Regulation of Canine Skeletal Muscle Phosphofructokinase-1 by Adenine Nucleotides

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ABSTRACT

Phosphofructokinase-1 (PFK-1) is the most important rate-controlling enzyme for glycolysis in both prokaryotes and eukaryotes. PFK-1 activity is regulated by multiple cellular metabolites, including nucleotides. Intracellular nucleotides are produced through various metabolic processes, including energy metabolism and intracellular signaling pathways. The activity of PFK-1, purified from canine skeletal muscle, was evaluated in the presence of various concentrations of adenine nucleotides to examine the regulation of glucose catabolism in canine skeletal muscle. Although UTP did not inhibit PFK-1 activity to the same extent as ATP, it substituted for ATP as a phosphate donor. cAMP functioned in a similar manner as AMP, as an activating effector of the PFK-1 reaction. ADP activated PFK-1 at low concentrations, but slightly inhibited PFK-1 at higher concentrations. The results suggested that canine PFK-1 had three different binding sites for adenine nucleotides acting as phosphate donors, activating effectors, and inhibitory effectors. Moreover, PFK-1 was shown to be activated by AMP and inhibited by ATP, while UTP and cAMP regulated PFK-1 activity. The results also suggested that ADP binds to the allosteric sites (for ATP and AMP) of PFK-1. Each adenine nucleotide functions as either an activating or inhibitory effector of PFK-1 reaction in canine skeletal muscle. Therefore, intracellular nucleotides may play an important role in regulating glucose metabolism by binding to the allosteric site of the enzyme.

INTRODUCTION

Glycolysis plays a central role in eukaryotic energy metabolism by producing NADH and ATP, along with pyruvate, which is converted to acetyl-coenzyme A for subsequent utilization in the tricarboxylic acid cycle. Phosphofructokinase-1 (EC: 2.7.1.11, PFK-1), the most important rate-controlling enzyme in glycolysis, catalyzes the phosphorylation of fructose 6-phosphate (F-6-P) by ATP to form fructose 1,6-bisphosphate. Three subunits of mammalian PFK-1 (M, L, and P) are present in various proportions in different tissues.[1] However, skeletal muscle is an exception, as it expresses only the M-type, specifically a homotetramer of M-type, while all other tissues express distinct levels of all three subunits.[2]

Recent studies reported the crystal struc-

tures of eukaryotic PFK-1 from yeast,[3, 4] rabbit muscle,[4] and human muscle,[5] which were generally based on crystallographic studies of PFK-1 purified from various eukaryotes.

PFK-1 is regulated by adenine nucleotides; the enzyme is inhibited by ATP and activated by AMP.[2, 6–11] Mammalian PFK-1 contains catalytic sites for binding to ATP and F-6-P, inhibitory sites for ATP and citrate, and activator sites for AMP/ADP and fructose-2,6-bisphosphate (F-2,6-P_2).[12]

In humans, muscle PFK deficiency, known as Tarui's disease, is an autosomal recessive disorder characterized by exercise-induced muscle weakness, pain, cramping, myoglobinuria, and hemolysis.[13–15] In animals, hereditary muscle PFK deficiency with chronic hemolysis and exertional myopathy, due to a single nonsense mutation in PFK, has been reported in English springer spaniels, whippets, and wachtelhunds.[16–19]

Very few physiological studies have been conducted on canine PFK-1, and thus the characteristics of canine PFK-1 remain unclear. Understanding the physiological regulation of canine skeletal muscle PFK-1 by adenine nucleotides is important for clarifying energy metabolism in canine skeletal muscle and the effects of abundant intracellular adenine nucleotides on glucose metabolism. In this study, regulation of canine muscle PFK-1 activity by adenine nucleotides was examined using partially purified PFK-1 from canine skeletal muscle.

MATERIALS AND METHODS

Materials

The chemicals and reagents used in this study were obtained from Wako Pure Chemicals (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA). ATP, ADP, AMP, UTP, cAMP, citrate, aldolase (EC: 4.1.2.13), and glycerol 3-phosphate dehydrogenase (EC: 1.1.1.8) were purchased from Wako Pure Chemicals. F-6-P, fructose 1,6-bisphosphate, D-fructose-1,2-cyclic-6-diphosphate, NAD, and triosephosphate isomerase (EC: 5.3.1.1) were purchased from Sigma-Aldrich. Blue Sepharose 6 Fast Flow as Cibacron Blue was purchased from GE Healthcare (Little Chalfont, UK). F-2,6-P2 was synthesized from D-fructose-1,2-cyclic-6-diphosphate as described previously.[20] The molar concentration of F-2,6-P2 was measured based on the correlated measurement of phosphorus concentration.[21]

Animals

The beagles were housed in appropriate facilities to ensure animal welfare. They were fed commercial dry food and had free access to water. The beagles were housed in adequately sized cages under a 12-h dark–light cycle (light from 06:00 to 18:00) in an air-conditioned environment. This study protocol was approved by the Nihon University Animal Care and Use Committee (permission number: AP13B074-1). Sodium pentobarbital (150 mg/kg body weight) was administered intravenously for sacrifice.

Purification of PFK-1

After sacrifice, canine skeletal muscle PFK-1 was purified as described previously with some modifications.[7] Briefly, fresh skeletal muscle was resected from sacrificed beagles and stored at -80°C until PFK-1 purification. All purification steps were carried out below 4°C, except for heat treatment. Fifteen grams of skeletal muscles, minced with scissors, were dissolved in homogenization buffer (50 mM Tris-phosphate buffer (pH 8), 1 mM PMSF, and 10 mM DTT), and homogenized 5 times for 1 min each with a Teflon homogenizer at 500 rpm. The homogenate was ultracentrifuged at 100,000 ×g for 30 min. The supernatant was heat-treated at 52°C for 3 min, and then centrifuged at 10,000 ×g for 10 min to separate the denatured protein. The heat-treated supernatant was loaded onto a Blue Sepharose 6 Fast Flow (Cibacron Blue) column (1 × 5 cm) equilibrated with buffer A (50 mM Tris-phosphate buffer (pH 8), 0.1 mM EDTA (pH 8), 0.05 mM fructose 1,6-bisphosphate, 10 mM DTT). After washing the column with 50 ml buffer A containing 0.15 mM ADP, PFK-1 was eluted with buffer B (50 mM Tris-phosphate buffer (pH 8), 0.1 mM EDTA (pH 8), 0.05 mM fructose 1,6-bisphosphate,

Table 1. *Purification of PFK-1 from canine skeletal muscle*

Step	Total activity (units)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Ultracentrifugation	38.03	1313.4	0.029	100.0	1.00
Heat treatment	28.80	421.47	0.068	75.7	2.36
Cibacron Blue affinity column	20.74	15.03	1.380	54.5	47.66
Dialysis	12.03	2.61	4.609	31.6	159.18

5 mM ATP, 2 mM F-6-P, 10 mM DTT) at a flow rate of 1 ml/min. Fractions with PFK-1 activity were collected, precipitated with 50% ammonium sulfate, and centrifuged at 10,000 ×g for 30 min. The precipitate was dissolved in 50 mM Tris-phosphate buffer with 10 mM DTT and dialyzed overnight to eliminate ammonium sulfate. The dialyzed solution was used as purified PFK-1.

Protein Estimation

Protein concentration after each purification step and that of purified PFK-1 were determined using the Bradford method[22] with bovine serum albumin as the standard.

PFK-1 Assay

The PFK-1 assay was conducted as reported previously.[7] The enzymatic reaction was initiated by adding enzyme solution at 25°C and monitoring the rate of NADH oxidation at 340 nm.

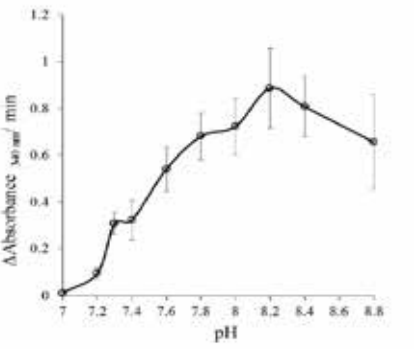
The pH-dependent activity profile was shown by the value of decreasing absorbance. PFK-1 activity regulation was determined by velocity standardized by maximum velocity (v) calculated based on the optimal condition (V_{max}) in each experiment, in order to eliminate interindividual differences or effects of the freeze-thaw process.[7]

Optimal PFK-1 activity was determined in a reaction mixture containing the following components in a final volume of 1 ml: 50 mM HEPES buffer (pH 8.2), 100 mM KCl, 6.5 mM $MgCl_2$, 1 mM NH_4Cl, 5 mM KH_2PO_4, 0.3 mM NADH, aldolase (0.5 U), glycerol 3-phosphate dehydrogenase (0.5 U), triosephosphate isomerase (5 U), 1 mM F-6-P, 5 mM ATP, and 0.1 mM AMP. One unit of PFK-1 activity was defined as the amount of enzyme that phosphorylated 1 µmol of F-6-P per min at 25°C.

The allosteric-kinetic properties of PFK-1 were determined in a reaction mixture containing the following components in a final volume of 1 ml: 50 mM HEPES buffer (pH 7.3), 100 mM KCl, 6.5 mM $MgCl_2$, 1 mM NH_4Cl, 5 mM KH_2PO_4, 0.3 mM NADH, aldolase (0.5 U), glycerol 3-phosphate dehydrogenase (0.5 U), triosephosphate isomerase (5 U), and varying concentrations of F-6-P, ATP, and/or AMP.

The PFK-1 activity of each enzyme solution was expressed as v/V_{max} for normalization, where v is the activity at pH 7.3 (intracellular physiological condition), and V_{max} is the maximum activity determined at pH 8.2 for excluding the allosteric effects of regulatory factors.

Figure 1. *pH-dependent activity profile of canine muscle PFK-1. PFK-1 activity was assayed using 5 µl of purified PFK-1 solution in a total volume of 1 ml containing 1 mM F-6-P, 5 mM ATP, and 0.1 mM AMP at various pH levels. Values show the decrease in absorbance at 340 nm in 1 min. The results are shown as the mean ± SEM of 3 independent experiments.*



RESULTS

Purification of Canine Skeletal Muscle PFK-1

Purity of PFK-1 was analyzed based on protein concentrations and PFK-1 activities after each step of purification (Table 1). PFK-1 was purified by 159-fold from canine skeletal muscle with a specific activity of 4.6 U/mg protein and yield of 31.6%.

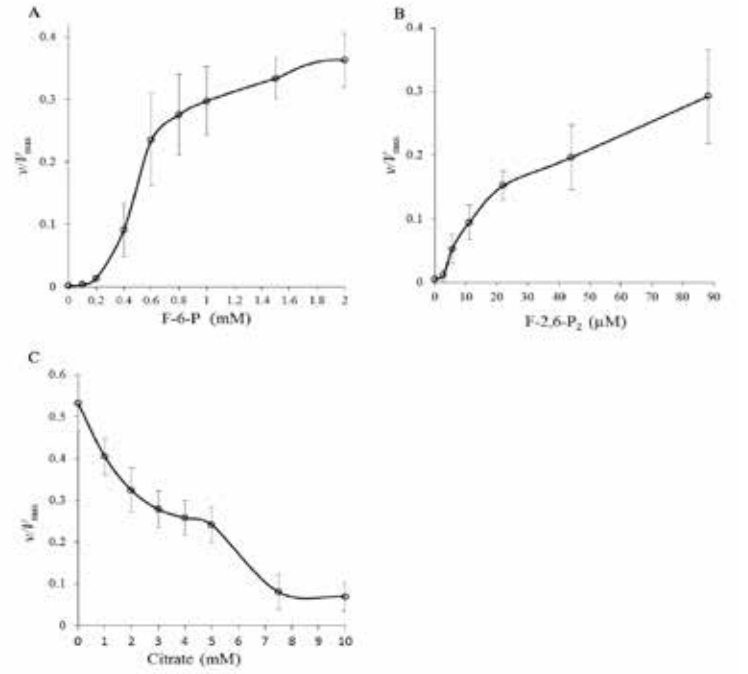
Fundamental Regulation of PFK-1 Activity

PFK-1 activity was determined over pH 7.0–8.8 to investigate pH-dependence of the reaction in 50 mM HEPES buffer, 1 mM F-6-P, 5 mM ATP, and 0.1 mM AMP. PFK-1 activity showed a peak at pH 8.2 (Figure 1). Thus, the optimal pH for canine muscle PFK-1 was determined to be 8.2. The velocity under this condition, which reflects PFK-1 activity, was determined as the maximum velocity (V_{max}).

Regulation of PFK-1 activity by F-6-P concentration is shown in Figure 2A. When PFK-1 was assayed with 5 mM ATP and 0.1 mM AMP, PFK-1 showed higher activation with increasing concentrations of F-6-P. The saturation curve was remarkably sigmoidal and nearly reached a plateau level at 1 mM F-6-P.

Regulation of PFK-1 activity by F-2,6-P_2 concentration is shown in Figure 2B. When PFK-1 was assayed with 0.1 mM F-6-P, 5 mM ATP, and 0.1 mM AMP, PFK-1 was found to be activated by increasing concentrations of F-2,6-P_2.

Figure 2. *Regulation of canine skeletal muscle PFK-1 activity with varying concentrations of effectors.*
PFK-1 activities were assayed under the following conditions: (A) with 5 mM ATP, 0.1 mM AMP, and varying concentrations of F-6-P, (B) with 0.1 mM F-6-P, 5 mM ATP, 0.1 mM AMP, and varying concentrations of F-2,6-P_2, and (C) with 0.1 mM F-6-P, 0.3 mM ATP, 0.1 mM AMP, and varying concentrations of citrate. Values correspond to enzyme activity expressed as v/V_{max}. Results are shown as the mean ± SEM of at least 3 independent experiments.



Regulation of PFK-1 activity by the citrate concentration is shown in Figure 2C. When assayed with 0.1 mM F-6-P, 0.3 mM ATP, and 0.1 mM AMP, PFK-1 activity was found to be inhibited with increasing citrate concentrations.

Regulation of PFK-1 by Adenine Nucleotides

The effects of ATP and UTP on the regulation of PFK-1 activity were compared, as shown in Figure 3. When assayed with 0.1 mM F-6-P and 0.1 mM AMP, PFK-1 was activated by low concentrations (<1 mM) of ATP. PFK-1 activity was inhibited by millimolar concentrations of ATP, with nearly complete inhibition at 5 mM ATP.

Figure 3. *Effect of ATP and UTP on regulation of canine skeletal muscle PFK-1 activity. PFK-1 activities were assayed with 0.1 mM F-6-P, 0.1 mM AMP, and varying concentrations of ATP or UTP. Values refer to the enzyme activity expressed as v/V_{max}. Results are shown as the mean ± SEM of 3 independent experiments.*

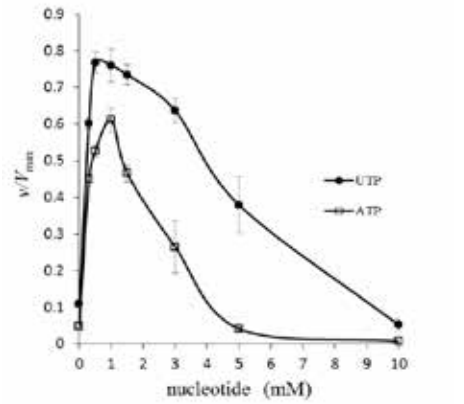


Figure 4. *Effect of AMP and cAMP on regulation of canine skeletal muscle PFK-1 activity. PFK-1 activities were assayed with 0.1 mM F-6-P, 2.5 mM ATP, and varying concentrations of AMP (solid line) or cAMP (dotted line). Values refer to the enzyme activity expressed as v/V_{max}. Results are shown as the mean ± SEM of 3 independent experiments.*

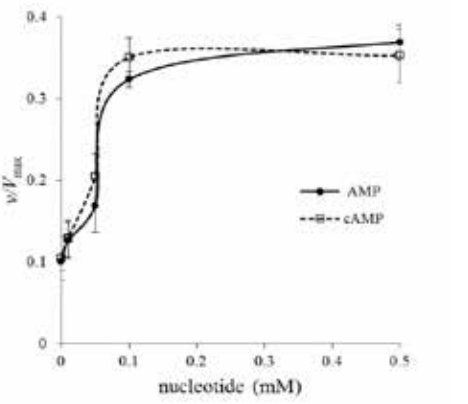
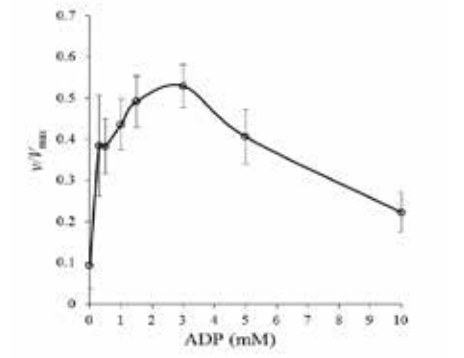


Figure 5. *Effect of ADP on regulation of canine skeletal muscle PFK-1 activity. PFK-1 activities were assayed with 0.1 mM F-6-P and varying concentrations of ADP without AMP and ATP. Values shown are the mean of enzyme activity expressed as v/V_{max}. Results are shown as the mean ± SEM of 5 independent experiments.*



When assayed following administration of UTP, rather than ATP, PFK-1, activation occurred at low concentrations (<0.5 mM) of UTP, followed by inhibition at millimolar concentrations of UTP. However, unlike ATP, millimolar concentrations of UTP only slightly inhibited PFK-1 activity, showing half-maximum activity at 5 mM UTP.

The effects of AMP and cAMP on the regulation of PFK-1 activity were also compared (Figure 4). PFK-1 activity was assayed with 0.1 mM F-6-P, 2.5 mM ATP, and varying concentrations of AMP and found to be activated by increasing AMP concentrations, finally reaching a plateau at 0.1 mM AMP. Rather than AMP, PFK-1 activity was next assayed using varying concentrations of cAMP and 1 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase. Regulation of PFK-1 activity by cAMP was similar to that by AMP.

Figure 5 shows the regulation of PFK-1 activity by ADP. Using 0.1 mM F-6-P without AMP and ATP, PFK-1 was found to be activated by low concentrations of ADP (<3 mM) and slightly inhibited by higher concentrations of ADP.

DISCUSSION

Following each step of purification, the specific activity of PFK-1 increased exponentially, as revealed by affinity chromatog-

raphy using Cibacron Blue. This suggests that intracellular PFK-1 is controlled by numerous small molecules that, in this study, were eliminated by affinity chromatography.

The fundamental regulation of PFK-1 activity was confirmed as described previously, although the method for PFK-1 purification was slightly modified.[2, 6–8, 11] Therefore, the purified PFK-1 in this study was appropriate for assaying enzyme activity.

In general, glycolysis produces ATP directly or indirectly via NADH. ATP is essential for the phosphate donor function of PFK-1, formation of the final products of glucose catabolism via glycolysis, and subsequent tricarboxylic acid cycle and oxidative phosphorylation. PFK-1 has three different binding sites for adenine nucleotides acting as phosphate donors, activating effectors, and inhibitory effectors.[6]

Based on the results of this study, UTP appears to bind the catalytic site of PFK-1 like ATP and functions as a more efficient phosphate donor than ATP. Additionally, a higher concentration of UTP was required to bind the inhibitory site of PFK-1 compared to that of ATP. These results suggest that UTP can readily bind the catalytic site, but has difficulty binding the inhibitory site of PFK-1 when compared to ATP. However, there was no functional difference between AMP and cAMP in regulating PFK-1 activity, suggesting that cAMP acted similarly to AMP for binding and activation of PFK-1. Thus, increasing intracellular cAMP concentrations can directly promote PFK-1 activation.

ADP, like ATP, may act as a phosphate donor at low concentrations and showed half-maximum activity at 0.3 mM. Because ADP triggered high activity without AMP, it may be considered as an activating effector like AMP. Additionally, a high concentration of ADP slightly inhibited PFK-1 activity by binding the inhibitory site of PFK-1. Therefore, ADP has the potential to bind to both the activation and inhibitory sites of PFK-1.

Intracellular nucleotides, including adenine nucleotides and UTP, are produced by various metabolic processes such as energy metabolism and intracellular signaling pathways, including DNA replication and transcription, and influence various biological activities. Various nucleotides may regulate PFK-1 activity in the cell: PFK-1 was shown to be activated by AMP and inhibited by ATP, whereas UTP and cAMP were shown to regulate PFK-1 activity. UTP and cAMP function in the skeletal muscle as a substrate of glycogenesis and second messenger in glycogenolysis, respectively. This suggests that glycolysis in the skeletal muscle is influenced by glycogen metabolism. ADP, as an intermediate of ATP and AMP, can both activate and inhibit PFK-1 activity. Affinity to the activation or inhibitory site of PFK-1 depends on the type of nucleotide. The concentrations of bases, nucleosides, and nucleotides in mammalian cells and fluids, based on various assay methods and samples, or from previously published reports, were summarized by T. W. Traut in a review.[23] However, the exact concentrations of intracellular nucleotides remained unclear, as the values in the original published data were irregular. Moreover, the mechanism underlying the regulation of the concentration and ratio of intracellular nucleotides remains unclear. Further studies are needed to evaluate the relationship between glycolysis and intracellular nucleotides.

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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