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Posttranslational Modification of 6-Phosphofructo-1-Kinase in *Aspergillus niger*

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Two different enzymes exhibiting 6-phosphofructo-1-kinase (PFK1) activity were isolated from the mycelium of *Aspergillus niger*: the native enzyme with a molecular mass of 85 kDa, which corresponded to the calculated molecular mass of the deduced amino acid sequence of the *A. niger pfkA* gene, and a shorter protein of approximately 49 kDa. A fragment of identical size also was obtained in vitro by the proteolytic digestion of the partially purified native PFK1 with proteinase K. When PFK1 activity was measured during the proteolytic degradation of the native protein, it was found to be lost after 1 h of incubation, but it was reestablished after induction of phosphorylation by adding the catalytic subunit of cyclic AMP-dependent protein kinase to the system. By determining kinetic parameters, different ratios of activities measured at ATP concentrations of 0.1 and 1 mM were detected with fragmented PFK1, as with the native enzyme. Fructose-2,6-biphosphate significantly increased the V_{max} of the fragmented protein, while it had virtually no effect on the native protein. The native enzyme could be purified only from the early stages of growth on a minimal medium, while the 49-kDa fragment appeared later and was activated at the time of a sudden change in the growth rate. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sequential purifications of PFK1 enzymes by affinity chromatography during the early stages of the fungal development suggested spontaneous posttranslational modification of the native PFK1 in *A. niger* cells, while from the kinetic parameters determined for both isolated forms it could be concluded that the fragmented enzyme might be more efficient under physiological conditions.

The filamentous fungus *Aspergillus niger* belongs to a group of the most important commercial organisms, since it can convert up to 80% of substrate such as sucrose to a final product, i.e., citric acid. A crucial prerequisite for overflow of citric acid from *A. niger* cells is an increased level of tricarboxylic acid cycle intermediates (19, 24) caused by anaplerotic reactions. One of the events that replenish tricarboxylic acid cycle intermediates is an enhanced metabolic flow through the glycolysis due to altered regulatory properties of 6-phosphofructo-1-kinase (PFK1), which is normally a key enzyme in control of glycolysis. During the cultivation of *A. niger* on a medium with a high glucose concentration, which enables a high yield of citric acid, glycolysis is no longer regulated at the fructose-6-phosphate step but regulation is shifted down to glyceraldehyde-3-phosphate dehydrogenase (13, 31).

ATP-dependent PFK1 (E.C. 2.7.1.11.) catalyzes the phosphorylation of fructose-6-phosphate from Mg-ATP to form fructose-1,6-biphosphate and Mg-ADP. The allosteric mechanism is one of the regulation strategies used by PFK1 to control catalysis in this normally highly regulated step of glycolysis. Activators increase, whereas inhibitors decrease, substrate binding affinity and concomitantly determine overall enzyme activity.

The enzyme can also be regulated by the covalent modification of the protein molecule. Phosphorylation of phosphofructo-1-kinases has been observed in many species (15, 37). PFK1 enzymes from various mammalian tissues (42), mollusks (4), and some nematodes (10) have been reported to be positively regulated by phosphorylation, while in the microbial world such control of the enzyme activity seems to be less common.

Because of the importance of 6-phosphofructo-1-kinase in regulating the primary metabolism of *A. niger*, its kinetic properties have been extensively studied (1, 8, 9, 20, 22).

PFK1 partially purified from *A. niger* cells was found to be up regulated by fructose-2,6-biphosphate (9), while citrate and ATP were reported to have an inhibitory effect on the enzyme (1, 20). Besides well-known organic effectors (AMP and fructose-2,6-biphosphate), ammonium ions as univalent cations were described to have a role in PFK1 regulation. It was suggested that they might antagonize inhibition by citrate in vivo (8, 9), which might be functionally linked with the lack of glycolytic control at the level of fructose-6-phosphate during citric acid accumulation (31). Another type of PFK1 regulation was observed in *A. niger* (22). It was suggested that the enzyme could be activated by the phosphorylation that is mediated by cyclic AMP (cAMP)-dependent protein kinase. While the enzyme that was phosphorylated had a molecular mass of about 48 kDa (22), the deduced amino acid sequence of the *pfkA* gene isolated from *A. niger* (EMBL accession number Z79690) indicated that the M_r of the native PFK1 should be 85,770, suggesting that specific proteases might be responsible for the in vivo processing of PFK1 in the fungal cells. In the present study we report on the posttranslational modification of PFK1, which leads to significant changes in allosteric regulation of the enzyme activity.

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MATERIALS AND METHODS

Microorganism. An *A. niger pfkA* multicopy strain (35) designated A459 and stored in the Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia, was used throughout all of the experiments. The strain was

obtained from the Section of Molecular Genetics of Industrial Microorganisms, Wageningen Agricultural University, Wageningen, The Netherlands. For inoculum preparation the spores were harvested from 7-day-old wort agar slants and suspended in 25 ml of 0.1% (wt/vol) Tween 80 solution.

Medium. The medium for growing the mycelium consisted of, per liter, 20 g of glucose, 5 g of $(NH4)_2SO_4$, 5 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of NaCl with the pH adjusted to 6.0. The mycelium was grown in a stirred tank bioreactor (IS-100; Infors, Bottmingen, Switzerland); 5 liters of the medium was inoculated with approximately 10^9 spores. The growth temperature was 30°C, and the medium was aerated with 5 liters of air per min. After the cells were taken out of the bioreactor, they were harvested by suction filtration on a linen cloth, extensively washed with ice-cold extraction buffer (50 mM sodium phosphate buffer [pH 7.8] with 5 mM dithioerythritol [DTE]), and immediately frozen under liquid nitrogen. The amount of dry biomass was determined gravimetrically after the washed cells were dried at 105°C to a constant weight.

Homogenate preparation. For homogenate preparation the frozen mycelium (about 100 g [dry mass]) was disrupted in a glass bead disintegrator (Braun, Melsungen, Germany) and the frozen powder was dissolved in 20 ml of extraction buffer containing 10 μl of protease inhibitor cocktail (Sigma Chemical Co., St. Louis, Mo.). After centrifugation at 15,000 rpm (SE-12 rotor; Sorval, Wilmington, Del.) for 15 min in a refrigerated centrifuge (Sorvall), the homogenate contained more than 5 mg of soluble protein per ml.

Partial purification of PFK1. The proteins in the crude enzyme extract were precipitated with ammonium sulfate, and a fraction between 50 and 75% saturation was taken for further purification. After the sample was dissolved and desalted on a Fast desalting column (Pharmacia, Uppsala, Sweden) with 50 mM sodium phosphate buffer (pH 8.0) in the presence of 5 mM DTE, it was applied to a triazyne dye affinity column equilibrated with sodium phosphate buffer. The Procion dye MX 4GD (Colors & Fine Chemicals, ICI, London, United Kingdom) was used as a ligand and was coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) as described by Atkinson et al. (2). After the sample was applied to the column containing 10 ml of matrix, unbound enzymes were removed by extensive washing with buffer. PFK1 was eluted from the column with 1 ml of 6 mM fructose-6-phosphate in buffer. Combined fractions containing activity were dialyzed overnight against buffer with 20% (vol/vol) glycerol and stored at 4°C. The enzyme remained active for several weeks. When the PFK1 fragment was purified, 10 μl of phosphatase inhibitor cocktail (Sigma) was added to the homogenate as well, and 100 mM sodium phosphate buffer (pH 7.8) instead of 50 mM buffer (pH 8.0) was used throughout all of the purification steps. After the final purification step (affinity chromatography), 5 mg of bovine serum albumin per ml was dissolved in the fractions exhibiting PFK1 activity.

Enzyme assay. 6-Phosphofructo-1-kinase activity was measured spectrophotometrically at 340 nm (DU-600 spectrophotometer; Beckman Instrument Co., Berkeley, Calif.), essentially as reported previously (35), using a coupled reaction system. Unless otherwise stated, the assay mixture contained, in a final volume of 1 ml, 25 mM Tris-HCl buffer (pH 7.8), 5 mM DTE, 100 mM KCl, 5 mM $MgCl_2$, 20 μl of enzyme sample, 1 mM ATP, 0.2 mM NADH, 0.9 U of aldolase (Roche Molecular Biochemicals, Indianapolis, Ind.) per ml, 5 U of triosephosphate isomerase per ml, and 2.5 U of glycerol-3-phosphate dehydrogenase (Roche Molecular Biochemicals) per ml. Before use the auxiliary enzymes were dialyzed against 25 mM Tris-HCl buffer (pH 7.8)–1 mM DTE overnight at 4°C, with one change of buffer after 8 h. The activity of the PFK1 fragment was determined in a buffer containing 5 mg of albumin per ml. All kinetic data presented are averages of results obtained in three or more independent measurements. Total protein concentrations in the samples were determined by bicinchoninic acid protein assays (36) performed with the Sigma kit.

Electrophoresis. Electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) was performed as described by Laemmli (21) in a minigel system (LKB, Bromma, Sweden). The molecular weight markers bovine albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), and α-lactalbumin (14,200) (Sigma) were used as standards.

Digestion of native PFK1 with proteases. An aliquot of 15 μl of partially purified native PFK1 (115 μg of protein/ml) in 50 mM sodium phosphate buffer (pH 8.0)–5 mM DTE was incubated with 5 μl of specific proteinase dissolved in 50 mM sodium phosphate buffer (pH 7.0). Subtilisin A and proteinase A were used at 1 mg/ml, while the proteinase K concentration was lower (0.1 mg/ml). Native PFK1 was digested for 1 h at 30°C (Thermomixer; Eppendorf). The reaction was stopped by adding the specific protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (final concentration, 1 mM) and pepstatin A (1 μM).

In vitro proteolytic degradation of native PFK1 followed by phosphorylation of the PFK1 fragment. For monitoring the successive proteolytic degradation of the native protein by measuring the residual enzyme activity, 10-fold-higher volumes than used in the previous experiment were originally taken. After 1 h of incubation at 30°C (Thermomixer; Eppendorf), PMSF at a final concentration of 1 mM was added to the system. Finally, 50 U of the catalytic subunit of cAMP-dependent protein kinase (PKA) (Promega, Madison, Wis.) together with 0.2 mM ATP and 5 mM $MgCl_2$ was introduced into the remaining 140 μl of PFK1 digest to induce the phosphorylation of the fragment. For measuring enzyme activity, 20-μl aliquots were periodically taken from the incubation mixture and measured as described under "Enzyme assay" above.

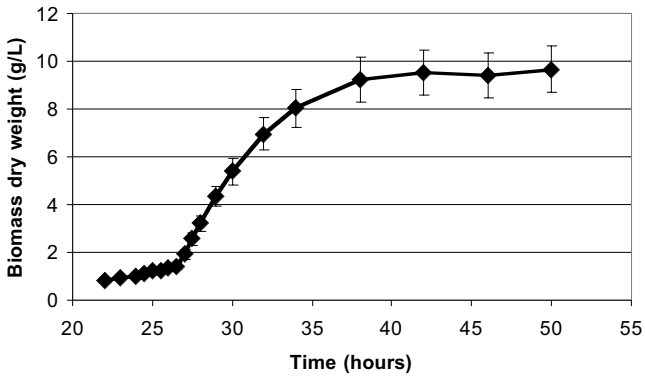


FIG. 1. Growth curve of *A. niger* mycelium in a minimal medium with ammonium ions as the sole nitrogen source. Error bars indicate standard errors of the means.

RESULTS

Growth curve. In the medium with 2% glucose and a high initial ammonium concentration, rapid growth of *A. niger* occurred, and within 35 to 40 h of growth the mycelium entered the stationary phase. However, two different specific growth rates were observed during the initial phase (Fig. 1). Up to about 26 h the biomass increased with a specific growth rate of about 0.09 h^{-1}, which is equal to a doubling time of 7.5 h, while later the specific growth rate increased to 0.19 h^{-1} and concomitantly the doubling time was reduced to 3.6 h. The specific growth rate then gradually decreased, and cells entered the stationary phase at approximately 42 h, when there was about 10 g (dry mass) of mycelium per liter of substrate. Characteristically, the change from one growth rate to another appeared instantly without any reduction and reestablishment of growth rate, which would appear in a typical diauxic growth curve. Morphologically no significant differences in the shape of hyphae or type of branching could be observed among the mycelium growing at lower or higher growth rates.

Enzymes isolated by affinity chromatography. When mycelium was grown for 16 h in a bioreactor and PFK1 was isolated by affinity chromatography as the final stage of purification, a partially purified enzyme exhibiting PFK1 activity that gave a major protein band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) corresponding to an M_r of about 85,000 was obtained (Fig. 2). According to the data on the nucleotide sequence of *A. niger* 6-phosphofructo-1-kinase (EMBL accession number Z79690), the molecular mass the native PFK1 was determined to be 85,770 Da from the deduced amino acid sequence. From a cell extract prepared from 4-h-older mycelium, an additional protein was isolated as observed on SDS-PAGE, apart from the native PFK1. In an extract from 24-h-old mycelium, a protein band with an approximate M_r of

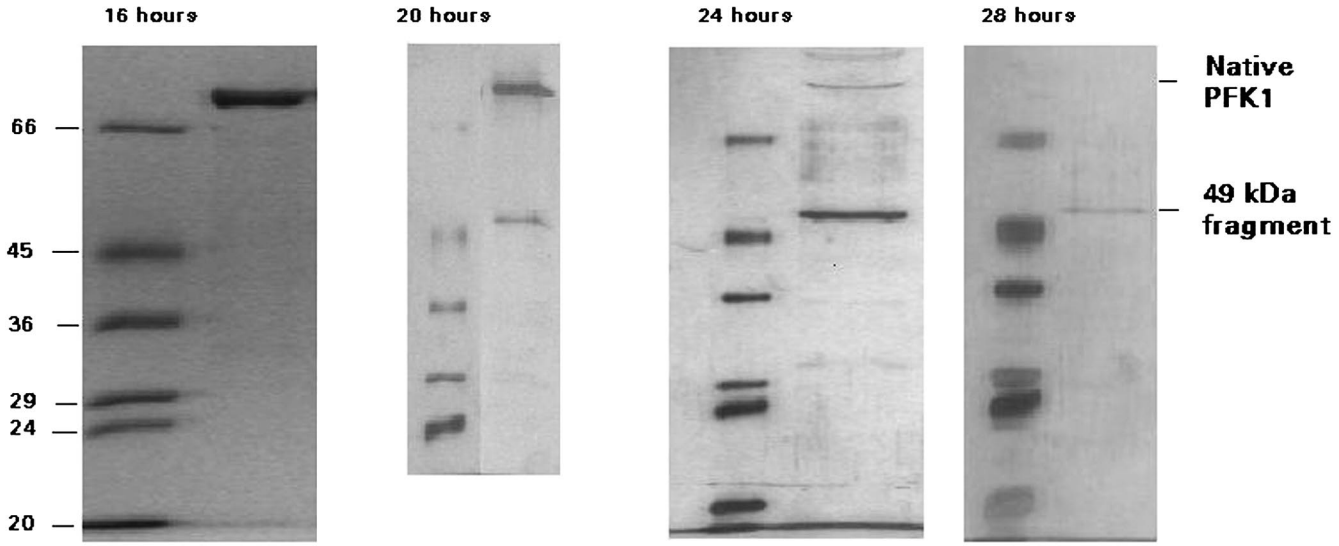


FIG. 2. SDS-PAGE of partially purified PFK1 from the mycelium at different ages, after elution from the affinity column. In the left lane of each gel the following standards are shown: bovine serum albumin (molecular weight, 66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and trypsin inhibitor (20,000) (all purchased from Sigma).

49,000 became predominant, while the band representing the native protein could hardly be observed. In the isolate from 28-h-old mycelium, again only one protein was predominant, exhibiting distinct PFK1 activity, while on SDS-PAGE the protein band with an M_r of 49,000 was detected. (Fig. 2).

Proteolytic degradation of native PFK1 in vitro. To find the protease that could be responsible for cleavage of the native protein to yield an active fragment of about 49 kDa, isolated native protein was digested with various proteases. After processing of the native protein with bacterial subtilisin A, a fragment of approximately 66 kDa predominated on SDS-PAGE, while proteinase A of yeast origin cleaved the native protein to a major fragment of about 45 kDa. Only when the PFK1 was incubated with proteinase K for 1 h was a fragment of about 49 kDa formed, as well as some other, shorter, proteins (Fig. 3).

Proteolytic degradation of the native PFK1 with proteinase K and phosphorylation with PKA. The degradation of the native PFK1, followed by activation by phosphorylation, was also examined by an in vitro procedure. When partially purified native PFK1 was digested with 25 μg of proteinase K per ml, PFK1 activity was lost within 1 h. After PMSF, a strong inhibitor of serine proteases, was added, PKA, ATP, and magnesium ions were introduced into the incubation system. Within next 30 min the PFK1 activity reestablished, showing different ratios of activities measured in the presence of 0.1 and 1 mM ATP compared to those of the native PFK1 protein. Fructose-2,6-biphosphate at 8 μM significantly activated the fragmented protein after proteolytic degradation, while it had virtually no effect on the native protein. Yet, after about 1 h of incubation of the enzyme with PKA, the activity started to decline again, which indicated the instability of the fragment under the dilute noncellular conditions (Fig. 4).

Kinetics. (i) Native 85-kDa PFK1. The native PFK1 was eluted from the affinity column as a single peak, which showed a single protein band on SDS-PAGE electrophoresis after silver staining, with an M_r of 85.000 (Fig. 2). By measuring the kinetic parameters with partially purified native PFK1, a sigmoidal increase in activity against higher concentrations of fructose-6-phosphate was found (see Fig. 6B), with maximal velocity reached at about 10 mM and a K_m of 4.5 mM substrate. The corresponding Hill coefficient (Fig. 5) was 3.6, which implies good cooperativity in substrate binding. ATP, which often acts as a feedback inhibitor on eukaryotic PFK1, was observed to increase enzyme velocity in the range from 0.1 to 1 mM, while at higher concentrations the activity was strongly reduced. At about 2 mM, which is close to the physiological value detected in *A. niger* cells (35), the enzyme showed only about one-third of its maximal activity (Fig. 6A). Among several effectors of eukaryotic PFK1, fructose-2,6-biphosphate is believed to have the most prominent activating role. It was found to be a strong inducer of the native *A. niger* enzyme as well, since it increased the affinity of the enzyme for the substrate. By adding the effector in micromolar concentrations, the K_m value was reduced below the value of 1 mM; however, there was no convincing evidence that the presence of fructose-2,6-biphosphate could increase the maximum velocity of the enzyme. Thus, at 12 mM fructose-6-phosphate, similar activities were detected regardless of the concentration of fructose-2,6-biphosphate in the system (Fig. 6B). No effect of fructose-2,6-biphosphate on the activation and inhibition of the enzyme by ATP was observed at substrate concentrations that enabled maximal velocities.

(ii) The 49-kDa PFK1 fragment. When PFK1 was isolated from the mycelium grown for 28 h in a minimal medium, a PFK1 fragment was obtained by using the same purification method as for the native protein. The enzyme was again eluted from the affinity column as a single peak, and on SDS-PAGE only one major protein band was detected, corresponding to an M_r of about 49,000 (Fig. 2). The fragmented PFK1 has been found to be extremely unstable upon dilution, but it was possible to retain its activity by using a buffer with increased ionic strength (100 mM phosphate buffer) and by adding proteins

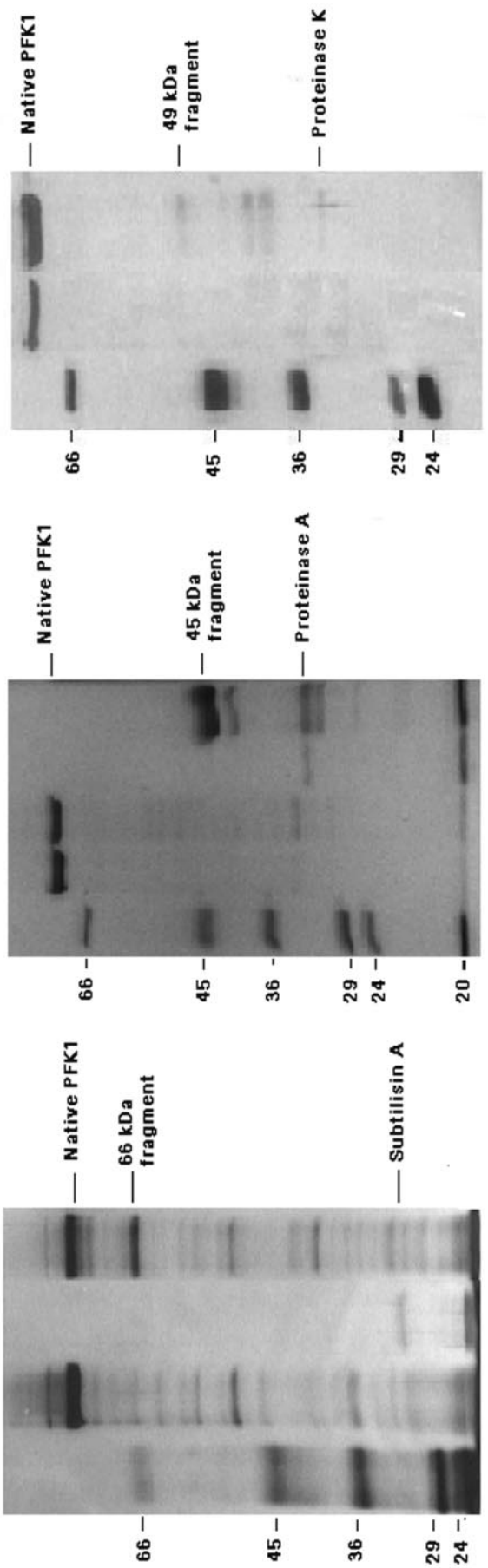


FIG. 3. SDS-PAGE of the partially purified native PFK1 protein digested with specific proteases. Subtilisin A (left panel) and proteinase A (middle panel) were used at 250 μg/ml, while proteinase K (right panel) was used at a lower concentration (25 μg/ml). The same standard markers as in Fig. 2 were used.

(albumin) to a final concentration of 5 mg/ml immediately after the elution of the fragment from the affinity gel.

There were some significant differences observed between the kinetic parameters of the PFK1 fragment and the native protein. First, the affinity constant (K_m) of the fragment was lower, and a value of 2.45 mM substrate was determined from the Hill plot (Fig. 5). From the same plot, a slightly lower level of cooperativity in substrate binding was calculated, giving a value of 2.95. However, the most significant results were obtained by adding fructose-2,6-biphosphate to the system. The effector at 4 μM efficiently prevented a strong inhibition of the fragment by ATP, which appeared in the range of 0.1 mM ATP upwards if only enzyme, substrate, Mg^{2+} ions, and ATP were in the system (Fig. 7A). Fructose-2,6-biphosphate exhibited another stimulating effect on the fragment. Besides increasing the affinity of the enzyme for the substrate, it significantly raised the maximal velocity of the enzyme. With 2 μM fructose-2,6-biphosphate in the system the activities were nearly doubled, while in the presence of 8 μM effector, up to threefold-higher activities were detected (Fig. 7B).

DISCUSSION

The sequence analysis of numerous bacterial and mammalian 6-phosphofructo-1-kinases indicated that eukaryotic enzymes evolved by a process of tandem gene duplication and fusion to yield a protein that is more than double the size of prokaryotic PFK1 (33). Although PFK1 is poorly regulated in bacteria (32), more allosteric sites developed in higher organisms (16). Such evolutionary processes resulted in the divergence of catalytic and effector binding sites of a prokaryotic ancestor (16, 27). By proteolytic cleavage of a part of such an enzyme, activity can be maintained; however, changes in allosteric properties are feasible. In the past many allosteric enzymes have been modified by limited proteolysis, and several 6-phosphofructo-1-kinases were included. Removal of 17 amino acid residues from the carboxyl terminus of *Staphylococcus aureus* PFK1 dramatically decreased inhibition by ATP (41). Emerk and Frieden (3) showed that trypsin cleaved rabbit muscle PFK1 into apparently two halves and had no effect on enzyme regulatory properties, while limited digestion of the same enzyme with subtilisin resulted in its inactivation (6). In fungal systems the yeast enzyme was studied in detail (5), and a complete loss of catalytic activity was observed after the exposure of PFK1 to a variety of proteolytic enzymes (18). Although no data about the proteolytic cleavage of *A. niger* PFK1 are available in the literature, our results on the digestion of the native enzyme by proteinase K showed that the removal of a part of the molecule also caused its deactivation.

On the other hand, 6-phosphofructo-1-kinases were often reported to be regulated by covalent modification in a form of phosphorylation-dephosphorylation of the protein molecule (37). Such a type of regulation was reported mostly for higher eukaryotes (14), where the kinase responsible for the phosphorylation of the protein molecule was found to be cAMP dependent. There are fewer reports about the phosphorylation of PFK1 in the microbial world. In *Dictyostelium discoideum* the treatment of the enzyme with cAMP-dependent protein kinase led to phosphorylation of the protein without a change in activity (28), and a similar situation was observed for yeast PFK1 (11). Recently the activation of phosphofructo-1-kinase by phosphorylation was observed in the bacterium *Myxococcus*

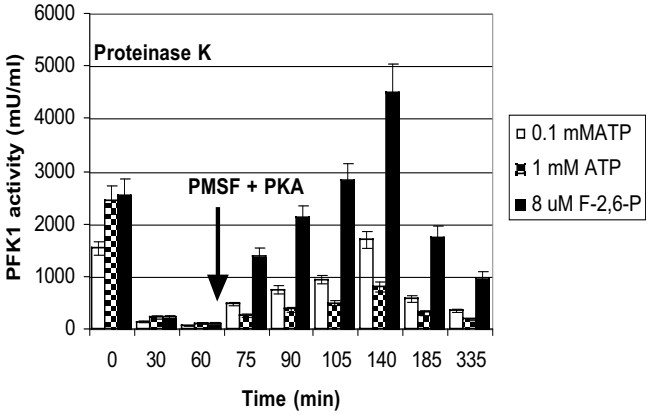


FIG. 4. When the native PFK1 was subjected to treatment with proteinase K (25 μg/ml) and the activity was measured during the incubation, virtually all activity was lost within 1 h. After addition of the serine protease inhibitor PMSF (1 mM) and the catalytic subunit of cAMP-dependent protein kinase (PKA) (10 U) to the system (arrow), the activity gradually reestablished. The activities were measured first with 0.1 mM ATP, then ATP was added to a final concentration of 1 mM, and finally fructose-2,6-biphosphate (F-2,6-P) was introduced into the system to 8 μM. The initial substrate (fructose-6-phosphate) concentration was 8 mM. The results are the means ± standard errors of the means from triplicate determination.

xanthus, with the serine/threonine kinase Pkn4 found to be responsible for covalent modification (29).

However, there were no reports of a spontaneous proteolytic degradation of PFK1 which deactivated the enzyme, while the fragment retained its activity after phosphorylation. In the experiment with proteinase K, which belongs to a family of serine proteases, a relatively low concentration of protease (25 μg/ml) was used in order to obtain a fragment of appropriate length. After in vitro phosphorylation of fragmented PFK1 by the commercially available catalytic subunit of cAMP-dependent protein kinase, the PFK1 activity was readily restored, whereas measurement of activity at 0.1 and 1 mM ATP as well as in the presence of 8 μM fructose-2,6-biphosphate clearly showed different kinetic properties of the isoenzymes. These

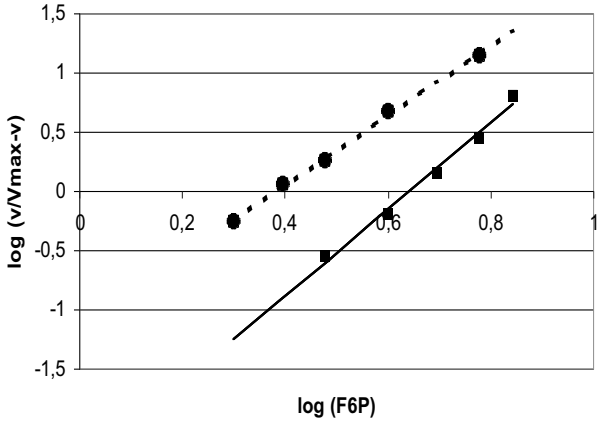
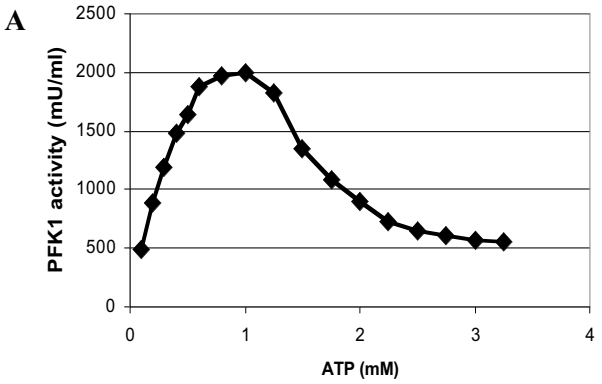


FIG. 5. Hill plot of PFK1 activities of the fragmented enzyme (●) and native protein (■) determined at different fructose-6-phosphate (F6P) concentrations without any effectors present in the measuring system.



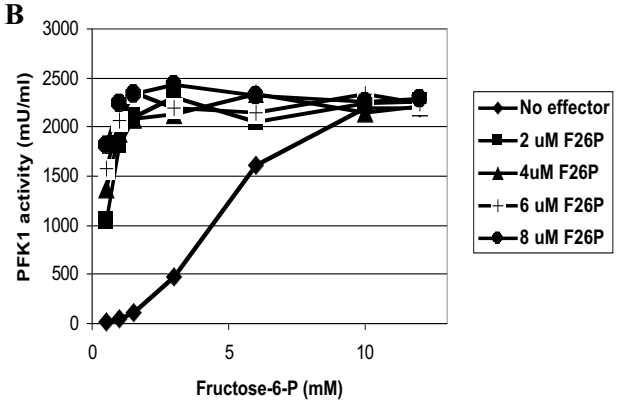


FIG. 6. Kinetic parameters of the native PFK1. (A) ATP profile of the native enzyme as detected in the presence of 8 mM fructose-6-phosphate in the system with no other effectors added. (B) Activities measured at different concentrations of fructose-2,6-biphosphate (F26P).

data are in accordance with the results obtained by measuring basic kinetic parameters, where fructose-2,6-biphosphate had a more pronounced effect on the fragment then on the native protein. Another feature of fragmented PFK1 can be observed from the results shown in Fig. 4. After approximately 1 h of incubation at 30°C, the enzyme activity started to decline rapidly. It was indicative of an instability, when the enzyme is removed from the cells. Since often only the loss of activity was observed, despite an SDS-PAGE protein band of identical molecular mass being detected (data not shown), deactivation of the enzyme was most probably due to disintegration of the tetrameric holoenzyme to dimers and monomers and not to digestion of protein by proteases. Deactivation was found to be accelerated by dilution; therefore, immediately after the final purification step for fragmented PFK1, albumin was added to the buffer at a final concentration of 5 mg/ml. The native protein appeared to be more resistant to dilution, yet particular care was taken during the kinetic measurements to keep the concentration of proteins in the assay system above 1 μg/ml.

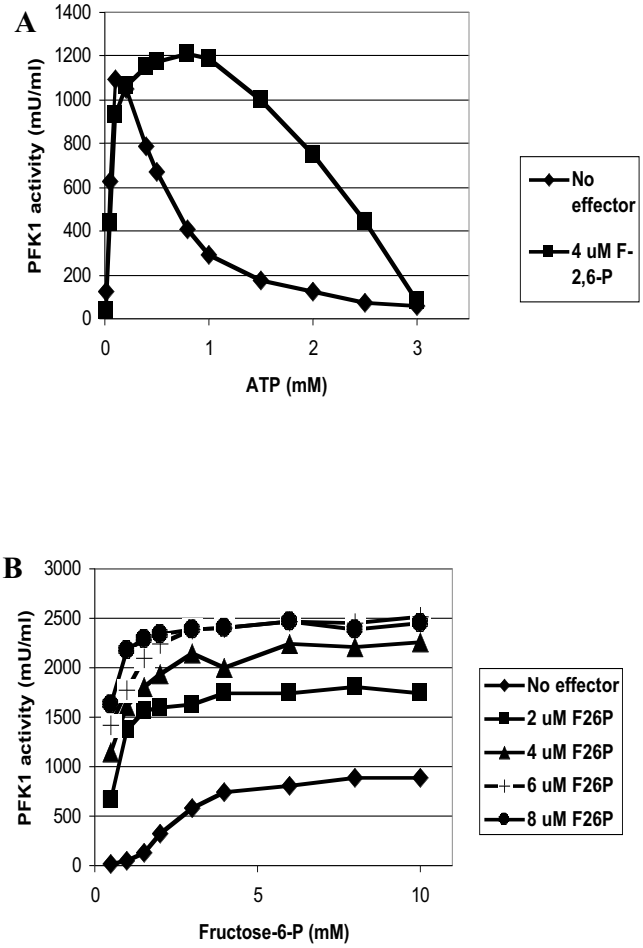


FIG. 7. Kinetic parameters of the partially purified 49-kDa fragment. (A) ATP profile of the fragment as detected in the presence of 8 mM fructose-6-phosphate in the system with and without 4 μM fructose-2,6-biphosphate (F-2,6-P) present. (B) Activities measured at different concentrations of fructose-2,6-biphosphate (F26P).

Inactivation of 6-phosphofructo-1-kinase by dissociation at low protein concentrations was previously reported and well characterized for the rat liver enzyme (34).

In *A. niger* the activity of specific proteases could be induced by a slight drop of the intracellular pH, which was recently reported to occur in a strain with impaired H^+-ATPase activity during growth in medium with ammonium salt as the sole nitrogen source (12). The data on the appearance of fragmented PFK1 presented in Fig. 2 indicate that the native protein was gradually digested to a shorter form in a period of 6 to 8 h. Under slightly acidic intracellular conditions, some proteases might exhibit low activities (43), just enough to cleave the native protein to the appropriate length. In vivo proteolytic degradation of the 85-kDa protein therefore seems to be a very delicate process, which depends both on the concentration of active serine protease and on the intracellular pH. On the other hand the activation of fragmented PFK1 by phosphorylation is believed to occur instantly at about 26 h. The phosphorylation and activation of a PFK1 of about 48 kDa from *A. niger* by cAMP-dependent protein kinase was previously shown in our lab by using labeled [γ-^{32}P]ATP (22). To induce cAMP-dependent protein kinase activity, a sudden increase in cAMP concentration must be stimulated. In *A. niger* a spontaneous rise in intracellular cyclic AMP was observed at the early stages of growth under conditions enabling citric acid excretion. The amount of cAMP formed was found to depend on the initial sucrose concentration in the medium; more specifically, under higher-sucrose conditions, the cAMP peak appeared earlier and was higher (7). In *A. niger*, a prominent elevation of cAMP levels could also be induced by a hypoosmotic shock. Simultaneously, a significant increase in the specific activity of 6-phosphofructo-1-kinase was detected in the cell-free homogenate (23). Intracellular acidification was often reported to cause an increase in cAMP concentration in fungal cells. The phenomenon was well studied in *S. cerevisiae* (38, 39) and was reported also for *Neurospora crassa* (30) and *Mucor racemosus* (40). Since in some *A. niger* strains a slight drop of intracellular pH was also reported to occur at the early stages of growth (12, 17), this might be responsible for triggering the cAMP synthesis.

Kinetic measurements obtained for the partially purified native protein and fragment showed some similarities but also significant differences. Both enzymes exhibited relatively good cooperativity in substrate binding; however, the fragment appeared to have a higher affinity for fructose-6-phosphate than the native protein. Fructose-2,6-biphosphate, as a potent inducer of eukaryotic PFK1, significantly increased the affinity of both enzymes. It had a strong stimulatory effect on the maximal velocity of the fragment but left the native protein unaffected. Since in the fragment the effector significantly increased V_{max}, higher activities were detected at low substrate concentrations in the presence of fructose-2,6-phopshate than at fructose-6-phosphate concentrations of several millimolar without the presence of the effector. On the other hand, this was not the case with the native enzyme, where at low substrate concentrations the activities never exceeded the V_{max} values of unaffected protein. This phenomenon might have an important physiological consequence, since in *A. niger* the intracellular concentration of fructose-6-phosphate was determined to be 0.23 mM and that of fructose-2,6-biphosphate was 6.7 μM (35), which suggests that the enzyme works at activities far from maximal but in a range where a slight change of substrate concentration might have significant consequences for the level of enzyme activity.

Posttranslational modification was also accomplished in an in vitro experiment. After digestion of partially purified native protein by serine protease (proteinase K), the PFK1 activity was reestablished upon the phosphorylation of the fragmented protein. By measuring the activities at 0.1 and 1 mM ATP and after the addition of 8 μM fructose-2,6-biphosphate to the system, characteristic ratios of the activities for the native protein and for the PFK1 fragment that corresponded well to the basic measurements of the kinetic parameters were observed. It is worth noting that at some point higher PFK1 activities were obtained for the fragmented protein in the presence of fructose-2,6-biphosphate than for the native protein. Although the fragment originated from a defined amount of the native protein, the conversion was unlikely to be complete, and yet higher relative activities were detected with the fragment,

which again implied better efficiency of the phosphorylated, cleaved protein under the specific conditions used.

During the growth of *A. niger* cells in medium with ammonium salts as the sole nitrogen source, the correlation between the increase in biomass and time was surprisingly linear for the first 24 h, indicating that the specific growth rate must have been steadily decreasing. This situation was repeated later, after the sudden increase of the growth rate, where again no typical exponential growth curve could be observed and characteristically, after the initial boost, the specific growth rate gradually decreased. Similarly, an instant increase in the growth rate of *A. niger* during the early stages of growth in a medium of similar composition was reported previously (25), while no PFK1 activity, measured at 0.1 mM ATP, could be detected immediately after spore germination but activity appeared later after the increase in the growth rate (26). In *A. niger* growing in a minimal medium with a higher initial sucrose concentration, not only the specific growth rate but also the specific rate of citric acid excretion were observed to decline during the second half of fermentation (23). An atypical growth curve is indicative of significant changes in metabolism, and proteolytic degradation of the native 6-phosphofructo-1-kinase might be responsible for the initial decrease during the first 24 h. After the phosphorylation of fragmented PFK1, the highly active enzyme could reestablish the metabolic flow through glycolysis, which might result in the sudden boost of biomass formation.

Fragmented PFK1 is believed to be activated by phosphorylation only once during batch growth, and no more active enzyme was synthesized later. On the other hand, the native protein which was constitutively formed might be simultaneously degraded if the pH_i remained low and intracellular proteases remained active. In the case of no extra induction of cAMP formation, specific PFK1 activity gradually decreased due to the turnover of fragmented protein, which could be a cause for a decrease in the specific growth rate at the later stage of fermentation.

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