Enzymatic Synthesis of Carbocyclic Analogue of Fructose 2,6-bisphosphate with 6-Phosphofructo-2-kinase

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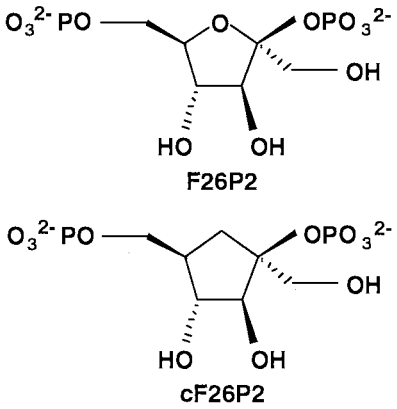
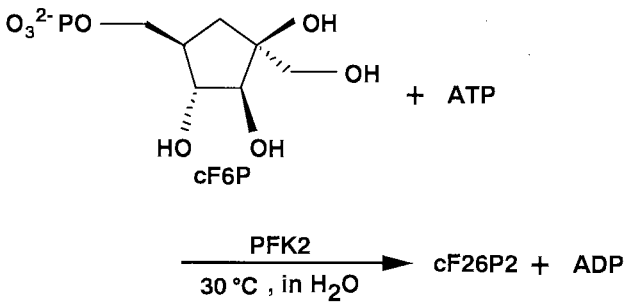
cF26P2 was synthesized enzymatically by the regioselective phosphorylation of cF6P with PFK2 and ATP. cF26P2 was found to be much stabler in the acidic condition than F26P2 and could activate the glycolytic pathway enzyme PFK.

Fructose 2,6-bisphosphate (F26P2) is an important regulatory metabolite in the glycolytic and glucogenic pathway and acts as an activator of 6-phosphofructo-1-kinase (PFK; EC 2.7.1.11) and an inhibitor of fructose-1,6-bisphosphatase (FDPase; EC 3.1.3.11).[1,2] F26P2 is therefore an interesting potential agent for controlling diseases caused by errors in regulation of glycolysis. F26P2 is formed in higher cells from fructose 6-phosphate (F6P) and ATP with 6-phosphofructo-2-kinase (PFK2; EC 2.7.1.105) and hydrolyzed to F6P and phosphoric acid with fructose-2,6-bisphophatase (EC 3.1.3.46).[3] However, since F26P2 is extremely acid-labile: its half-life is about 15 min at pH 2 and at 0 °C, and 5 min at pH 4 at 37 °C,[4] the derivatives of fructose phosphate have been studied in order to stabilize F26P2.[5]

This paper describes the enzymatic synthesis of carbocyclic analogue (cF26P2) of F26P2 with PFK2 from ATP and the carbocyclic analogue (cF6P)[6] of F6P prepared chemically. Scheme 1 shows the structures of F26P2 and cF26P2. The synthetic route of cF26P2 is shown in Scheme 2. The design of cF26P2 as a stable analogue of F26P2 and the chemical synthetic method of cF6P were reported previously by C. S. Wilcox et al.[6] Because the keto group is easy to form at the 2-position of F26P2 by tautomerization, the phosphate group at the 2-position is liable to be relieved. On the other hand, since no formation of the keto-form of cF26P2 results in no elimination of the phosphate group at the 2-position, cF26P2 is expected to be stabler than F26P2. In addition, since cF26P2 is very similar in structure to F26P2 from energy minimization by MOPAC



Scheme 1. Structures of F26P2 and cF26P2.



Scheme 2. Synthetic route of cF26P2.

method with CAche work system, it is predicted that cF26P2 could activate PFK in analogy with F26P2.

DEAE Sepharose FF and Blue Sepharose CL-6B columns were purchased from Pharmacia. Asahipak ES-502N and DIAION WA10 columns were obtained from Showa Denko and Mitsubishi Chemicals, respectively. ATP and F6P were obtained from Boehringer. Pyrophosphate-dependent 6-phosphofructo-1-kinase (PPi-PFK; EC 2.7.1.90) and F26P2 were purchased from Sigma. All reagents and solvents for cF6P synthesis were of high purity commercial available and were used without further purification.

cF6P was prepared from 2,3,5-tri-O-benzyl-D-arabinofuranose in 1.1% overall yield in 9 steps.[6] PFK2 was expressed in *Saccharomyces cerevisiae* strain DFY658 containing recombinant plasmid pMK11.[7] One hundred grams of cells was resuspended in 200 ml of 50 mM TrisHCl pH 7.5, 20 mM KCl, 2mM EDTA, and 2 mM mercaptoethanol (buffer A) and disrupted with glass beads. After cell debris was removed from centrifugation, the extract was applied to a DEAE Sepharose FF column and washed with 300 ml of buffer A. The active fractions were pooled and applied to a Blue Sepharose CL-6B column. After washing with 50 ml of buffer A, PFK2 was eluted with 2 M KCl in 40 ml of buffer A to obtain 2.6 units as activity for F26P2 synthesis. The specific activity increased from 2.7 to 190 mU mg^{-1} by the purification procedure. The PFK2 activity was assayed in literature method.[7]

The synthesis of cF26P2 was performed from 10 mM cF6P and 15 mM ATP with 2.1 units PFK2 in 20 ml of 150 mM TrisHCl pH 8.0 containing 20 mM $MgCl_2$ at 30°C. After 24 h, the reaction was stopped by heating at 90°C for 3 min to obtain approximately 6.2 mM cF26P2 in 62.0% yield. The yield was calculated from HPLC peak area corresponding to cF26P2. HPLC analysis was performed with an anion exchange column Asahipak ES-502N in 150 mM sodium phosphate pH 7.0 buffer. Although the hydroxy group at the 1-position has higher chemical reactivity than that at the 2-position in phosphorylation of F6P, F26P2 is synthesized with PFK2.[7] Similarly the hydroxy group at the 2-position for cF6P was predominantly phophorylated with

PFK2 and that at the 1-position was not reacted. Consequently, cF6P was found to act as a substrate for PFK2 and the regioselectivity of PFK2 was maintained in the phosphorylation of cF6P.

The reaction mixture was applied to an activated carbon column in order to remove ATP, ADP, and AMP, and the eluted solution was collected since the adenosine compounds adsorbed to the activated carbon column. The solution was applied to an anion exchange column DIAION WA10 and cF26P2 was eluted with 0.02 M NaOH as shown in Figure 1. After the fractions which were free from cF6P were collected and desalted with a membrane having a molecular weight cutoff of 100, the solution was lyophilized to give 0.10 mmol of cF26P2 as a white powder.[8]

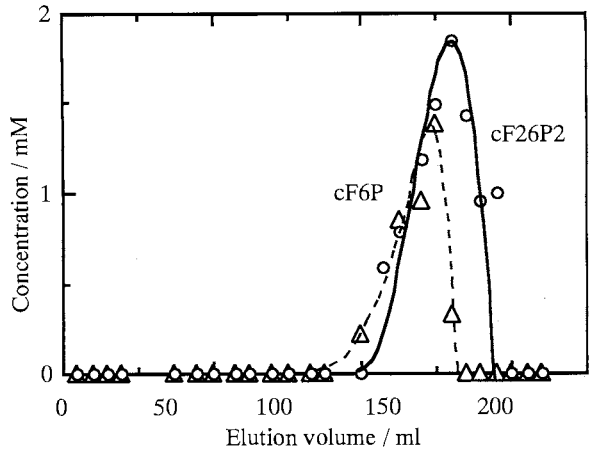


Figure 1. Elution profile of cF26P2 from anion exchange column DIAION WA10. cF26P2 (O) and cF6P (△).

Figure 2 shows comparison of pH stabilization for F26P2 and cF26P2. The half-life of F26P2 was approximately 1 day at pH 6.0 at 25 °C and 10 min at pH 4.0 at 25 °C, whereas cF26P2 scarcely decomposed for 40 days at pH 4.0 and 6.0. Therefore, cF26P2 was much stabler in the acid conditions than F26P2.

The regulatory effect of cF26P2 was tested on the rate of F6P phosphorylation by PPi-PFK. Although cF26P2 was found to act as a regulator of PPi-PFK, cF26P2 activated PPi-PFK catalyzed phosphorylation as effectively as F26P2 at approximately 200 times the concentration. This indicates that the oxygen of the fructose ring is significant in the activation of PFK. The other F26P2 analogue, β-D-arabinose 1,5-diphosphate prepared by B. E. Maryanoff et al. activated PFK at a concentration 20 times higher than F26P2.[5]

In conclusions, cF6P was phosphorylated regioselectively with PFK2, and the stability of cF26P2 was superior to that of F26P2 in the acidic conditions.

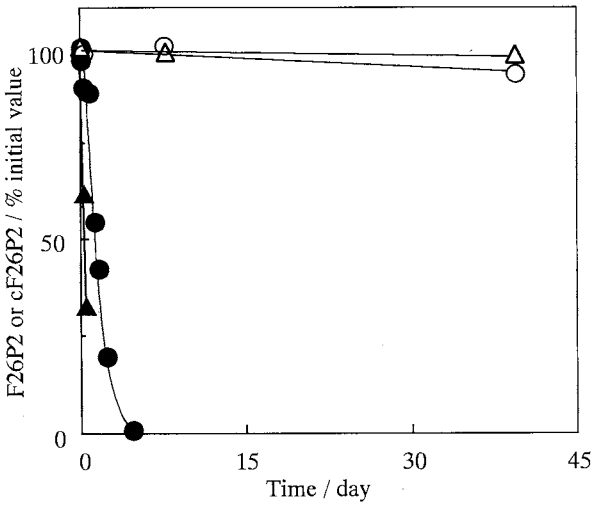


Figure 2. Time course of F26P2 or cF26P2 stabilization at pH 6.0 and 4.0 at 25 °C. F26P2 and cF26P2 was assayed by HPLC peak area. (●) F26P2 at pH 6.0; (▲) F26P2 at pH 4.0; (○) cF26P2 at pH 6.0; (△) cF26P2 at pH 4.0.

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References

1 E. Van Schaftingen and H. G. Hers, *Biochem. Biophys. Res. Commun.*, **96**, 1524 (1980).
2 H. G. Hers and L. Hue, *Annu. Rev. Biochem.*, **52**, 617 (1983).
3 S. J. Pilkis, T. H. Claus, P. Kountz, and M. R. El-Maghrabi, *Enzymes*, **28**, 3 (1987).
4 E. Van chaftingen and H. G. Hers, *Eur. J. Biochem.*, **192**, 319 (1981).
5 B. E. Maryanoff, A. B. Reitz, G. F. Tutwiler, S. J. Benkovic, P. A. Benkovic, and S. J. Pilkis, *J. Am. Chem. Soc.*, **106**, 7851 (1984).
6 C. S. Wilcox and J. J. Gaudino, *J. Am. Chem. Soc.*, **108**, 3102(1986).
7 M. Kretschmer, C. Langer, and W. Prinz, *Biochemistry*, **32**, 11143 (1993).
8 ^{1}H NMR (300 MHz, D_2O) δ 3.96 (1 H, C-3 position) 3.92 (1 H, C-1) 3.83 (2 H, C-6) 3.71 (1 H, C-1) 3.64 (1 H, C-4) 2.14 (1 H, at carbon which substitute for oxygen of fructose ring (C-c)) 1.98 (1 H, C-5) 1.92 (1 H, C-c). ^{13}C NMR (75 MHz, D_2O) δ 85.19, 83.12, 82.29, 69.57, 69.34, 43.67, 36.56.