FEBS Letters 459 (1999) 448–452 FEBS 22743

Evidence that fructose 1,6-bisphosphate specifically protects the α-subunit of pyrophosphate-dependent 6-phosphofructo-1-phosphotransferase against proteolytic degradation

Yue-Hao Wang*, Jiao-Nai Shi

Shanghai Institute of Plant Physiology, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, PR China

Received 30 August 1999

Abstract Pyrophosphate-dependent 6-phosphofructo-1-phosphotransferase (PFP) consists of α (regulatory) and β (catalytic) subunits. The α-subunit was previously reported to be much more susceptible to tryptic digestion than the β-subunit. In this study, ligand-induced protection of PFP subunits against proteolysis by subtilisin was investigated in vitro and the data obtained demonstrated that fructose 1,6-bisphosphate (Fru-1,6-P_2), while exerting negligible effect on the β-subunit, remarkably protected the α-subunit against proteolytic degradation. Western blot analysis revealed a good correlation between the Fru-1,6-P_2 concentration and the degree of corresponding protection on the α-subunit against proteolysis. In contrast, none of other examined ligands including fructose 2,6-bisphosphate, fructose 6-phosphate and pyrophosphate had such protection on the α-subunit. This finding (1) indicates that the stability of the α-subunit can be selectively increased by Fru-1,6-P_2, and (2) suggests that Fru-1,6-P_2 is likely a special effector of the α-subunit.

Key words: Pyrophosphate-dependent 6-phosphofructo-1-phosphotransferase; Potato tuber; Subunit; Proteolysis; Subtilisin; Fructose-1,6-bisphosphate

1. Introduction

Pyrophosphate-dependent 6-phosphofructo-1-phosphotransferase (PFP, EC2.7.1.90), catalyzing the interconversion of fructose 6-phosphate (Fru-6-P) to fructose 1,6-bisphosphate (Fru-1,6-P_2), exists in a wide range of plant tissues [1–3], propionibacterium [4] and entamoeba [5] but not in animals. Interestingly, in plants, PFP instead of ATP-dependent 6-phosphofructo-1-kinase (PFK) shares an important modulator Fru-2,6-P_2 with animal PFK. Fru-2,6-P_2 strongly activates PFP, but potently inhibits fructose-1,6-bisphosphatase (FBPase), controlling the flux between Fru-6-P and Fru-1,6-P_2 [6].

Plant PFP generally consists of α- and β-subunits [7]. However, the enzyme exists in multiple forms with different subunit compositions. β_2 and $\alpha_2\beta_2$ are usually the major forms [7,8]. Comparison between the kinetic and regulatory properties of these isoforms has been made and remarkable differences in basal activity (V_0), Fru-2,6-P_2 stimulated activity ($V_{Fru\text{-}2,6\text{-}P2}$) and the activity ratio ($V_{Fru\text{-}2,6\text{-}P2}$ versus V_0) were identified [8]. Much smaller V_0 and greater activity ratio have been recognized to be the characteristics of β_2, and the α-subunit is proposed to act as the regulatory component conferring the β-subunit, catalytic subunit, the greater basal activity [9].

The reasons for the existence of multiple PFP isoforms remain unknown. Recently, differential proteolysis of PFP subunits has been reported indicating that the α-subunit is much more susceptible to tryptic digestion than the β-subunit [9]. Thus, instability of the α-subunit was considered part of the reasons. Besides, the subunit composition of PFP was also affected by the other factors. It was reported that phosphate starvation can selectively induce accumulation of the α-subunit in suspension cells of *Brassica nigra* [10]. However, it remains to be determined whether such accumulation of the α-subunit is due to increased de novo synthesis or due to decreased proteolytic degradation of this protein. Anyhow, changing the subunit composition of PFP (e.g. through selective accumulation of the α-subunit) seems to underlie an important mechanism by which the enzyme is regulated in vivo. Incidentally, there are some precedents in the literature that demonstrate the similar regulatory mechanism of other enzymes [11,12].

In order to gain insight into this intriguing mechanism, we investigated the ligand-induced protection of PFP subunits by analyzing limited proteolytic digestion of purified potato tuber PFP in vitro. Interestingly, the data obtained demonstrated that in marked contrast to the effects of other examined ligands including Fru-2,6-P_2, Fru-6-P and pyrophosphate (PPi), Fru-1,6-P_2 remarkably and specifically protected the α-subunit against proteolytic degradation while exerting negligible effect on the β-subunit. Therefore, this finding (1) indicates that the stability of the α-subunit can be selectively increased by Fru-1,6-P_2 and (2) suggests that Fru-1,6-P_2 is likely a special effector of the α-subunit.

2. Materials and methods

2.1. Materials

Subtilisin was obtained from Boehringer Mannheim. Fru-1,6-P_2, Fru-6-P, Fru-2,6-P_2, aldolase, glycerol-3-phosphate dehydrogenase, triose phosphate isomerase, goat anti-rabbit immunoglobulin antibody conjugate to horseradish peroxidase, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), molecular weight markers: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa) were purchased from Sigma. Cellulose phosphate P-11 and DEAE-Cellulose DE52 were from Whatman.

*Corresponding author. Fax: (86) (21) 64042385.
E-mail: yhwang@iris.sipp.ac.cn

Abbreviations: PFP, pyrophosphate-dependent 6-phosphofructo-1-phosphotransferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PPi, pyrophosphate; Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; PMSF, phenylmethylsulfonyl fluoride

0014-5793/99/$20.00
PII: S0014-5793(99)01304-6

2.2. PFP activity assay

PFP activity was measured spectrophotometrically as described earlier [8]. 1 ml assay mixture consisted of 50 mM Tris-HCl, pH 7.5, 2 mM Fru-6-P, 1 mM PPi, 5 mM $MgCl_2$, 0.14 mM NADH, 1 unit aldolase, 10 units triose phosphate isomerase, 2.5 units glycerol-3-phosphate dehydrogenase and 1 μM Fru-2,6-P_2 (unless indicated otherwise).

2.3. Purification of PFP.

PFP was rapidly purified from potato tuber through 6–14% polyethylene glycol (PEG, average M_r 6000) fractionation and sequential column chromatography on DEAE-Cellulose DE52 and cellulose phosphate P-11. The protocol was similar to that described in [13] except for the following modifications: (1) heat treatment step was omitted, and (2) dialysis of PFP samples was replaced by 20% PEG precipitation. All the purification steps were carried out at 4°C in the presence of protease inhibitors.

2.4. Preparation of monospecific antibodies against PFP subunits

The α- and β-subunits of purified potato tuber PFP were separated by preparative SDS-PAGE (8.5% separation gel). After brief staining, the gels containing the α- and β-subunits were separately excised and extensively washed in phosphate-buffered saline (PBS, 20 mM phosphate buffer, pH7.4, 0.15 M NaCl) with agitation. Then the gel pieces were smashed following six cycles of freezing-thawing and emulsified in Freund's complete or incomplete adjuvant for immunizing New Zealand white rabbits following a standard protocol [14]. After four injections, antisera were collected and antibodies(IgG) were purified through 0–50% saturation ammonium sulfate fractionation and chromatography on DEAE-Cellulose DE52 [14]. Monospecific antibodies against the α- and β-subunits were further prepared by affinity purification following the protocol described in [15], respectively.

2.5. SDS-PAGE and Western blotting

SDS-PAGE was performed using 10% separation gel (unless indicated otherwise) [16]. The resolved polypeptides were either visualized by silver stain [17] or transferred electrophoretically onto nitrocellulose (NC) membrane [18]. Western blotting was performed according to the method described earlier [14] except for the following modifications: after primary antibody incubation and subsequent washes, the NC membrane was incubated with the horseradish peroxidase-conjugated goat anti-rabbit antibodies; finally, the blot was either developed using 4-Cl-1-naphthol and H_2O_2 [14] or visualized by enhanced chemiluminescent (ECL) method following the manufacturer's instructions (Amersham). For reprobing, the blotted NC membrane was treated with two changes of 2% (w/v) SDS solution at 80°C for 30 min to wash away the antibodies of initial blot completely. Following extensive washes with PBS, the membrane was reblotted as above. For reblotting, the antigen-antibody complexes were visualized by the ECL method through exposure of X-ray film.

2.6. Limited proteolytic digestion of PFP

Purified potato tuber PFP was desalted through a Sephadex G-25 column (1.6×15 cm) at 4°C into buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM dithiothreitol). Before proteolysis, the desalted enzyme was preincubated without or with appropriate ligands (substrates, effector or a cocktail of them as indicated) at 30°C for 20 min. Then subtilisin was added to start proteolysis. Digestion was carried out at 30°C for indicated lengths of time, then terminated by addition of 100 mM phenylmethylsulfonyl fluoride (PMSF) to make a final concentration of 2 mM. Aliquots of the samples were analyzed by SDS-PAGE and Western blotting. The residual PFP activity was also assayed. Protein concentrations were determined as described by Bradford [19] using crystalline bovine serum albumin as a standard. The experiments reported in this paper were repeated at least twice and the same results were obtained.

3. Results

PFP used in all the experiments of this report was purified from potato tuber following a simplified purification protocol with special attentions paid to minimizing the potential proteolysis of the enzyme during preparation [20,21]. The purified enzyme was a heterotetramer of $\alpha_2\beta_2$ as determined by chromatography on a calibrated Sephacryl S-300 column (data not shown) and SDS-PAGE (Fig. 1). For partial proteolysis of PFP, subtilisin instead of trypsin [9] was chosen because it is an unspecific protease and presumably able to sense more signals reflecting conformational changes of substrate protein (i.e. PFP) than a specific protease like trypsin [9].

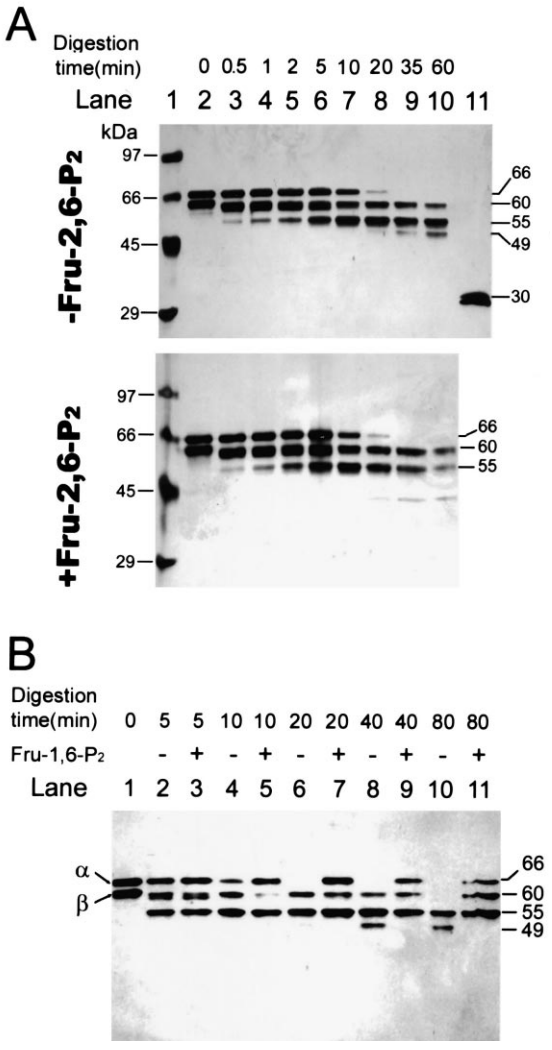

Fig. 1. Limited proteolytic digestion of PFP in the presence of Fru-2,6-P_2 (A) or Fru-1,6-P_2 (B). A: Purified potato tuber PFP (150 μg/ml) was preincubated without (upper panel) or with (lower panel) 500 μM Fru-2, 6-P_2 at 30°C for 20 min, then proteolysis was carried out with 5 μg/ml subtilisin at 30°C for indicated lengths of time. Lane 1, molecular weight markers (values in kDa); lane 11 in upper panel, subtilisin (15 μg). B: Purified potato tuber PFP (150 μg/ml) was preincubated without (lanes 2,4,6,8,10) or with (lanes 3,5,7,9,11) 10 mM Fru-1,6-P_2/10 mM $MgSO_4$ at 30°C for 20 min. Proteolysis was carried out with 10 μg/ml subtilisin at 30°C for indicated lengths of time. Aliquots of samples were analyzed by SDS-PAGE and the resolved polypeptides were visualized by silver stain. Indicated on the right side are the deduced molecular weights of the corresponding polypeptides (values in kDa).

As shown in Fig. 1A, the α-subunit (66 kDa band) was readily degraded by subtilisin and completely disappeared after being digested for 35 min. A 55 kDa band, which was identified later by Western blotting (see Fig. 2B) as proteolytic product of the 60 kDa β-subunit, appeared and accumulated gradually. Except for some minor differences, the proteolytic profiles of purified potato tuber PFP in the absence (Fig. 1A,

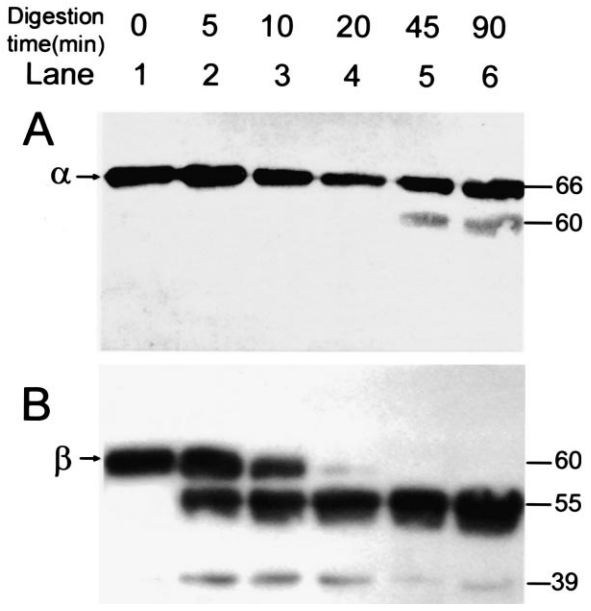

Fig. 2. Western blot analysis of proteolytic products of PFP in the presence of Fru-1,6-P2. Purified potato tuber PFP (100 μg/ml) was preincubated with 10 mM Fru-1,6-P_2/10 mM $MgSO_4$ at 30°C for 20 min, then proteolysis was carried out with 10 μg/ml subtilisin at 30°C for indicated lengths of time. Polypeptides were resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. Then the membrane was blotted with monospecific antibodies against the α (A) or the β (B) subunits, respectively. Indicated on the right side are the deduced molecular weights of the corresponding polypeptides (values in kDa).

upper panel) and presence (Fig. 1A, lower panel) of Fru-2,6-P_2 share high similarity, indicating that Fru-2,6-P_2 virtually did not exert significant protection on the α-subunit as well as the β-subunit against proteolysis. Fig. 1B shows a comparison between the proteolytic profiles of PFP in the absence (lanes 2,4,6,8,10) and presence (lanes 3,5,7,9,11) of Fru-1,6-P_2. The results demonstrated that in marked contrast to the effect of Fru-2,6-P_2, Fru-1,6-P_2 remarkably protected the α-subunit against proteolytic degradation. This observation was confirmed by Western blot analysis (Fig. 2A). As shown in Fig. 2A, under protection of Fru-1,6-P_2, the majority of the 66 kDa α-subunit remained intact in the period of 90 min proteolytic digestion; in contrast, the α-subunit of the control sample was completely degraded within 20 min (Fig. 1B, lane 6). Roughly, the half life of the α-subunit was increased about 10-fold by Fru-1,6-P_2. A 60 kDa proteolytic product of the α-subunit was identified by the corresponding monospecific antibody (Fig. 2A). This band, which has the same molecular weight as the β-subunit, virtually existed in lots of the PFP samples purified from various plant tissues [9]. Such coincidence suggests the chemical resemblance of the present in vitro partial proteolysis to the natural proteolytic process in vivo. In marked contrast to the α-subunit, the 60 kDa β-subunit was degraded into a predominant 55 kDa band and a minor 39 kDa band (Fig. 2B), which was found to be virtually invariable among the proteolytic profiles of PFP in the absence or presence of tested ligands as assessed by electrophoretic and Western blot analyses (Figs. 1–4). These results collectively indicate that the stability of the α-subunit instead of the β-subunit actually can be greatly and specifically increased by Fru-1,6-P_2.

PFP activity was assayed to evaluate the influence of proteolytic digestion on the biological functions of the subunits. Before proteolysis, the purified potato tuber PFP had an activity ratio ($V_{Fru\text{-}2,6\text{-}P2}/V_0$) of 6. After 30 min proteolytic digestion, the residual V_0, $V_{Fru\text{-}2,6\text{-}P2}$ and activity ratio of the Fru-1,6-P_2-protected sample (see Fig. 3, lane 6 for the proteolytic profile) virtually remained unchanged, indicating that Fru-1,6-P_2 not only protected the α-subunit from proteolysis but also effectively protected the biological functions of PFP subunits; in contrast, the residual V_0 of the control sample (see Fig. 3, lane 2 for proteolytic profile) became barely detectable while about 35% of initial $V_{Fru\text{-}2,6\text{-}P2}$ was retained, the activity ratio rose sharply to about 35. Apparently, these changes in V_0, $V_{Fru\text{-}2,6\text{-}P2}$ and activity ratio of the control sample were due to proteolytic degradation of the α-subunit, implying that the α-subunit plays an important role in conferring the β-subunit the greater activity and mediating regulation of PFP by Fru-2,6-P_2. This result provides additional support for the proposal of the previous report [9]. Interestingly, this implies that the 55 kDa truncated β-subunit, despite missing 5 kDa, was still fully functional.

The kinetics of Fru-1,6-P_2-induced protection of the α-subunit against proteolytic degradation was determined. The results presented in Fig. 3A and B demonstrated that the Fru-1,6-P_2 concentration was apparently proportional to the amount of the residual intact α-subunit after limited proteo-

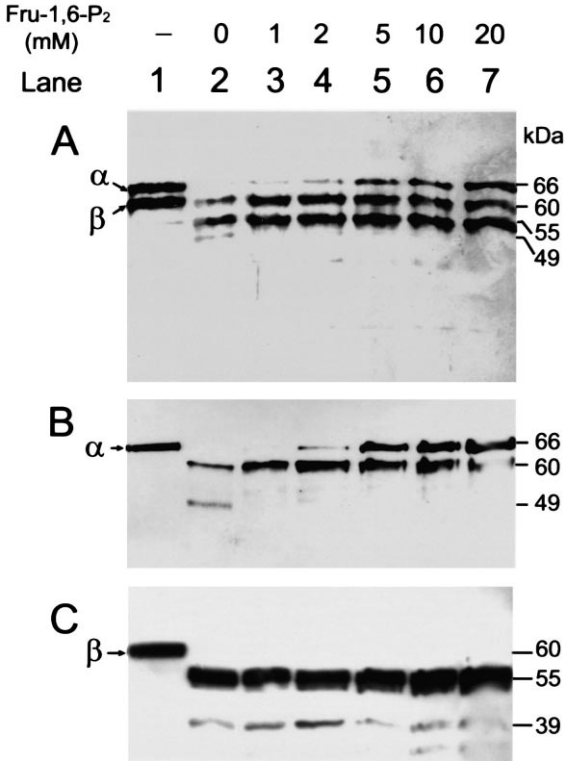

Fig. 3. Correlation between the Fru-1,6-P_2 concentration and the degree of corresponding protection on the α-subunit. Purified potato tuber PFP (100 μg/ml) was preincubated with indicated concentrations of Fru-1,6-P_2 at 30°C for 20 min, then proteolysis was carried out with 10 μg/ml subtilisin at 30°C for 30 min. Digested samples were analyzed as in Fig. 2. A: Silver stained gel. B,C: Western blot for the α (B) and β (C) subunits, respectively. Lane 1, purified potato tuber PFP (no digestion control). Indicated on the right side are the deduced molecular weights of the corresponding polypeptides (values in kDa).

lytic digestion, revealing a good correlation between the Fru-1,6-P_2 concentration and the degree of the corresponding protection on stability of the α-subunit. In contrast, Fig. 3C showed that the 55 kDa polypeptide was invariably the predominant proteolytic product of the β-subunit in all the samples tested, unambiguously demonstrating that Fru-1,6-P_2 exerted a negligible effect on the β-subunit.

In addition to Fru-1,6-P_2 and Fru-2,6-P_2, we further examined the effects of Fru-6-P and PPi on the proteolytic profiles of potato tuber PFP. The results presented in Fig. 4A and B indicate that Fru-1,6-P_2 was virtually the only ligand that significantly protected the α-subunit from proteolysis (Fig. 4, lanes 5–7). In contrast, the proteolytic profiles of the β-subunit in all the analyzed samples were strikingly similar (Fig. 4C), consistently demonstrating the high specificity of Fru-1,6-P_2-induced protection on the α-subunit. By the way, it is noteworthy (1) that Fru-1,6-P_2 alone (Fig. 4, lane 5) seemed to have the comparable degree of protection on the α-subunit to Fru-1,6-P_2/Mg^{2+} (Fig. 4, lane 6), indicating that Mg^{2+} is not indispensable for this effect; and (2) that inclusion of Fru-2,6-P_2 into the Fru-1,6-P_2/Mg^{2+} sample (Fig. 4, lane 7) did not make significant difference in the effect.

In order to gain further insight into the nature of PFP subunits, we investigated the susceptibility of the SDS-denatured PFP subunits to proteolytic digestion. The denatured PFP was found to be much more susceptible to proteolysis by subtilisin than the native enzyme. The protease concentration used to digest the denatured PFP (0.1 μg/ml) (Fig. 5) was

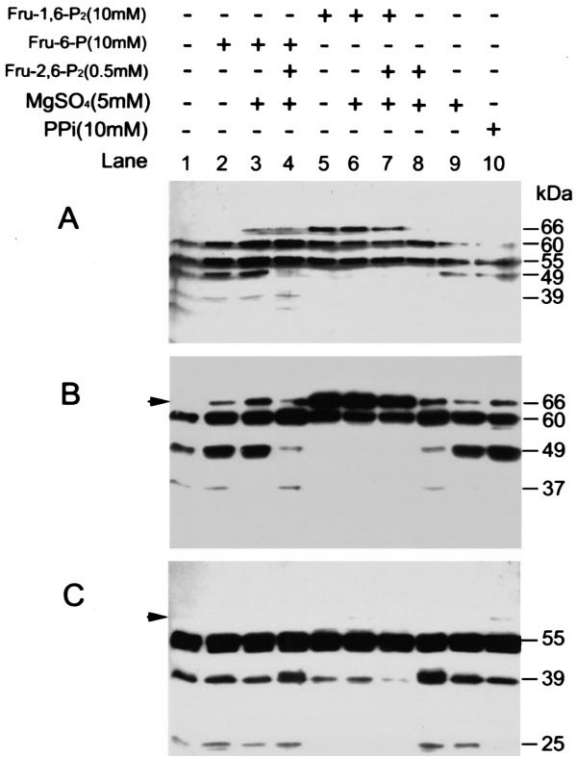

Fig. 4. Limited proteolytic digestion of PFP in the presence of different ligands. Purified potato tuber PFP (100 μg/ml) was preincubated with indicated ligand(s) at 30°C for 20 min. Then proteolysis was carried out with 10 μg/ml subtilisin at 30°C for 30 min. Digested samples were analyzed as in Fig. 3. A: Silver stained gel. B,C: Western blot for the α (B) and β (C) subunits, respectively. Indicated on the right side are the deduced molecular weights of the corresponding polypeptides (values in kDa).

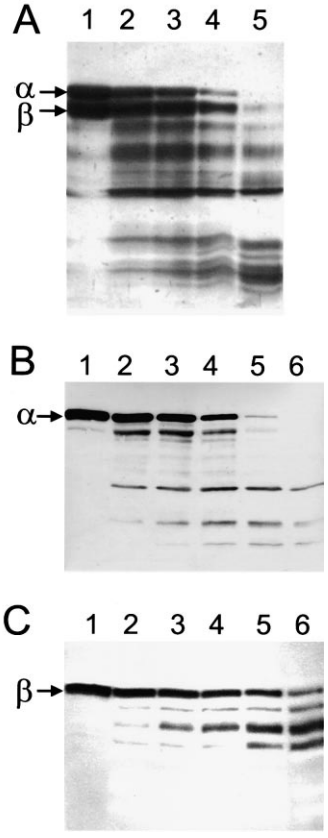

Fig. 5. Limited proteolytic digestion of the SDS-denatured PFP subunits. Purified potato tuber PFP (200 μg/ml) was denatured by 2% SDS at 100°C for 5 min, then proteolysis was carried out with 0.1 μg/ml subtilisin at 30°C for indicated lengths of time. Digested samples were analyzed as in Fig. 3. A: Silver stained gel. B,C: Western blot for the α (B) and β (C) subunits, respectively. Lanes 1–6 represent samples digested for 0, 10, 20, 30, 45 and 60 min, respectively.

about two magnitude orders smaller than that used to digest the native enzyme (10 μg/ml) (Fig. 1B). Obviously, peptide folding somehow greatly stabilizes the PFP subunits. More notably, the data presented in Fig. 5A and B demonstrated that the denatured α-subunit was still much more susceptible to proteolytic digestion than the denatured β-subunit (Fig. 5C). These results are in excellent agreement with the observations on the native proteins (Fig. 1B [9]), implying that the primary structure of the α-subunit underlies the instability of this protein.

4. Discussion

We report with convincing data the important discovery that Fru-1,6-P_2 can significantly and specifically protect the α-subunit of potato tuber PFP against proteolytic degradation by subtilisin in a concentration-dependent manner. Previously the α-subunit was found to be much more susceptible to tryptic digestion than the β-subunit [9] and instability of the α-subunit was considered part of the reasons for the existence of multiple PFP forms. The presented data now, on one hand, convince us that the instability of the α-subunit is determined to some degree by its primary structure. On the other hand, these data indicate that the stability of the α-subunit actually can be selectively increased by Fru-1,6-P_2.

Under protection of Fru-1,6-P_2, the α-subunit was much more resistant to proteolytic degradation than the β-subunit. We believe that the increased stabilization of the α-subunit solely results from its conformational changes caused by Fru-1,6-P_2, which lead to alterations in the accessibility of proteolytic sites thereon to subtilisin, since (1) to date no one has reported that Fru-1,6-P_2 is an inhibitor of subtilisin activity, and more notably (2) the β-subunit, whose susceptibility to proteolytic digestion was not affected by Fru-1,6-P_2, virtually served as an excellent control for the whole set experiments in this report. Similar phenomena of other proteins (enzymes) have been studied previously. For instance, (1) the conformation and stability of vitamin D receptor are affected by binding of its ligands [22,23]; (2) the accessibility of the phosphorylation site near the N-terminus of maize C_4 phosphoenolpyruvate carboxylase to its light-regulated serine/threonine protein kinase is altered by its feedback inhibitor L-malate [24,25]. In light of those studies, the striking specificity of Fru-1,6-P_2-induced protection of the α-subunit strongly suggests that Fru-1,6-P_2 acts through its direct interaction with the α-subunit, or in another word, that Fru-1,6-P_2 is likely a special effector of the A-subunit. Thus Fru-1,6-P_2 is possibly a dual-functional ligand of PFP: (1) as a substrate in gluconeogenic direction and (2) as an effector stabilizing the α-subunit.

By virtue of this finding, we can look into an intriguing mechanism by which the subunit composition of PFP is regulated by metabolites like Fru-1,6-P_2. Such regulatory mechanism is of obvious importance to PFP for its operation in gluconeogenesis under limiting concentration of cytoplasmic Fru-2,6-P_2. It is well known to us that PFP was studied mostly as a glycolytic enzyme in the past [26], whose activity is greatly stimulated by Fru-2,6-P_2. However, the Fru-2,6-P_2 level in plant cells is under tight control by many metabolites such as 3-phosphoglycerate (3-PGA) and dihydroxyacetone phosphate (DHAP) through activating Fru-2,6-bisphosphatase and inhibiting Fru-6-P,2-kinase [6]. When carbon metabolism shifts from glycolysis to gluconeogenesis, increases in pool size of 3-PGA and DHAP, which usually form the driving force for gluconeogenesis, will consequently cause a significant decrease in the cytoplasmic Fru-2,6-P_2 level through inhibition of its synthesis and activation of its degradation. This metabolite-mediated regulation of Fru-2,6-P_2 level in plant cells is physiologically necessary to relieve the severe inhibition of FBPase, an important enzyme for gluconeogenesis catalyzing the conversion of Fru-1,6-P_2 to Fru-6-P, and to deactivate the glycolytic enzymes. However, before the Fru-2,6-P_2 level decreases until it is low enough to activate FBPase, The Fru-1,6-P_2 pool size will presumably fluctuate significantly. During this period, increased stabilization of the α-subunit by the elevated level of Fru-1,6-P_2 and ensuing changes in subunit composition of PFP due to selective accumulation of the α-subunit would enable the enzyme to operate in gluconeogenesis or in PPi turnover despite lack of activator.

Acknowledgements: This work was supported in part by State Key Basic Research and Developmental Plan (China) Grant G1998010100 and a grant from NSF (China). Authors thank Y.K. Shen, C.H. Xu, J. Gao, M.S. Jin and other helpful colleagues for their kind helps during preparation of the manuscript. Y.H.W. is very grateful to C.L. Xu and J. Wang for their support.

References

[1] Carnal, N.W. and Black, C.C. (1979) Biochem. Biophys. Res. Commun. 86, 20–26.
[2] Kruger, N.J., Kombrink, E. and Beevers, H. (1983) FEBS Lett. 153, 409–412.
[3] Carnal, N.W. and Black, C.C. (1983) Plant Physiol. 71, 150–155.
[4] Botha, F.C., de Vries, C. and Small, J.G.C. (1988) Plant Cell Physiol. 29, 223–229.
[5] Yuan, X-H., Kwiatkowska, D. and Kemp, R.G. (1988) Biochem. Biophys. Res. Commun. 154, 113–117.
[6] Stitt, M. (1990) Annu. Rev. Plant Physiol. 41, 153–185.
[7] Kruger, N.J. and Dennis, D.T. (1987) Arch. Biochem. Biophys. 256, 273–279.
[8] Yan, T.F.Y. and Tao, M. (1984) J. Biol. Chem. 259, 5087–5092.
[9] Cheng, H.F. and Tao, M. (1990) J. Biol. Chem. 265, 2173–2177.
[10] Theodorou, M.E., Cornel, F.A., Duff, S.M.G. and Plaxton, W.C. (1992) J. Biol. Chem. 267, 21901–21905.
[11] Loulakakis, K.A. and Roubelakis-Angelakis, K.A. (1992) Planta 187, 322–327.
[12] Glotzer, A.H., Murray, A.W. and Kirschner, M. (1991) Nature 349, 132–138.
[13] Li, L, Pan, L. and Xu, G.J. (1990) Acta Biochim. Biophys. Sin. 23, 277–284.
[14] Wang, Y.H., Chen, J.Z. and Shi, J.N. (1991) Acta Phytophysiol. Sin. 17, 207–213.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Heukeshoven, J. and Dernick, R. (1988) Electrophoresis 9, 28–32.
[18] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–253.
[20] Wang, Y.H., Duff, S.M.G., Lepiniec, L., Cretin, C., Sarath, G., Condon, S.A., Vidal, J., Gadal, P. and Chollet, R. (1992) J. Biol. Chem. 267, 16759–16762.
[21] Wang, Y.H. and Chollet, R. (1993) FEBS Lett. 328, 215–218.
[22] Bemd, G.T., Pols, H.A.P., Birkenhager, J.P. and Leeuwen, T.M. (1996) Proc. Natl. Acad. Sci. USA 93, 10685–10690.
[23] Vaisanen, S., Rouvinen, J. and Maenpaa, P.H. (1998) FEBS Lett. 440, 203–207.
[24] Wang, Y.H. and Chollet, R. (1993) Arch. Biochem. Biophys. 304, 496–502.
[26] Theodorou, M.E. and Plaxton, W.C. (1993) Plant Physiol. 101, 339–344.