Biochemistry and Biophysics Reports 12 (2017) 151–157

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

A novel mammalian glucokinase exhibiting exclusive inorganic polyphosphate dependence in the cell nucleus

Antasar Ali[a], D. Claire Wathes[b], Angelina Swali[c], Helena Burns[a], Shamus Burns[a,*]

[a] Biological Sciences, University of Huddersfield, Huddersfield UK HD1 3DH, United Kingdom
[b] Department of Pathobiology and Population Sciences, The Royal Veterinary College, London UK NW1 0TU, United Kingdom
[c] School of Biosciences, University of Nottingham, Nottingham UK LE12 5RD, United Kingdom

ARTICLE INFO

Keywords:
Glucokinase
Hexokinase
Polyphosphate
Evolution

ABSTRACT

Background: Hexokinase and glucokinase enzymes are ubiquitously expressed and use ATP and ADP as substrates in mammalian systems and a variety of polyphosphate substrates and/or ATP in some eukaryotic and microbial systems. Polyphosphate synthesising or utilizing enzymes are widely expressed in microbial systems but have not been reported in mammalian systems, despite the presence of polyphosphate in mammalian cells. Only two micro-organisms have previously been shown to express an enzyme that uses polyphosphate exclusively.

Methods: A variety of experimental approaches, including NMR and NAD-linked assay systems were used to conduct a biochemical investigation of polyphosphate dependent glucokinase activity in mammalian tissues.

Results: A novel mammalian glucokinase, highly responsive to hexametaphosphate (HMP) but not ATP or ADP as a phosphoryl donor is present in the nuclei of mammalian hepatocytes. The liver enzyme exhibited sigmoidal kinetics with respect to glucose with a $S_{0.5}$ of 12 mM, similar to the known kinetics of mammalian ATP-glucokinase. The Km for HMP (0.5 mM) was also similar to that of phosphoryl donors for mammalian ATP-glucokinases. The new enzyme was inhibited by several nucleotide phosphates.

Conclusions: We report the discovery of a polyphosphate-dependent enzyme system in mammalian cells with kinetics similar to established ATP-dependent glucokinase, also known to have a nuclear location. The kinetics suggest possible regulatory or redox protective roles.

General significance: The role of polyphosphate in mammalian systems has remained an enigma for decades, and the present report describes progress on the significance of this compound in intracellular metabolism in mammals.

1. Introduction

Hexokinases are ubiquitously expressed and have a central role in metabolism phosphorylating hexoses (e.g. glucose), raising the hexose free-energy content and facilitating subsequent reactions central to metabolic energy production and biosynthesis via NADPH production. Hexokinases have undergone extensive evolution resulting in highly specialized enzymes and isoenzymes that have distinct intracellular location, substrate specificity and kinetics [1,2]. This has resulted in highly specialized functions and accessory actions such as the key components of the glucose sensor in the pancreas and brain [3,4]. The majority of hexokinases studied use ATP exclusively as the phosphoryl donor, though a novel mammalian hexokinase, expressed in rat and man, with a different evolutionary origin uses ADP [5]. Many bacteria and archaea also synthesise and use a variety of polyphosphate substrates [6,7] which are considered evolutionary starting points for the ATP-dependent enzymes. Polyphosphate chains may contain 1000 inorganic phosphates joined by high energy phospho-anhydride bonds; they are readily formed under high temperatures, or by dehydration of inorganic phosphate, and would have been an available source of energy before ATP became ubiquitous [8]. Polyphosphate is present at varying concentration in all mammalian cells studied, although no enzymes have been identified to date responsible for either its synthesis or utilization [9]. Two organisms, a phosphate accumulating bacterium, *Microlunatus phosphovorous* [10] and a nitrogen-fixing cyanobacterium, *Anabaena sp.* PCC 1720, express a hexokinase which exclusively uses inorganic polyphosphate and, importantly, cannot phosphorylate hexoses with ATP or ADP [11]. We now report on a mammalian

Abbreviations: HK, hexokinase; ATP-GK, glucokinase (mammalian ATP utilizing); PPGKm, polyphosphate dependent glucokinase (mammalian)

* Corresponding author.
E-mail address: s.p.burns@hud.ac.uk (S. Burns).

http://dx.doi.org/10.1016/j.bbrep.2017.09.004
Received 19 August 2017; Received in revised form 13 September 2017; Accepted 15 September 2017
Available online 20 September 2017

hexokinase which phosphorylates glucose exclusively using inorganic polyphosphate, and which is inhibited by ATP. Inorganic polyphosphate is often concentrated in the hepatocyte nucleus [12], with a variety of roles proposed, such as acting as a chaperone for nuclear proteins, but until now it has not been shown to be of metabolic significance [12]. The enzyme reported here is the first mammalian enzyme shown to utilize polyphosphate in a biochemical reaction. The enzyme is expressed predominantly in the nucleus of hepatocytes, but is also present in cardiac and striated muscle. Because of the enzyme's kinetics we have named it polyphosphate dependent GlucoKinase, mammalian, or PPGKm.

2. Methods

2.1. Tissue samples

Tissue samples were used immediately after being transported on ice, or from frozen – no significant difference was found in activity or kinetics from Hexokinase (HK), Glucokinase (GK) or polyphosphate dependent glucokinase (PPGKm) using frozen tissue. Only tissues from adult animals were studied: rat, ovine, bovine and porcine liver was investigated for hexokinase activity as well as a variety of ovine tissues including, heart, muscle, lung, kidney, spleen and adipose tissue. Local and National procedures were followed using University Ethics committee approval for the use of animal tissues, and fulfilling the 2010/63/EU directive and UK Home Office regulations.

2.1.1. Tissue homogenates

Tissue homogenates (10% w/v) were prepared in 50 mM HEPES buffer containing 2.5 mM DTE, 7.5 mM $MgCl_2$, 100 mM KCl, adjusted to PH 7.4 and kept on ice or frozen at −80 °C. Homogenates were ultra-centrifuged at 100,000 g in a Beckman Coulter ultra-centrifuge model Ti 50,000 at 4 °C, and the supernatant removed and stored on ice or frozen at −80 °C. For direct enzyme assay the supernatant was used without modification. For separation procedures the supernatant was then concentrated using Vivaspin® Centrifugal Concentrators at 4 °C with a 30 kDa MW cut-off, and stored at −80 °C for further purification steps (below).

2.2. NAD-linked hexokinase/glucokinase assay

Enzyme activity was determined as previously described exploiting the well established difference in K_m and $S_{0.5}$ between hexokinases and glucokinase respectively to obtain their activities. ATP, ADP, CTP, GTP or HMP (sodium hexametaphosphate, 96% Sigma-Aldrich) were used at 5 mM where indicated (all reagents sourced from Sigma-Aldrich). In previous work, the reaction blank for both HK and GK was the reaction system without ATP using 0.5 and 100 mM glucose respectively. Because of the native polyphosphate present in various tissues, the blank for HK was used for the 'GK' and PPGKm assays - the rationale being that if the enzyme under consideration is ATP dependent then the concentration of glucose in the system should be irrelevant in the absence of ATP. The fact that increased activity was present in the absence of ATP, but with increasing glucose concentration clearly indicates an additional activity. This activity was lost when G6PDH was removed from the system, on heating and on omission of magnesium. The section on Nuclear Magnetic Resonance (NMR) identifies the mechanism of this reaction. G6PDH from *Leuconostoc mesenteroides* (Sigma-Aldrich) was used which uses NAD as a co-factor. All enzyme activity assays were conducted in triplicate at 37 °C.

2.3. Purification steps and procedures

Precast Run Blue tris-glycine 4–12% gradient SDS-PAGE gels and the relevant running and loading buffers were purchased from Expedion Ltd. Spectra™ Multicolour Broad Range Ladders were purchased from Life Technologies. The protein ladder was thawed at room temperature, with 6 µl samples loaded directly into the first lane of the gradient SDS-PAGE gel. All samples were mixed 1:4 with the loading buffer, heated to 100 °C for 10 min and loaded onto the SDS-PAGE gel. The samples were electrophoresed for 1 h at a 120v using an XCell *Surelock*® Mini-Cell from Life Technologies at room temperature.

2.3.1. Chromatography

All enzyme purification steps were conducted with AKTA smart FPLC system (GE Healthcare Life Sciences). Each step was examined for separation suitability and fractions were further analysed for HK and PPGKm activity. All columns were run at 1 ml/min with the exception of the size exclusion column (0.5 ml/min) at room temperature.

2.3.1.1. Anion exchange chromatography. A strong anion exchange media, Q HP, acquired as a pre-packed 1 ml column (GE Health care Life Sciences), was equilibrated with 10 column volumes of buffer A (50 mM HEPES, PH 7.4). Concentrated sheep liver extract (5 ml) was injected onto the column at a rate of 1 ml/min. An initial wash step with homogenization buffer was carried out for a further 5 ml, followed by a gradient elution of protein fractions using the same buffer over 30 min.

2.3.1.2. Size exclusion chromatography. HiPrep 16/60 Sephacryl S-200 h pre-packed gel filtration columns with a bed volume of 120 ml were used. The columns were equilibrated with two column volumes of homogenization buffer. A spin concentrated 500 µl sample was injected into the column and automatic peak elution was set at 30 mAU running for 8 h at 4 °C.

2.3.1.3. Hydrophobic interaction chromatography. HiTrap Butyl FF prepacked Butyl Sepharose 4 Fast Flow columns were equilibrated with 10 column volumes of buffer (3 M NaCl, 50 mM HEPES, PH 7.4). The test samples were buffer exchanged with this buffer before loading the column. An initial wash step with the same buffer was followed by gradient elution of protein fractions with NaCl-free buffer (50 mM HEPES, PH 7.4) over 30 min.

2.4. Substrate depletion on purified fractions

Supernatant was polyphosphate substrate depleted by incubation with 100 mM glucose, 5 mM NAD and 8 units G6PDH in homogenization buffer until activity tended to zero (approx.12 h at 37 °C). Addition of 2 mM HMP restored activity.

2.5. Cell fractionation

Three buffers all adjusted to pH 7.4 were used in the cell fractionation procedures as follows: **Buffer 1** contained 0.25 M sucrose, 5 mM Tris, 7.5 mM $MgCl_2$ and 1 mM Dithiothreitol (DTT). **Buffer 2** was identical to Buffer 1 but contained 2 M sucrose instead of 0.25 M sucrose. **Buffer 3** contained 7.5 mM $MgCl_2$, 2.5 mM DTT, 100 mM KCl and 50 mM Na HEPES. Liver (10 g) was homogenized at 4 °C in 50 ml Buffer 1 using a pestle and mortar for 10 min. The suspension was filtered through muslin cloth and the homogenate spun in a 50 ml centrifuge tube at 600 g for 10 min. The supernatant was retained (cytoplasmic fraction) and the pellet re-suspended (nuclear fraction) in 6 ml of Buffer 2. The nuclear fraction was centrifuged at 800 g (Beckman coulter Avanti J-26XPI centrifuge S/N JXT08C02) for 15 min. The supernatant was discarded and the nuclear pellet re-suspended in Buffer I, and kept on ice (nuclear fraction). The cytoplasmic fraction was spun at 8000 g (using Beckman coulter Avanti J-26XPI centrifuge S/N JXT08C02) for 10 min: the supernatant was saved and the pellet re-suspended (mitochondrial fraction) in 3 ml of homogenization Buffer I. The remaining supernatant was then centrifuged at 15,000 g (Beckman coulter Avanti J-26XPI centrifuge S/N JXT08C02) for 10 min, The