Analytical Biochemistry 447 (2014) 1–5

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Capillary electrophoresis-based assay of phosphofructokinase-1

Andrew Malina [a,b], Sherrisse K. Bryant [b], Simon H. Chang [a], Grover L. Waldrop [a], S. Douglass Gilman [b,*]

[a] Division of Biochemistry and Molecular Biology, Louisiana State University, Baton Rouge, LA 70803, USA
[b] Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA

ARTICLE INFO

Article history:
Received 15 April 2013
Received in revised form 14 October 2013
Accepted 17 October 2013
Available online 26 October 2013

Keywords:
Phosphofructokinase-1
Inhibition
Capillary electrophoresis
Enzyme assay

ABSTRACT

An assay was developed for phosphofructokinase-1 (PFK-1) using capillary electrophoresis (CE). In the glycolytic pathway, this enzyme catalyzes the rate-limiting step from fructose-6-phosphate and magnesium-bound adenosine triphosphate (Mg–ATP) to fructose-1,6-bisphosphate and magnesium-bound adenosine diphosphate (Mg–ADP). This enzyme has recently become a research target because of the importance of glycolysis in cancer and obesity. The CE assay for PFK-1 is based on the separation and detection by ultraviolet (UV) absorbance at 260 nm of Mg–ATP and Mg–ADP. The separation was enhanced by the addition of Mg^{2+} to the separation buffer. Inhibition studies of PFK-1 by aurintricarboxylic acid and palmitoyl coenzyme A were also performed. An IC_{50} value was determined for aurintricarboxylic acid, and this value matched values in the literature obtained using coupled spectrophotometric assays. This assay for PFK-1 directly monitors the enzyme-catalyzed reaction, and the CE separation reduces the potential of spectral interference by inhibitors.

© 2013 Elsevier Inc. All rights reserved.

Phosphofructokinase-1 (PFK-1)[1] is an allosteric enzyme that catalyzes the adenosine 5′-triphosphate (ATP)-dependent phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate (F1,6P), as shown in Scheme 1. This reaction is one of the principal regulatory steps in glycolysis [1], and as such PFK-1 is a homotetramer, which allows the activity of the enzyme to be controlled allosterically by the cellular energy level or ATP/AMP (adenosine 5′-monophosphate) ratio [1]. Citrate and ATP act as feedback inhibitors of PFK-1, whereas AMP, adenosine 5′-diphosphate (ADP), and fructose 2,6-bisphosphate activate the enzyme [2]. The mammalian form of the enzyme has three different isozymes: M for muscle, L for liver, and P for platelets (also called PFK-C) [2,3]. Whereas most PFK-1 research has focused on its regulatory properties [1–3], very little effort has been put into developing inhibitors of the enzyme. This is unfortunate given that there are potential medical applications for the inhibition of PFK-1.

PFK-1 is a potential target for the treatment of obesity and infectious disease. A recent report by Getty-Kaushik and coworkers [4] found that mice deficient in the M isozyme of PFK-1 had significantly decreased fat stores. This suggests that inhibition of muscle PFK-1 could aid in the treatment of obesity, diabetes, and metabolic syndrome. In addition, PFK-1 activity is increased in cancer cells [5], suggesting that PFK-1, as well as the other enzymes involved in glycolysis, is a potential target for anticancer drugs [6]. For instance, inhibition of lactate dehydrogenase resulted in inhibited cell growth in both lymphoma and pancreatic cancer by inducing oxidative stress [7]. Glycosylation of PFK-1 with *N*-acetylglucosamine also plays a role in cancer cell metabolism [8]. Finally, PFK-1 is being explored as a possible target for the treatment of the parasitic disease, African sleeping sickness (trypanosomiasis) [9].

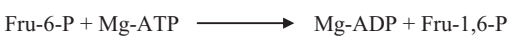
Despite the fact that inhibition of PFK-1 is therapeutically relevant to several areas of medicine, there are very few known PFK-1 inhibitors that are suitable for pharmaceutical applications. This will likely change now that the first three-dimensional structure of a mammalian PFK-1 is available [10] and will enable structure-based drug design to be used for inhibitor development.

One reason for the shortage of PFK-1 inhibitors could be the lack of suitable assays to screen for such inhibitors. Coupled enzyme assays are routinely used to measure PFK-1 activity. For example, the rate of ADP production is determined using pyruvate kinase and lactate dehydrogenase [1], whereas production of F1,6P can be coupled to aldolase, glycerol-3-phosphate dehydrogenase, and triosephosphate isomerase [11]. Although these two assays have proven to be very useful for the study of the allosteric behavior of PFK-1, they have one major shortcoming when it comes to screening for inhibitors. Both assays rely on the absorbance of NADH at 340 nm as the basis for detection. This is problematic for screening molecules that might inhibit PFK-1 because many of those molecules absorb strongly around 340 nm. Therefore, an

* Corresponding author. Fax: +225 578 3465.
E-mail address: sdgilman@lsu.edu (S.D. Gilman).

[1] *Abbreviations used:* PFK-1, phosphofructokinase-1; ATP, adenosine 5′-triphosphate; F6P, fructose 6-phosphate; F1,6P, fructose 1,6-bisphosphate; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; CE, capillary electrophoresis; SDS, sodium dodecyl sulfate; ATA, aurintricarboxylic acid; PCoA, palmitoyl coenzyme A; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; UV, ultraviolet; Mg–ADP, magnesium-bound ADP; Mg–ATP, magnesium-bound ATP.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.10.028



Scheme 1. Reaction catalyzed by phosphofructokinase-1.

assay for PFK-1 that obviates the spectral interference of potential inhibitors would have wide utility. In addition, there is always a possibility that an inhibitor can act on one of the reactions only used for detection purposes in a coupled enzyme assay.

Capillary electrophoresis (CE) is a separation technique based on differences in the ratio of charge to hydrodynamic radius for analytes in a conductive solution and can be used to analyze enzyme kinetics and inhibition [12,13]. Most important, because CE is an electrophoretic separation technique, the potential for spectral interference by inhibitors is greatly reduced. Moreover, CE is also a rapid technique that consumes only a few nanoliters of sample per measurement, and it can be used to screen for and study enzyme inhibitors [12,14,15]. A CE assay was developed recently by Meades and coworkers to study inhibitors of the carboxyltransferase component of acetyl-CoA carboxylase in cases where the inhibitor spectrally interfered with a coupled enzyme assay based on NADH absorbance at 340 nm [16]. Here we report the development of a CE assay for PFK-1 and show that the assay can detect inhibition of PFK-1 activity with known inhibitors.

Materials and methods

Chemicals

ATP, ADP, F6P, sodium dodecyl sulfate (SDS), aurintricarboxylic acid (ATA), and palmitoyl coenzyme A (PCoA) were obtained from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) was obtained from Promega (Madison, WI, USA). Magnesium chloride was obtained from Fisher Scientific (Pittsburgh, PA, USA). All structures are shown in the online Supplementary material (Fig. S1).

Preparation of PFK-1

The rabbit muscle PFK-1 used in these experiments was expressed and purified as described by Banaszak and coworkers [10]. The final purified enzyme was suspended in a 50.0-mM solution of Tris at pH 8.2. The pH of the Tris buffer used to suspend the enzyme was adjusted to 8.2 by the addition of concentrated phosphoric acid. Small aliquots were made and stored at 4 °C. Using a Bradford assay [17], the final concentration of the PFK-1 was determined to be 0.5 mg/ml.

Capillary electrophoresis

A simple laboratory-constructed CE instrument was used for all experiments and was similar to instruments described previously [18]. Fused-silica capillary (50 μm i.d., 360 μm o.d.) was purchased from Polymicro (Phoenix, AZ, USA) and cut to 60.0 cm. The polyimide coating was burned (~1 cm) to make a detection window at 40.0 cm using The Window Maker (MicroSolv Technology, Eatontown, NJ, USA). The electrophoretic potential was applied with a Spellman CZE 1000R high-voltage power supply (Hauppauge, NY, USA). A potential of 25.0 kV (417 V/cm) was used for all experiments. All injections were electrokinetic (3.0 s at 25.0 kV).

Absorbance detection was performed at 260 nm using an Acutect 500 UV/Vis (ultraviolet–visible) detector (Scientific Systems, State College, PA, USA) with an on-column capillary cell. A computer program was written in LabView (version 7.1, National Instruments) and used for data acquisition at 100 Hz. Data were analyzed using OriginLab 7.5 (Northampton, MA, USA). All separation buffers used were prepared to contain 15.0 mM Tris–HCl and 30.0 mM SDS. The pH was adjusted to 8.0 with 1.0 M KOH. The sample buffer used for separation development was the same as the enzyme assay buffer described below except that no PFK-1 was added to the solution. All solutions used for CE experiments were prepared with ultrapure water (>18 M Ωcm) obtained from a Modulab water purification system (U.S. Filter, Palm Desert, CA, USA). All buffers used for CE were filtered using a 0.2-μm nylon membrane filter prior to use (Whatman, Hillsboro, OR, USA).

CE assays

The sample buffer used for CE enzyme assays was based on 15.0 mM Tris–HCl at pH 8.0 and did not contain SDS. The sample buffer also contained 5.0 mM $MgCl_2$, 1.00 mM ATP, and 1.00 mM F6P. To initiate the reaction, 5.0 μl of 0.5 mg/ml PFK-1 was added to give a final concentration of 5×10^{-3} mg/ml (typically) in a total sample volume of 500 μl. All reactions were performed in 600-μl polypropylene microcentrifuge tubes. For inhibition studies with ATA, the ATA was first suspended in water, and then KOH was added to a final concentration of 0.02 M. The pH of the solution after KOH addition was 8.0. When performing the PFK-1 assay with ATA, the ATA was added to the reaction mixture, and then PFK-1 was added to initiate the reaction.

The capillary was conditioned between each run by successively injecting 0.5 M NaOH, ultrapure water, and then the separation buffer for 5 min each. The Tris–HCl, SDS, and magnesium chloride buffer was then placed into both the inlet and outlet vials. To ensure a stable current in the capillary and a stable baseline in the electropherogram, electrophoresis was performed at 25.0 kV for 5 min with no analyte before each injection.

Data analysis

The dose dependence of inhibition of PFK-1 by ATA was fitted by nonlinear regression analysis to Eq. (1) to determine the concentration of ATA that inhibits the enzyme activity by 50%:

$$v_i/v_0 = 1/(1 + ([I]/IC_{50})) \quad (1)$$

In Eq. (1), v_i is the enzyme activity at a particular ATA concentration and v_0 is the activity in the absence of ATA. The concentration of ATA is $[I]$, and IC_{50} is the concentration of ATA that results in 50% inhibition. Enzyme activity was defined as the ratio of CE peak areas for Mg–ADP/(Mg–ATP + Mg–ADP), where Mg–ADP is magnesium-bound ADP and Mg–ATP is magnesium-bound ATP.

Results and discussion

Separation and detection of Mg–ATP and Mg–ADP

The overall goal of this study was to develop a simple CE assay with UV absorbance detection for the reaction catalyzed by PFK-1 that directly measures substrate depletion and product formation. The first step in the development of this assay was to separate and detect the substrates and products for the PFK-1-catalyzed reaction (Scheme 1). F6P and F1,6P exhibit only weak absorbance in the UV and would be difficult to detect without derivatization [19]. In contrast, both ATP and ADP have a strong absorption band near 260 nm, and analysis of both molecules by CE has been reported previously [20]. An initial unsuccessful attempt to separate 1.0 mM ATP and 1.0 mM ADP for this assay using absorbance detection at 260 nm is presented in the Supplementary material (Fig. S2).

The separation buffer for this assay represents a compromise between ideal conditions for the PFK-1-catalyzed reaction and optimal conditions for the CE separation. The first separation buffer

Download English Version:

https://daneshyari.com/en/article/1173927

Download Persian Version:

https://daneshyari.com/article/1173927

Daneshyari.com