Kinetic analysis of PPi-dependent phosphofructokinase from *Porphyromonas gingivalis*

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Abstract

We have previously cloned the gene encoding a pyrophosphate-dependent phosphofructokinase (PFK), designated PgPFK, from *Porphyromonas gingivalis*, an oral anaerobic bacterium implicated in advanced periodontal disease. In this study, recombinant PgPFK was purified to homogeneity, and biochemically characterized. The apparent \( K_m \) value for fructose 6-phosphate was 2.2 mM, which was approximately 20 times higher than that for fructose 1,6-bisphosphate. The value was significantly greater than any other described PFKs, except for *Amycolatopsis methanolica* PFK which is proposed to function as a fructose 1,6 bisphosphatase (FBPase). The PgPFK appears to serves as FBPase in this organism. We postulate that this may lead to the gluconeogenic pathways to synthesize the lipopolysaccharides and/or glycoconjugates essential for cell viability. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

*Porphyromonas gingivalis*, an anaerobic, Gram-negative, rod-shaped bacterium, has been reported to be associated with advanced adult periodontal disease [1,2]. *P. gingivalis* is an asaccharolytic organism that is dependent on nitrogenous substrates for energy production [3,4]. Although sugars such as glucose can be utilized by this organism, these compounds are not converted to metabolic end products but are rather used for the biosynthesis of intracellular macromolecules [3–5]. However, the detailed mechanism of their synthesis remains to be determined.

Our final goal is to develop therapeutic agents for periodontal disease by means of interfering with vital energy metabolism in bacteria without affecting the metabolism of the human host: phosphofructokinase (PFK) would be one of the candidates. It is well known that PFK phosphorylates fructose 6-phosphate (F6-P) to fructose 1,6-bisphosphate (F1,6-BP) and thus occupies a key position in glycolysis [6]. Two major types of PFKs have been described with respect to phosphoryl donor specificity: the more widespread ATP-dependent PFK (ATP-PFK) (EC 2.7.1.11) and pyrophosphate-dependent PFK (PPi-PFK) (EC 2.7.1.90). PPi-PFK catalyzes a low-energy reversible reaction and can function in both glycolysis and gluconeogenesis [7].

Recently, the PPi-PFK gene from *P. gingivalis*, designated the PgPFK gene, was cloned and sequenced [8]. PgPFK exhibited significant similarity to the 62 kDa PPi-PFK from *Borrelia burgdorferi* (gene number, BB0020) and the PFK β-subunit from *Treponema pallidum* (gene number, TP0542). Although glycolysis is the primary mechanism for energy production, the molecular biology of glycolysis has been poorly understood in oral bacteria such as *P. gingivalis*. Our aims are to examine the biochemical traits of PgPFK, a key enzyme in this pathway. In this report, we present data which indicate that PgPFK might serve as an enzyme in the gluconeogenic pathway.
2. Materials and methods

2.1. Expression of the PgPFK gene in Escherichia coli

The open reading frame (ORF) was subcloned into the T7-based bacterial expression plasmid pET21a (Novagen) as previously described [8]. The PCR product was restricted and cloned into the same sites of pET21a as previously described [9]. The resulting clone, pET21a-PgPFK, was confirmed by DNA sequencing and introduced into E. coli BL21(DE3)/pLysS. Growth of the transformant, induction with isopropyl-1-thio-D-galactopyranoside (IPTG), and lysis with lysozyme were essentially as previously described [10]. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of proteins was performed with 16% acrylamide (acrylamide/bisacrylamide ratio, 30:0.4) gel.

2.2. Purification of PgPFK

The crude enzyme extract from the sonicate of the induced cells was purified as previously described [8]. Briefly, sonicated crude extract was applied to columns of Mono SP (Pharmacia Biotech), Mono Q (Pharmacia Biotech), and Mono SP again. These columns were pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The Ppi-PFK activity was eluted in a stepwise fashion using increasing concentrations of NaCl (0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 M) in 50 mM Tris–HCl buffer (pH 7.5). The enzyme was purified 32-fold with a final specific activity of 278 U mg⁻¹ [8]. The purified enzyme was frozen and stored at −80°C.

2.3. Enzyme assays

Ppi-PFK activities in the forward reaction (F1,6-BP formation from F6-P and Ppi) were assayed in triplicate at 37°C by a modification of the method of Alves et al. [11]. Assays for the reverse reaction (F6-P formation from F1,6-BP and Pi) were also conducted in triplicate by a modification of the method of Alves et al. [11]. Ppi-PFK activities were determined by monitoring the oxidation of NADH in the forward reaction or production of NADPH in the reverse reaction at 340 nm. For the substrate-dependent assay, in the forward reaction, Ppi was held at fixed concentrations of 0.7, 1 or 2 mM, and the F6-P concentration was varied at each Ppi concentration from 0.2 to 10 mM. In the converse set of experiments, Pi (K2HPO4) was held at either 2.5, 5 or 10 mM, and F1,6-BP was varied at each Pi concentration from 0.01 to 1 mM. The $K_m$ and $V_{max}$ values for F6-P or F1,6-BP were determined by the Lineweaver–Burk Plot.

2.4. Nucleotide sequence accession number

The GenBank accession number for the nucleotide sequence of the PgPFK gene reported in this paper is AB039836.

3. Results and discussion

3.1. Kinetic analysis

The effects of the substrate concentrations upon the initial velocity of the forward and reverse reactions were determined. A double-reciprocal plot of the initial velocity of both F1,6-BP formation and F6-P formation produced converging non-parallel lines (Fig. 1A). This pattern indicates a sequential mechanism, and more specifically a random, single-displacement reaction. The $K_m$ for the purified PgPFK with F6-P was found to be $2.2 \pm 0.1$ mM (Ppi, 1 mM) and the $V_{max}$ was $278 \pm 34 \mu$mol min⁻¹ mg⁻¹ of enzyme. When the $K_m$ value of PgPFK was compared with that of all bacterial Ppi-PFKs reported thus far, PgPFK had the highest $K_m$ as follows: Acholeplasma laidlawii (0.65 mM) [12], Amycolatopsis methanolica (0.4 mM).

![Fig. 1. Kinetic analysis of PgPFK. (A) Double-reciprocal plots of the initial velocities of F1,6-BP formation from F6-P and Ppi catalyzed by purified PgPFK. Variable concentrations of F6-P (0.2–10.0 mM) at a fixed Ppi concentration of 0.7, 1 or 2 mM were used. The assays were performed as described in Section 2, except for the substrate concentrations. The data were plotted by the Lineweaver–Burk method and subjected to a linear regression. (B) Double-reciprocal plots of the initial velocities of F6-P formation from F1,6-BP and Pi catalyzed by purified PgPFK. Variable concentrations of F1,6-BP (0.01–1 mM) at a fixed Pi concentration of 2.5, 5 or 10 mM were used.](https://academic.oup.com/femsle/article-abstract/207/1/35/572064)
The same enzyme solution was used to study the kinetics of the reverse reaction (Fig. 1B). From these plots, the $K_m$ for the purified PgPFK with F1,6-BP was determined to be 0.11 ± 0.03 mM (Pi, 5 mM) and the $V_{max}$ was 190 ± 14 μmol min$^{-1}$ mg$^{-1}$. The $K_m$ value for F1,6-BP was slightly higher than those of the other PPi-PFKs (A. methanolicus (0.025 mM) [13], E. histolytica (0.018 mM) [14], G. lamblia (0.072 mM) [16], H. inflata (0.02 mM) [17], T. tenax (0.033 mM) [19], and T. pallidum (0.267 mM) [20]). The $K_m$ with F 6-P is about 20 times greater than that with F 1,6-BP. Likewise, the ratios of the other PPi-PFKs are as follows: A. methanolicus is 16 times; E. histolytica is 2.1 times; T. tenax is 1.6 times; and T. pallidum is 2.0 times. These reports indicate that A. methanolicus PPi-PFK and PgPFK have very similar kinetic properties. Alves et al. suggested that A. methanolicus PPi-PFK is readily reversible and may serve as fructose 1,6-bisphosphatase (FBPase), one of the gluconeogenic enzymes, since this organism has other pathways to catalyze glucose metabolism [11]. Similarly, considering that P. gingivalis does not utilize sugars such as glucose as an energy source [3,4], it is possible that PgPFK also serves as FBPase in P. gingivalis.

We have previously confirmed by RT-PCR analysis that the PgPFK gene was expressed in P. gingivalis by RT-PCR analysis [8]. In order to suggest the role of PgPFK, we tried to identify the FBPase gene in the data from the P. gingivalis Genome Project (http://www. tigr. org/tigr-scripts/CMR2/GeneNameSearch.spl?db = gpg). These websites revealed that P. gingivalis has FBPase significantly similar to FBPases from Clostridium acetobutylicum (a member of the Bacillus group: E value by PSI-BLAST, 0.0) and Bacillus subtilis (E value, e−157), but its characteristics are still unknown. Interestingly, Fujita et al. [21] reported that B. subtilis can bypass FBPase and that FBPase is not needed for the sporulation of B. subtilis. It is also suggested that the B. subtilis FBPase gene might have arisen by convergent evolution independently of other members of the FBPase family [22]. It is therefore tempting to speculate that, as in B. subtilis, PgPFK rather than P. gingivalis FBPase found in the websites might be involved in gluconeogenesis. As shown in Fig. 2, we tried to identify the components of the P. gingivalis glycolysis pathway by running a BLAST search with the ORF initially identified from pairwise comparisons with enzymes from E. coli, B. subtilis or other organisms such as B. burgdorferi and Thermotoga maritima. P. gingivalis mainly utilizes peptides instead of single amino acids as a source of energy and cell material [4,23]. In contrast, little is known about glucose metabolism in this organism. From the model, we could propose as follows: (a) PgPFK may have an important physiological role in supplying F6-P and hence G6-P and G1-P; (b) this role may lead to the gluconeogenic pathways to synthesize macromolecules such as the lipopolysaccharides and/or glycoconjugates essential for cell viability. As the intracellular concentrations of F6-P, F1,6-BP, Pi or Pi$^-$ were not measured in this study, we could not determine whether this organism has enough substrates for PgPFK to function effectively. Further studies using radioactive tracers and specific enzyme-deficient mutants would be valuable to investigate these pathways and their metabolic regulation.

In conclusion, biochemical studies of PgPFK suggest that P. gingivalis might preferentially utilize PgPFK as an enzyme of the gluconeogenetic pathway to synthesize lipopolysaccharides and/or glycoconjugates for cell viability.
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