosteric regulation involving an equilibrium between two forms: the active dimer and the inactive hexamer. For the first time, it was demonstrated that the two forms can be separated by CE after introducing GlcN6P in the sample for pre-incubation and in the BGE. A sodium phosphate based BGE plus 20 mM GlcN6P at pH 7.2 and an ionic strength of 100 mM leads to the best apparent resolution between the hexamer and dimer forms. Nevertheless, the displacement of the dimer/hexamer equilibrium during the time frame of the analysis prevents any improvement of the resolution when varying the effective separation length or the analysis temperature. Therefore, the use of a short-end CE method allowed the decrease in the analysis time to about 1 min. Some parameters such as the temperature and the time of incubation and the ratio of the inhibitor and enzyme concentrations were studied. Then, it was also possible to test, very rapidly and using minute amounts, some molecules having an inhibition potential for the GlmS enzyme.

REFERENCES

- [1] S. Milewski, Glucosamine-6-phosphate synthase – the multi-facets enzyme, *Biochim. Biophys. Acta* 1597 (2002) 173-192.
- [2] P. Durand, B. Golinelli-Pimpaneau, S. Mouilleron, B. Badet, M.-A. Badet-Denisot, Highlights of glucosamine-6P synthase catalysis, *Arch. Biochem. Biophys.* 474 (2008) 302-317.
- [3] N. Flocquet, C. Richez, P. Durand, B. Maigret, B. Badet, M.-A. Badet-Denisot, Discovering new inhibitors of bacterial glucosamine-6P synthase (GlmS) by docking simulations, *Bioorg. Med. Chem. Lett.* 17 (2007) 1966-1970.
- [4] J. Ramakrishnan, S.S. Rathore, T. Raman, Review on fungal enzyme inhibitors - potential drug targets to manage human fungal infections, *RSC Adv.* 6 (2016) 42387-42401.
- [5] S. Mouilleron, M.-A. Badet-Denisot, L. Pecqueur, K. Madiona, N. Assrir, B. Badet, B. Golinelli-Pimpaneau, Structural basis for morpheein-type allosteric regulation of *Escherichia coli* glucosamine-6-phosphate synthase – Equilibrium between inactive hexamer and active dimer, *J. Biol. Chem.* 287 (2012) 34533-34546.
- [6] D. Pawlak, M. Schielmann, M. Wojciechowski, R. Andruszkiewicz, Synthesis and biological activity of novel ester derivatives of N³-4-metoxymethyl-2,3-diaminopropanoic acid containing amide and keto function as inhibitors of glucosamine-6-phosphate synthase, *Bioorg. Med. Chem. Lett.* 26 (2016) 3586-3589.
- [7] G. Zong, H. Zhao, R. Jiang, J. Zhang, X. Liang, B. Li, Y. Shi, D. Wang, Design, synthesis and bioactivity of novel glycosylthiadiazole derivatives, *Molecules* 19 (2014) 7832-7849.
- [8] M. Valerio-Lepiniec, M. Aumont-Nicaise, C. Roux, B. Raynal, P. England, B. Badet, M.-A. Badet-Denisot, M. Desmadril, Analysis of the *Escherichia coli* glucosamine-6-phosphate synthase activity by isothermal titration calorimetry and differential scanning calorimetry, *Arch. Biochem. Biophys.* 498 (2010) 95-104.
- [9] F. Gaucher-Wieczorek, V. Guérineau, D. Touboul, S. Thétiot-Laurent, F. Pelissier, M.-A. Badet-Denisot, B. Badet, P. Durand, Evaluation of synthase and hemisynthase activities of glucosamine-6-phosphate synthase by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Anal. Biochem.* 458 (2014) 61-65.

470 [10] X. Ma, E.C.Y. Chan, On-line chromatographic screening of matrix
 471 metalloproteinase inhibitors using immobilized MMP-9 enzyme reactor, J. Chromatogr.
 472 B 878 (2010) 1777-1783.

473 [11] H. Chen, I. Garrido Arias, E. Adams, A. Van Schepdael, HPLC-UV method for
 474 determining phosphorylated peptide and for Abl1 tyrosine kinase inhibition study,
 475 Chromatographia 77 (2014) 241-247.

476 [12] K.D. Greis, Mass spectrometry for enzyme assays and inhibitor screening: an
 477 emerging application in pharmaceutical research, Mass Spectrom. Rev. 26 (2007) 324-
 478 339.

479 [13] J.J. Bao, J.M. Fujima, N.D. Danielson, Determination of minute enzymatic
 480 activities by means of capillary electrophoretic techniques, J. Chromatogr. B 699 (1997)
 481 481-497.

482 [14] Z. Glatz, Determination of enzymatic activity by capillary electrophoresis, J.
 483 Chromatogr. B 841 (2006) 23-37.

484 [15] Y. Fan, G.K.E. Scriba, Advances in-capillary electrophoretic enzyme assays, J.
 485 Pharm. Biomed. Anal. 53 (2010) 1076-1090.

486 [16] X. Wang, K. Li, E. Adams, A. Van Schepdael, Recent advances in CE-mediated
 487 microanalysis for enzyme study, Electrophoresis 35 (2014) 119-127.

488 [17] D.M. Liu, Y.P. Shi, J. Chen, Application of capillary electrophoresis in enzyme
 489 inhibitors screening, Chinese J. Anal. Chem. 43 (2015) 775-782.

490 [18] S. Mouilleron, M.-A. Badet-Denisot, B. Golinelli-Pimpaneau, Glutamine binding
 491 opens the ammonia channel and activates glucosamine-6P synthase, J. Biol. Chem. 281
 492 (2006) 4404-4412.

493 [19] C. Lecamus, M.-A. Badet-Denisot, B. Badet, Arabinose-5-phosphate oxime vs its
 494 methylenephosphonate mimetic as high energy intermediate of the glucosamine-6P
 495 synthase catalyzed reaction, Tetrahedron Lett. 39 (1998) 2571-2572.

496 [20] M.-A. Badet-Denisot, C. Leriche, F. Massiere, B. Badet, Nitrogen transfer in E.
 497 coli glucosamine-6P synthase. Investigations using substrate and bisubstrate analogs,
 498 Bioorg. Med. Chem. Lett. 5 (1995) 815-820.

499 [21] J. Gonzalez-Ibarra, S. Milewski, J.C. Villagomez-Castro, C. Cano-Canchola, E.
 500 Lopez-Romero, *Sporothrix schenckii*: purification and partial biochemical
 501 characterization of glucosamine-6-phosphate synthase, a potential antifungal target,
 502 Med. Mycol. 48 (2010) 110-121.

- [22] B.J. de Kort, G.J. de Jong, C.W. Somsen, Potential of capillary electrophoresis with wavelength-resolved fluorescence detection for protein unfolding studies using β -lactoglobulin B as a test compound, *Analyst* 138 (2013) 4550-4557.
- [23] M. Cordechi, L. Di Paola, L. Marelli, M. Maschietti, Net proton charge of β - and κ -casein in concentrated aqueous electrolyte solutions, *Biophys. Chem.* 103 (2003) 77-88.
- [24] H.H. Lauer, G.P. Rozing, High performance capillary electrophoresis, Agilent Technologies, A Primer, Germany, 2009.