Inhibition of phosphofructokinases by copper(II)

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Abstract

The biochemical inhibition by Cu²⁺ on eight phylogenetically and biochemically different phosphofructokinases (PFKs) was investigated. The enzymes screened included representatives from thermophilic and mesophilic bacteria, a hyperthermophilic archaeon and a eukaryote, covering all three phosphoryl donor subtypes (ATP, ADP and pyrophosphate). The sensitivities of the enzymes to Cu²⁺ varied greatly, with the archaeal ADP-PFK being the least and the eukaryote ATP-PFK being the most sensitive. The bacterial ATP- and pyrophosphate-dependent PFKs showed intermediate sensitivity with the exception of the Spirochaeta thermophila enzyme (pyrophosphate-dependent) which was relatively resistant. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Phosphofructokinase (PFK) is a key enzyme of the Embden–Meyerhof glycolytic pathway. Since it is found in members of the three domains of life Archaea, Bacteria and Eukarya, phylogenetic analyses of different PFKs can be used to investigate the origin of the pathway [1,2]. During a study that involved the biochemical characterisation of PFKs from a broad range of thermophiles [3], it was found that the pyrophosphate-dependent PFK (PPᵢ-PFK) from Dictyoglomus thermophilum was extremely sensitive to Cu²⁺. Testing a range of cations on several PFKs that included the PPᵢ-PFK and ATP-dependent PFK (ATP-PFK) from Thermotoga maritima, Cu²⁺ inhibition was found to be exceptional, and was only matched by Zn²⁺ inhibition in some cases [3,4].

While copper is essential for the functioning of several proteins, its intracellular concentration needs to be closely regulated, usually mediated through special transport mechanisms, to avoid toxic effects [5,6]. Similar to heavy metal ions, copper(II) is able to bind to several electron donor groups of proteins, e.g. sulfhydryl or amino groups, and can thus irreversibly inhibit their function [7]. In lower and higher eukaryotes, the presence of a copper chaperone system maintains the intracellular copper concentrations at very low levels [6].

Even though extensive research has been conducted on the various effects of copper on whole organisms, only a few references can be found relating to its effects on a specific enzyme. In this study, we compared the inhibitory effect of Cu²⁺ on eight different PFKs with a broad phylogenetic range. PFKs from a hyperthermophilic archaeon (Thermococcus zilligii), hyperthermophilic (T. maritima), extremely thermophilic (D. thermophilum), thermophilic (Spirochaeta thermophila and Bacillus stearothermophilus) and mesophilic (Propionibacterium freudenreichii) bacteria and an eukaryote (rabbit muscle) were chosen. Among the above mentioned enzymes all three phosphoryl donors described thus far (ADP, ATP and PPᵢ) are represented [1].

2. Materials and methods

2.1. Enzymes

Aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, the ATP-PFKs from B. stearothermophilus and rabbit muscle and the PPᵢ-PFK from P. freudenreichii were purchased from Sigma (St. Louis,
MO, USA). PPi-PFKs from *S. thermophila*, *D. thermophilum* (recombinant enzyme expressed in *Escherichia coli*) and *T. maritima*, the ATP-PFK from *T. maritima*, and the ADP-PFK from *T. zilligii* were obtained from the Thermophile Research Unit [1,3,4,8,9].

2.2. Enzyme assays and determination of copper inhibition

PFK activity was assayed by a variation of the discontinuous method used by Hansen and Schönheit with fructose-6-phosphate, an empirically determined aliquot of PFK (described below), MgCl2 and appropriate phosphoryl donor [10]. Reactions were incubated for 5 min (*Dictyoglomus* PPi-PFK and rabbit muscle ATP-PFK for 10 min) at the optimal temperature for the respective PFK and stopped by transfer to a 0°C bath and addition of 50 mM EDTA. The reaction volume was 100 μl. Formation of fructose-1,6-diphosphate (F-1,6-P2) was measured at 37°C by addition of 0.2 mM NADH, 0.07 U aldolase, 2.1 U triosephosphate isomerase and 0.27 U α-glycerophosphate dehydrogenase to an aliquot of the reaction mixture to a final volume of 100 μl. Complete oxidation of NADH was followed at 340 nm (εNADH = 6.22 mM⁻¹ cm⁻¹), using an Ultrascan 3000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden), and the difference in optical density between the start of the reaction and the end (ΔOD340) was calculated. For the assay of *Thermococcus* ADP-PFK, 0.4 mM NADH was added and NADH oxidation followed at 365 nm (εNADH = 3.4 mM⁻¹ cm⁻¹).

Assay mixtures contained: for the *Bacillus* ATP-PFK: 50 mM TAPS (pH 8.2 at 60°C), 1 mM fructose-6-phosphate, 2.5 mM ATP, 10 mM MgCl2 and 5 mM NH4Cl (incubation at 60°C) [11]; for the *Propionibacterium* PPi-PFK: 50 mM TES (pH 7.4 at 30°C), 1.5 mM fructose-6-phosphate, 1 mM PPi, 3 mM MgCl2 and 50 mM KCl (incubation at 30°C) [12]; for the *Spirochaeta* PPi-PFK: 30 mM Bis-Tris (pH 6.0 at 65°C), 5 mM fructose-6-phosphate, 1 mM PPi, 3.5 mM MgCl2 and 100 mM KCl (incubation at 65°C) [8]; for the *Thermococcus* ADP-PFK: 50 mM Bis-Tris (pH 6.4 at 75°C), 10 mM fructose-6-phosphate, 5 mM ADP, 600 mM KCl and 10 mM KCl (incubation at 75°C) [1]; for the *Dictyoglomus* PPi-PFK: 30 mM Bis-Tris (pH 5.7 at 70°C), 2.5 mM fructose-6-phosphate, 0.75 mM PPi and 3 mM MgCl2 (incubation at 70°C) (variation of [3,9]); for the *Thermophile* ATP-PFK: 30 mM Tris–HCl (pH 7.6 at 80°C), 5 mM fructose-6-phosphate, 1 mM ATP, 3.5 mM MgCl2 and 175 mM KCl (incubation at 80°C) [4]; for the *Thermotoga* PPi-PFK: 25 mM phosphate buffer, (pH 6.4 at 80°C), 5 mM fructose-6-phosphate, 1 mM PPi, 3.5 mM MgCl2 and 350 mM KCl (incubation at 80°C) [4]; for the rabbit muscle ATP-PFK: 50 mM Tris–HCl (pH 7.5 at 37°C), 1.5 mM fructose-6-phosphate, 0.6 mM ATP, 5 mM MgCl2 and 1 mM fructose-2,6-diphosphate (incubation at 37°C) [13]. Each PFK was assayed in the absence and presence of CuCl2 under non-reducing conditions, using concentrations ranging from 1 μM to 1 mM. The remaining enzyme activity at the different concentrations was calculated in percent from the ΔOD340 or ΔOD365 measured in the absence of copper. Assays of the PFKs from *Spirochaeta*, *Thermococcus* and the PPi-PFK from *Thermotoga* were conducted in duplicate, those of the *Dictyoglomus* PPi-PFK in triplicate, and those of the remaining enzymes in triplicate.

To determine the amount of PFK per reaction that would produce a suitable concentration of F-1,6-P2 during the incubation time (i.e. giving ΔOD340 or ΔOD365 values between 0.4 and 0.8 for the control), continuous assays were performed at 50°C for the thermophilic enzymes, or at 37°C for the mesophilic enzymes by a variation of the method of Janssen and Morgan [14]. Reaction mixtures contained the same concentrations of substances as above except for lower concentrations of the linker enzymes (0.03 U aldolase, 1.05 U triosephosphate isomerase, 0.13 U α-glycerophosphate dehydrogenase).

The PFKs were diluted in 20–50 mM of the respective buffer, including 0.1% Triton X-100. Dilutions of the rabbit muscle ATP-PFK also contained 0.1 mg ml⁻¹ bovine serum albumin.

3. Results and discussion

3.1. Controls

Control experiments were carried out to ensure that other components of the assay system were not affected by copper(II). Incubation of various concentrations of F-1,6-P2 (10, 50 and 100 μM) with 1 mM CuCl2 at 75°C for 5 and 10 min did not show any change in ΔOD340 as compared to incubation in the absence of copper, indicating that no interaction between the two substances occurred. However, the linker enzymes, particularly the aldolase, were affected by copper(II) concentrations of 50 μM and higher (data not shown). To avoid this effect, we used the discontinuous method to assay for the inhibition of PFKs. The addition of EDTA to stop the reaction led to the chelation of Cu²⁺ ions but did not affect the linker enzymes.

Preincubation of the *Thermotoga* ATP-PFK with 0.2 mM CuCl2 (the level at which 50% inhibition was obtained) for up to 10 min at room temperature did not show any significant change as compared to non-preincubated enzyme. This indicated that the inhibitory effect of copper(II) did not show a significant dependence on the incubation time.

To ensure that the reactions did not reach equilibrium during the time of incubation, reactions of several enzymes were stopped at 2-min intervals over 10 min,
showing linearity over at least 10 min for the rabbit muscle ATP-PFK, and over 6 min for Thermotoga ATP- and PPi-PFKs. In the presence of 0.1 mM CuCl₂, the curve was still linear over 6 min for the Thermotoga ATP-PFK.

3.2. Copper inhibition

The eight PFKs tested here showed strongly varying degrees of sensitivity to copper(II) (Fig. 1). The differences are especially prominent at low concentrations of Cu²⁺. While 1 μM of CuCl₂ was sufficient to decrease the activity of the rabbit muscle ATP-PFK to 35%, 50% inhibition of the two bacterial ATP-PFKs from Thermotoga and Bacillus required approximately 0.2 mM of CuCl₂. A recent investigation of the effects of copper(II) on glycolytic enzymes from mouse muscle tissue has also shown this ATP-PFK to be strongly inhibited [15]. All three of these enzymes belong to Group I PFKs as defined by Siebers et al. [2], which includes all bacterial and eukaryal ATP-PFKs (properties of examined PFKs are summarised in Table 1). A gene duplication in the eukaryal enzymes led to a larger enzyme with a higher complexity of regulation and could possibly also be the cause of the increased sensitivity to Cu²⁺ as compared to the two bacterial ATP-PFKs [2,9]. The PPi-PFKs of Propionibacterium, Thermotoga and Dictyoglomus were inhibited to similar degrees with the former being slightly less sensitive to Cu²⁺. While the enzymes of Propionibacterium and Thermotoga are members of Group II PFKs, which contain most bacterial and eukaryal PFKs (both primitive eukaryotes such as Entamoeba, Trichomonas and Naegleria and higher plant PPi-PFKs), the Dictyoglomus PPi-PFK belongs to Group III PFKs. Group III PFKs are indicated to have diverged early from Group II and III enzymes and contain the PPi-PFKs from the archaeon Thermoproteus tenax as well as Amycolatopsis methanolica and Mycobacterium tuberculosis, in addition to the ATP-PFK of Streptomyces coelicolor [2,9]. Despite the presence of these three groups of relatively divergent PFKs, the residues in the active sites of enzymes of the groups show a similar pattern and are well conserved, and it is therefore not too surprising that these three enzymes are inhibited to a comparable degree [2,9]. An exception to this was the Spirochaeta PPi-PFK, also belonging to group II [8,9], that retained almost half its
activity even in the presence of 1 mM copper. However, the enzyme is also not severely inhibited by other cations, implying a generally higher resistance compared to the other three PPi-PFKs tested [8]. The least sensitive enzyme was the archaeal ADP-PFK of *Thermococcus*. No effect was to be seen with CuCl2 concentrations up to 0.1 mM, and at the highest concentration tested (1 mM CuCl2), 60% of the activity remained. The enzyme is also not particularly sensitive to other cations [1]. The *T. zilligii* ADP-PFK has been suggested to be unrelated to the Group I, II and III PFKs and is likely to have evolved independently [15]. The data presented here support the uniqueness of the enzyme.

Overall, the results of this study support a correlation between the pattern of sensitivity to copper(II) and to the phylogenetic origin of the enzymes. A good example for this is *Thermotoga*, where two phylogenetically distinct enzymes in the same organism show a noticeably different sensitivity to copper(II). Since there seems to be no consistent correlation between the inhibitory effect of copper(II) and the molecular mass or structure of the enzymes, the amino acid sequence (and resulting three-dimensional structure of the active site of the enzymes) and therefore the electron donor groups available for reaction with Cu2+ ions are likely responsible for determining some of the sensitivity of the enzymes. Copper(II) has also been shown to coordinate with the phosphate groups of nucleoside triphosphates and to markedly enhance their rate of hydrolysis [16]. This latter effect and the binding of Cu2+ ions to phosphates in general could also contribute to its inhibitory effect, possibly via the phosphoryl donor binding sites of the PFKs [16].

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**Table 1**

Summary of the PFKs and some of their properties

<table>
<thead>
<tr>
<th>Organism</th>
<th>Phylogenetic group</th>
<th>Domain</th>
<th>Phosphoryl donor</th>
<th>Molecular mass (kDa)</th>
<th>Structure</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. zilligii</em></td>
<td>–</td>
<td>Archaea</td>
<td>ADP</td>
<td>52.7</td>
<td>tetramer</td>
<td>[1]</td>
</tr>
<tr>
<td><em>D. thermophilum</em></td>
<td>III</td>
<td>Bacteria</td>
<td>PPi</td>
<td>37</td>
<td>dimer</td>
<td>[3,9]</td>
</tr>
<tr>
<td><em>T. maritima</em></td>
<td>I</td>
<td>Bacteria</td>
<td>ATP</td>
<td>34.5</td>
<td>tetramer</td>
<td>[4]</td>
</tr>
<tr>
<td><em>S. thermophila</em></td>
<td>II</td>
<td>Bacteria</td>
<td>PPi</td>
<td>60</td>
<td>dimer</td>
<td>[8]</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>II</td>
<td>Bacteria</td>
<td>ATP</td>
<td>35</td>
<td>tetramer</td>
<td>[11]</td>
</tr>
<tr>
<td><em>P. freudenreichii</em></td>
<td>II</td>
<td>Bacteria</td>
<td>PPi</td>
<td>45</td>
<td>dimer</td>
<td>[12]</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>I</td>
<td>Eukarya</td>
<td>ATP</td>
<td>80</td>
<td>tetramer</td>
<td>[13]</td>
</tr>
</tbody>
</table>

**References**


