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Citrate Inhibition-Resistant Form of 6-Phosphofructo-1-Kinase from *Aspergillus niger*

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Two forms of *Aspergillus niger* 6-phosphofructo-1-kinase (PFK1) have been described recently, the 85-kDa native enzyme and 49-kDa shorter fragment that is formed from the former by posttranslational modification. So far, kinetic characteristics have never been determined on the enzyme purified to near homogeneity. For the first time, kinetic parameters were determined for individual enzymes with respect to citrate inhibition. The native 85-kDa enzyme was found to be moderately inhibited by citrate, with the K_i value determined to be 1.5 mM, in the system with 5 mM Mg^{2+} ions, while increasing magnesium concentrations relieved the negative effect of citrate. An identical inhibition coefficient was determined also in the presence of ammonium ions, although ammonium acted as a strong activator of enzyme activity. On the other hand, the shorter fragment of PFK1 proved to be completely resistant to inhibition by citrate. Allosteric citrate binding sites were most probably lost after the truncation of the C-terminal part of the native protein, in which region some binding sites for inhibitor are known to be located. At near physiological conditions, characterized by low fructose-6-phosphate concentrations, a much higher efficiency of the shorter fragment was observed during an in vitro experiment. Since the enzyme became more susceptible to the positive control by specific ligands, while the negative control was lost after posttranslational modification, the shorter PFK1 fragment seems to be the enzyme most responsible for generating undisturbed metabolic flow through glycolysis in *A. niger* cells.

Phosphofructokinase (EC 2.7.1.11) catalyzes the first essentially irreversible reaction of glycolysis, the phosphorylation of fructose-6-phosphate to form fructose-1,6-bisphosphate using MgATP as the phosphoryl donor and releasing MgADP as a by-product. The eukaryotic enzyme has complex regulatory properties that are mediated by the interaction of allosteric ligands with a number of distinct binding sites and presents one of the principal regulatory steps in glycolysis (4, 30). Eukaryotic phosphofructo-1-kinases (PFK1s) are more than twice the size of prokaryotic PFKs and are under regulatory control by a wider array of effectors than seen with the simpler bacterial enzymes. Sequencing data of amino acid residues indicated an evolutionary relationship between bacterial and eukaryotic PFKs that suggests duplication, tandem fusion, and divergence of catalytic and effector binding sites of a prokaryotic ancestor to yield in eukaryotes a total of six organic ligand binding sites (22). One of these allosteric ligands is citrate, which acts as a potent allosteric inhibitor of the eukaryotic PFK enzymes. Studies on citrate allosteric sites of rabbit muscle PFK concluded that they developed from the phosphoenolpyruvate (PEP)/ADP allosteric site of *Escherichia coli* PFK and are scattered both on N-terminal and C-terminal parts of the enzyme (11, 18). On the other hand, the strict conservation between active site residues in the N-terminal half of the eukaryotic enzyme and those of bacterial PFKs suggests that the active site of the eukaryotic PFK is located in the N-terminal half (22).

In *Aspergillus niger*, which is an important commercial microorganism, undisturbed metabolic flow through glycolysis was found to be a key prerequisite during citric acid production (24). The regulatory properties of PFK1 attracted the interest of investigators in order to explain the phenomenon (1, 5, 6), since this enzyme was shown to be a major control point of *A. niger* glycolysis (19, 27). By studying the kinetics on a partially purified enzyme, it was proposed that the citrate inhibition of PFK1 was counteracted by NH_4^+ ions and AMP in *A. niger* cells (5, 6), while fructose-2,6-bisphosphate significantly stimulated the activity of the enzyme and relieved the inhibition caused by ATP (1). A posttranslational modification of *A. niger* PFK1 that was recently described resulted in formation of a shorter 49-kDa fragment by proteolytic cleavage of the native protein, which retained its activity after phosphorylation of the protein molecule by cyclic AMP-dependent protein kinase (8, 20). Preliminary kinetic studies indicated different kinetic characteristics of both PFK1 forms.

In the present study, we report the effect of citrate and other effectors on activities of both forms of *A. niger* PFKs after the proteins were purified to a high degree of homogeneity. By evaluating the results, we estimated which form of the enzyme might play the most prominent role in deregulating metabolic flow through glycolysis during the process of citric acid overflow in *A. niger*.

MATERIALS AND METHODS

Microorganism. *A. niger pfk*A multicopy strain (25) designated as strain A459 in the Culture Collection of the National Institute of Chemistry, Ljubljana, was used throughout all the experiments. For the inoculum, the spores were harvested from 7-day-old wort agar slants and suspended in 25 ml of 0.1% (wt/vol) Tween 80 solution, which contained approximately 1×10^7 spores/ml.

Medium. Five milliliters of spore suspension was used to inoculate 100 ml of the medium (20) in 500-ml Erlenmeyer flasks with baffles. For the isolation of the native PFK1 enzyme, the mycelium was grown on a rotary shaker (New

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Brunswick, Edison, N.J.) at 27°C for 16 h, while for obtaining the PFK1 fragment, the mycelium was grown 24 h at 30°C. Significantly reduced intracellular proteolytic degradation of the native protein was observed when the mycelium was grown at slightly reduced cultivation temperatures (27°C). Afterwards, the cells were collected by vacuum filtration, washed three times with a cold extraction buffer (50 mM sodium phosphate buffer, pH 7.8, with 1 mM of dithioerythritol [DTE]), frozen under liquid nitrogen, and stored at −70°C until used.

Homogenate preparation. For homogenate preparation, frozen mycelium (about 100 g of dry weight) was disrupted in a glass bead disintegrator (Braun, Melsungen, Germany), and the final frozen powder was dissolved in 20 ml of extraction buffer (50 mM sodium phosphate buffer, pH 8.0, plus 1 mM DTE for the native enzyme and 100 mM sodium phosphate buffer, pH 7.8, plus 1 mM DTE for the shorter PFK1 fragment) containing 10 μl of protease and 10 μl of phosphatase inhibitor cocktail (Sigma Chemical Co., St. Louis, Mo.). The higher ionic strength of the buffer prolonged the stability of the shorter fragment, while phosphatase inhibitor prevented deactivation of the fragment by dephosphorylation of the enzyme molecule (20). After centrifugation at 22,000 × *g* for 15 min in the refrigerated centrifuge (Sorvall, Wilmington, Del.), the homogenate contained more than 5 mg of soluble proteins per ml.

Purification of PFK1. The crude enzyme extract was precipitated with ammonium sulfate, and a fraction between 50 to 75% of 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 1 mM DTE, the sample was loaded onto a column containing 1 ml of aminophenyl-ATP-Sepharose (Jena Bioscience), previously equilibrated with sodium phosphate buffer. After the sample was applied on the column, unbound enzymes were removed by extensive washing with buffer. PFK1 was eluted from the column with 1 ml of 6 mM fructose-6-phosphate and 1 mM ADP in the buffer. Additionally, the fraction exhibiting PFK1 activity was applied on a column filled with Sephacryl S-400, equilibrated with 50 mM phosphate buffer, pH 7.8, and 1 mM DTE. The fraction with distinct PFK1 activity was dialyzed overnight against buffer containing 20% (vol/vol) glycerol and stored at 4°C. The enzyme remained active for 2 months.

When the 49-kDa fragment was isolated, 10 μl of phosphatase inhibitor cocktail (Sigma) was added to the homogenate, and 100 mM sodium phosphate buffer (pH 7.8), instead of 50 mM buffer, was used throughout all the purification steps. After the final purification step (affinity chromatography), 5 mg/ml of bovine serum albumin was dissolved in the fraction exhibiting PFK1 activity, while the gel filtration step was necessarily omitted because of the instability of the shorter fragment under the diluted conditions (20).

Enzyme assay. PFK1 activity was measured spectrophotometrically at 340 nm (DU-600 spectrophotometer; Beckman Instrument Co., Berkeley, Calif.), essentially as reported previously (25), using a coupled reaction system. Unless otherwise stated, the assay mixture contained the following in a final volume of 1 ml: 50 mM HEPES buffer (pH 7.8), 1 mM DTE, 100 mM KCl, 5 mM $MgCl_2$, 20 μl of enzyme sample, 1 mM ATP, 0.2 mM NADH, 0.9 U/ml aldolase (Roche Molecular Biochemicals, Indianapolis, Ind.), 5 U/ml triosephosphate isomerase, and 2.5 U/ml glycerol-3-phosphate dehydrogenase (Roche Molecular Biochemicals, Indianapolis, Ind.). Before use, the auxiliary enzymes were dialyzed against 50 mM HEPES 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. Since the order of the addition of reaction components might affect the kinetics measurement due to lags in the initial phases of reaction (3), the reactions started with fructose-6-phosphate while the effectors were added 4 min later, and the extent of inhibition/activation was determined from the rate after 3 to 4 min.

All presented kinetic data are averages obtained from a minimum of three replicate measurements.

Total protein concentrations of the samples were determined by bicinchoninic acid protein assays (28) performed with a Sigma kit (St. Louis, Mo.).

Electrophoresis. Electrophoresis in 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) was performed according to Laemmli (16) in a mini-gel system (LKB, Bromma, Sweden). Molecular weight markers used as standards were phosphorylase B (97, 000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400), all purchased from Sigma (St. Louis, Mo.).

Multiple sequence alignments were constructed with the ClustalW program available from EMBnet-CH (http://www.ch.embnet.org/software/ClustalW.html).

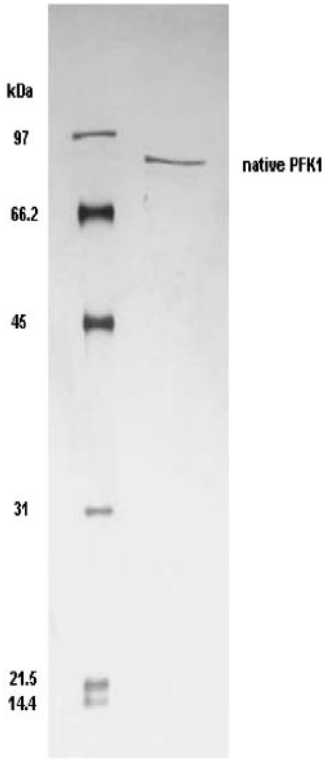

FIG. 1. SDS-PAGE of the native PFK1 enzyme purified to near homogeneity. In the left lane of the gel, the following standards are shown: phosphorylase (molecular weight, 97,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400).

RESULTS

For the first time, kinetic analyses of the native PFK1 enzyme isolated from *A. niger* cells were performed on the protein purified to near homogeneity. The three-step purification method was used by successive application of fractional ammonium sulfate precipitation, affinity chromatography, and gel filtration. By running the sample that exhibited PFK1 activity on SDS-polyacrylamide gel electrophoresis (PAGE) gels, a single band corresponding to a protein of 85 kDa was detected after the protein band was visualized by silver staining (Fig. 1).

Citrate inhibition of the native PFK1 enzyme. The activity of the purified 85-kDa PFK1 has been measured in the presence of 5 mM $MgCl_2$ in the buffer at pH 7.8. Citrate was added in the form of trisodium salt; however, the pH value of the system was not affected by the citrate concentrations used. Increasing the concentrations of citrate gradually decreased the activity of the native enzyme, and virtually no activity could be detected at 10 mM concentrations (Fig. 2). The K_i value for the citrate as an inhibitor was estimated from a Dixon plot (graph not shown) to be 1.5 mM, while the inhibition mode was clearly noncompetitive as determined from a Lineweaver-Burk plot (graph not shown). For both plots data from Fig. 2 were used.

Citrate inhibition of the native PFK1 enzyme at various Mg^{2+} concentrations. In the previous kinetic measurements, the concentration of Mg^{2+} ions was kept at 5 mM; however, by increasing the magnesium concentration in the measuring sys-

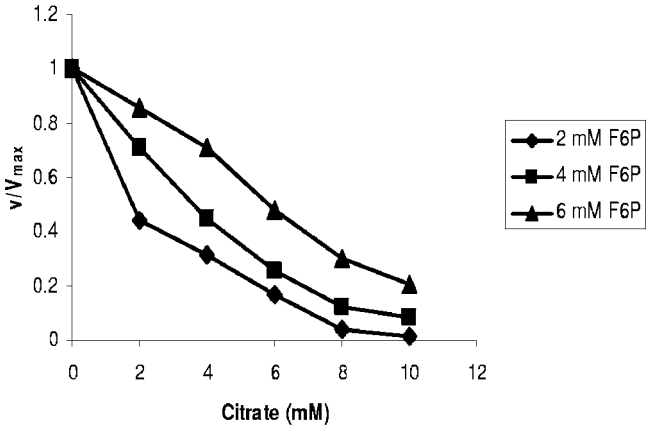

FIG. 2. Citrate inhibition of the native PFK1 enzyme as measured in the presence of 5 mM Mg^{2+} ions and 1 mM ATP in the system and different concentrations of fructose-6-phosphate (F6P) as indicated on the figure.

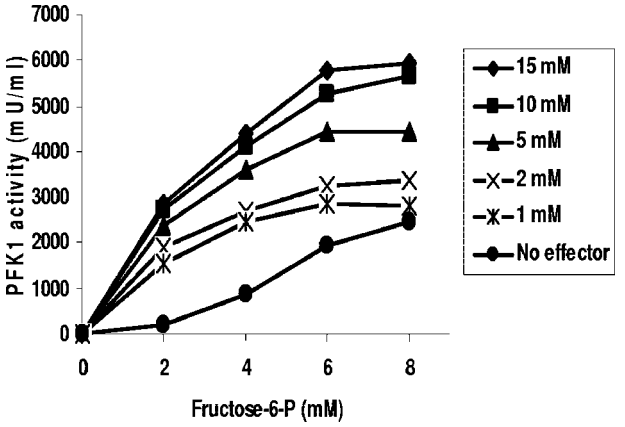

FIG. 4. Activities of the native PFK1 enzyme measured with and without various concentrations of ammonium ions as recorded in the system with 1 mM ATP and 5 mM Mg^{2+} ions.

tem, a reduction in the inhibition of PFK1 activity by citrate could be detected. The K_i value significantly decreased even in the presence of 10 mM Mg^{2+} ions, reaching the value of 5 mM, while at a 20 mM concentration of metal ions, inhibition was almost negligible (Fig. 3).

Ammonium ions as activators of the native PFK1 enzyme. Ammonium ions added to the measuring system in the form of ammonium nitrate have proven to be a strong enhancer of the enzyme activity affecting both the V_{max} and the affinity of the enzyme toward the substrate (Fig. 4). Concentrations as low as 1 mM significantly increased the enzyme activity especially at lower substrate concentrations (below 4 mM), since a change from the shape of the sigmoidal plot of reaction velocities against substrate concentrations to a hyperbolic shape could be observed in the presence of the ammonium ions. The sigmoidal shape of the saturation velocity indicated that the kinetic properties for fructose-6-phosphate cannot be accounted for by the Michaelis-Menten mode but belonged to those of an allosteric enzyme. The effector also exhibited a positive effect on V_{max} activity at higher fructose-6-phosphate concentrations, and up to threefold higher velocities were recorded with a 15 mM concentration of ammonium ions in the system with respect to the activities measured without the activator. Analyzing data from Fig. 4 by the Hill equation showed no cooperativity in NH_4^+ ion binding. The value of the Hill coefficient, which was calculated to be close to 1 (Fig. 5), indicated that one bound molecule of ligand has no significant effect on the binding of additional effector molecules to the oligomeric holoenzyme.

Citrate inhibition of the native PFK1 enzyme in the presence of ammonium ions. In contrast to previous reports (1, 5, 6), citrate inhibition of the native PFK1 enzyme isolated from *A. niger* cells could be recorded also in the presence of ammonium ions (5 mM). Although the specific activities detected were higher due to the activator present, a clear reduction of the velocities could be observed after a stepwise increase of the citrate concentration in the vial (Fig. 6). From the Dixon plot of the data presented in Fig. 6, the K_i value could be estimated to be 1.5 mM, which was identical to that obtained in the system without NH_4^+ ions. No reduction in citrate inhibition at higher ammonium ion concentrations could be observed.

Citrate-resistant shorter fragment of PFK1 enzyme. The shorter fragment of the PFK1 enzyme was partially purified from the cell-free homogenate of *A. niger* cells as reported previously (20). No traces of the native PFK1 enzyme could be

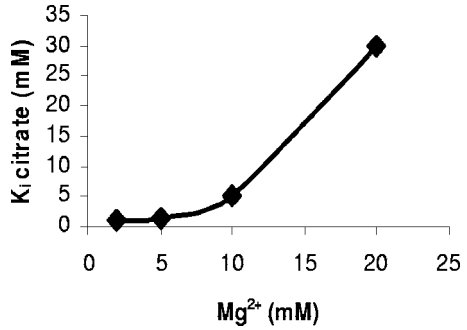

FIG. 3. Inhibition coefficient (K_i) for citrate as determined at different concentrations of magnesium ions in the systems and under the conditions described in the legend of Fig. 2.

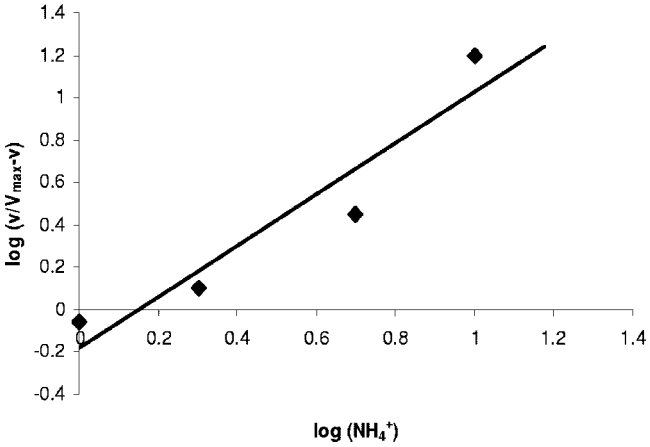

FIG. 5. Hill plot of PFK1 activities of the native PFK1 protein as determined at different ammonium ion concentrations.

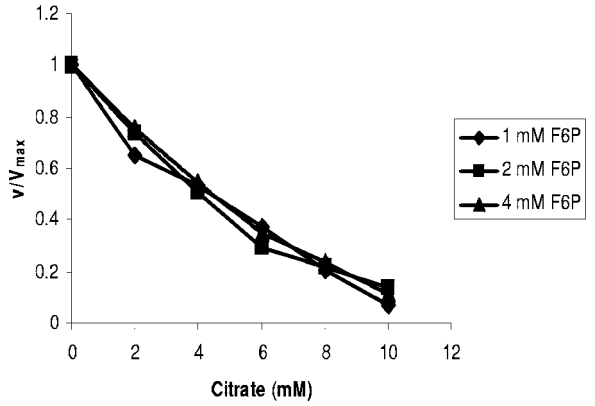

FIG. 6. Citrate inhibition of the native PFK1 enzyme measured in the presence of ammonium ions (1 mM), 1 mM ATP, 5 mM Mg^{2+}, and different concentrations of fructose-6-phosphate (F6P) as indicated on the figure.

detected in the isolate by SDS-PAGE. Due to the instability of the fragment, which was enhanced by diluting the protein homogenate during the purification steps, all measurements were done in the buffers with 5 mg/ml bovine serum albumin added. Inactivation of PFK1 by dissociation at low protein concentrations was previously reported and well characterized for the rat liver enzyme (23).

No significant decrease in enzyme velocity could be observed by measuring the activity of the isolated shorter PFK1 fragment in the system with stepwise increases of citrate up to 10 mM (Fig. 7).

There are reasons to believe that the shorter fragment was completely resistant to inhibition by citrate at physiological citrate concentrations, and no negative effect of the effector in the form of tri-sodium salt could be detected.

Unfortunately, due to the extreme instability of the shorter fragment, the ligand binding coefficient (K_d) for citrate could not be determined (10), since the enzyme completely lost its activity during the gel filtration technique.

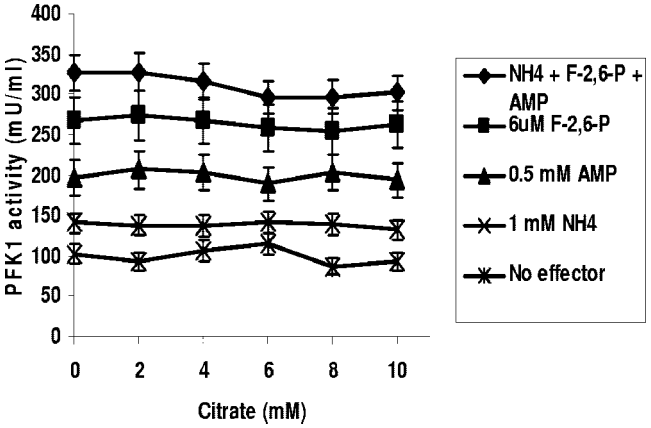

FIG. 7. Activities of the shorter 49-kDa PFK1 fragment measured at increasing concentrations of citrate and various activators. The measuring system contained 0.5 mM ATP, 6 mM fructose-6-phosphate, and 5 mM Mg^{2+} ions.

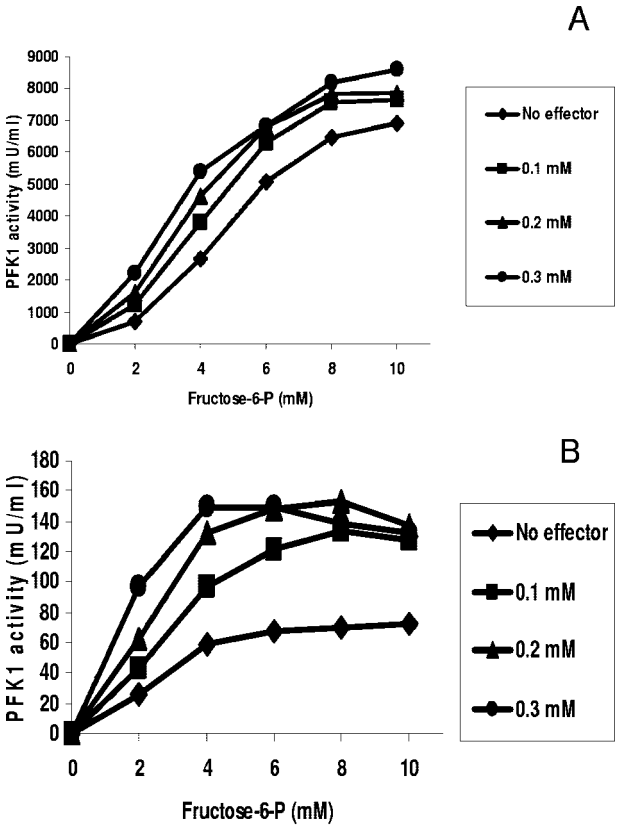

FIG. 8. Activities of the native PFK1 (A) and a shorter 49-kDa fragment (B) measured without and in the presence of various concentrations of AMP as indicated on the figure.

Activity of the shorter fragment of PFK1 enzyme in the presence of various effectors. In the presence of other activators such as fructose-2,6-bisphosphate (F-2,6-P), ammonium ions, and AMP, no inhibition by citrate could be observed either; only the specific activities detected were correspondingly higher. The activities shown in Fig. 7 were recorded at physiological concentrations of the effectors as reported to be present in *A. niger* mycelium (6, 21, 25), yet the substrate concentration permitted activities close to V_{max}.

Effect of other effectors on activities of *A. niger* enzymes. A number of other effectors were reported to influence activities of PFK1s from various sources (2), among which some adenosine phosphate species might have the most noteworthy effect. AMP has proven to increase the activity of both forms of *A. niger* PFK1, yet a more prominent positive effect was recorded at the shorter fragment. AMP increased the affinity of the fragment toward the substrate and significantly enhanced the maximal velocity of the enzyme (Fig. 8B); with the native enzyme, however, saturation curves remained sigmoidal, and maximal activity was only moderately elevated (Fig. 8A). When we examined enzyme activities of both enzymes in the presence of cyclic AMP (1 mM) and PEP (up to 5 mM), a strong inhibitor of *E. coli* PFK1 enzyme (15), no positive or negative effect could be detected on either the native enzyme or the shorter fragment.

Activities of both forms of *A. niger* PFK1 detected in a system with substrate and effectors at near physiological conditions. At physiological concentrations of fructose-6-phos-

phate, which were reported to be as low as 0.23 ± 0.07 mM (25), about 15-fold higher activities of the shorter fragment were measured in the presence of physiological concentrations of activators (6 μM fructose-2,6-bisphosphate, 1 mM ammonium ions, and 0.1 mM AMP) and inhibitors (1.5 mM ATP and 5 mM citrate) than the activities detected by the short fragment in the measuring system without any effectors. With the native enzyme, the addition of all effectors in their physiological concentrations increased the activities by only a factor of 2 ± 0.5 when measured at a 0.25 mM concentration of fructose-6-phosphate.

DISCUSSION

Much has been reported on PFK1 characteristics from the filamentous fungus *A. niger* in the past (1, 5, 6, 17, 20, 27). The enzyme attracted the interest of investigators, due to its ability to maintain a high glycolytic flux in spite of elevated intracellular concentrations of citrate during the process of the secretion of citric acid, which was reported to reach a concentration between 4 mM (23) and 7 mM (13). Kinetics were studied on partially purified enzyme, and initially the phenomenon of elevated concentrations of intracellular ammonium ions was regarded as significant; concentrations as high as 15 mM were determined (6), although much lower levels of ammonium ions were recently reported in *A. niger* mycelium (21). Increased levels of NH_4^+ ions were first postulated to be the crucial factor counteracting citrate inhibition of the enzyme (5, 6, 23), while later the model was extended to the simultaneous positive effect of fructose-2,6-bisphosphate, AMP, and NH_4^+ ions (1). Although the increase in enzyme affinity toward the substrate, mediated by fructose-2,6-bisphosphate and ammonium ions, must diminish the negative effect of citrate on the native PFK1 enzyme, especially at low physiological concentrations of fructose-6-phosphate, an important phenomenon of *A. niger* PFK1 enzyme has been overlooked and ignored. Recently, it has been reported that PFK1 can undergo a posttranslational modification in *A. niger* cells, resulting in the formation of a shorter 49-kDa fragment that exhibits different kinetics with respect to the native enzyme (20). Since both forms of the enzyme can be eluted from the affinity chromatography column simultaneously, partly purified PFK1 used for kinetics studies in the past might well contain both isoenzymes. Moreover, some investigators have reported (6) that the preparation obtained by affinity chromatography in the final step showed one major and two minor bands on SDS-PAGE, while some data assigned to the PFK1 enzyme of *A. niger* (1) clearly showed kinetic characteristics of the shorter fragment (20).

The present study of the kinetics of the purified *A. niger* native PFK1 with respect to citrate inhibition showed moderate inhibition of the enzyme by this effector, while activators like ammonium ions merely increased the overall activity of the enzyme but left the K_i values for citrate unchanged. In eukaryotes, there were numerous reports of PFK1 enzymes that exhibited moderate inhibition by citrate, and just a slight decrease of activity was recorded at physiological citrate concentrations (7, 9, 29, 31). Among fungal species, the enzyme studied in *Saccharomyces cerevisiae* also showed weak inhibition with about 25% of initial activity left at a 10 mM concentration of citrate (26). Moreover, citrate inhibited the yeast enzyme in the presence and absence of ammonium ions (32). On the other hand, relieved inhibition of PFK1 caused by magnesium ions was reported also for the ATP-dependent enzyme isolated from grapefruit juice sacs, where a 12 mM concentration of Mg^{2+} acetate was found to prevent the inhibition of the enzyme by citrate (31). Citrate might decrease PFK1 activity also indirectly by chelating Mg^{2+} ions and therefore preventing the formation of MgATP complex, which is a biologically active form for phosphorylation. Vice versa, increasing concentrations of Mg^{2+} ions induce the formation of Mg-citrate complex, which apparently cannot thereafter act as an inhibitor.

Most of the mammalian PFK1 enzymes so far studied have shown a strong inhibition by citrate. For the type C rabbit isoenzyme, strong inhibition by citrate was reported, with 2 mM concentrations completely deactivating the enzyme (18). The rabbit enzyme was used as a model protein for determination of allosteric binding sites for citrate that were determined by site-directed mutagenesis (11, 12, 18). The ligand binding sites were found to be located on both N-terminal and C-terminal parts of the enzyme, and the following relevant amino acid residues were determined to play a key role in binding: R-44, R-48, W-79, K-567, D-601, and K-627 (18). The rabbit PFK1 amino acid residues and the alignment of corresponding residues from *E. coli* isoenzyme 1, two yeast isoforms, and *A. niger* PFK1 are shown in Fig. 9, where the putative residues involved in citrate binding are marked in gray. In *E. coli* corresponding amino acid residues play a role in PEP binding. Detailed studies concluded that the allosteric sites for citrate in eukaryotic PFK1s developed from the PEP/ADP allosteric sites of bacterial ancestors (12). By comparing the aligned *A. niger* PFK1 amino acid sequence with rabbit type C isoenzyme, identical residues could be found on the N-terminal part of the enzyme, while no conserved sites for the allosteric binding of citrate could be detected on the C-terminal part. In yeast PFK1, the enzyme is a hetero-octamer composed of two types of subunits in an equimolar ratio. Alignment of the primary structure of both subunits with the amino acid sequence of rabbit type C PFK1 showed identical residues at the putative citrate binding sites on the N-terminal part of the molecule, while on the C-terminal part, two out of three binding sites differed. The yeast PFK1 enzyme exhibited similar kinetics with respect to citrate inhibition as *A. niger* native enzyme, and ammonium ions increased the rate of the enzyme significantly but could not prevent the inhibition by citrate (32). Therefore, it remains possible that the mutations at the C-terminal part of the enzyme resulted in the decreased negative effect of citrate in both fungal enzymes.

Proteolytic cleavage of the native PFK1 enzyme in *A. niger* cells led to the formation of a shorter fragment composed of approximately 450 amino acid residues and lacking the C-terminal part of the protein (20). Since the active site of the eukaryotic PFK is believed to be located at the N-terminal half (22), the enzyme was not deactivated by truncation, though the severe structural change effected its kinetic characteristics.

In the native enzyme, it seems that citrate is trapped in a gap between the N- and C-terminal parts of the protein (12), since binding sites on both halves play a role in its allosteric effect. By the cleavage of the C-terminal part, three citrate binding sites were lost, leading to ineffective binding of the inhibitor to

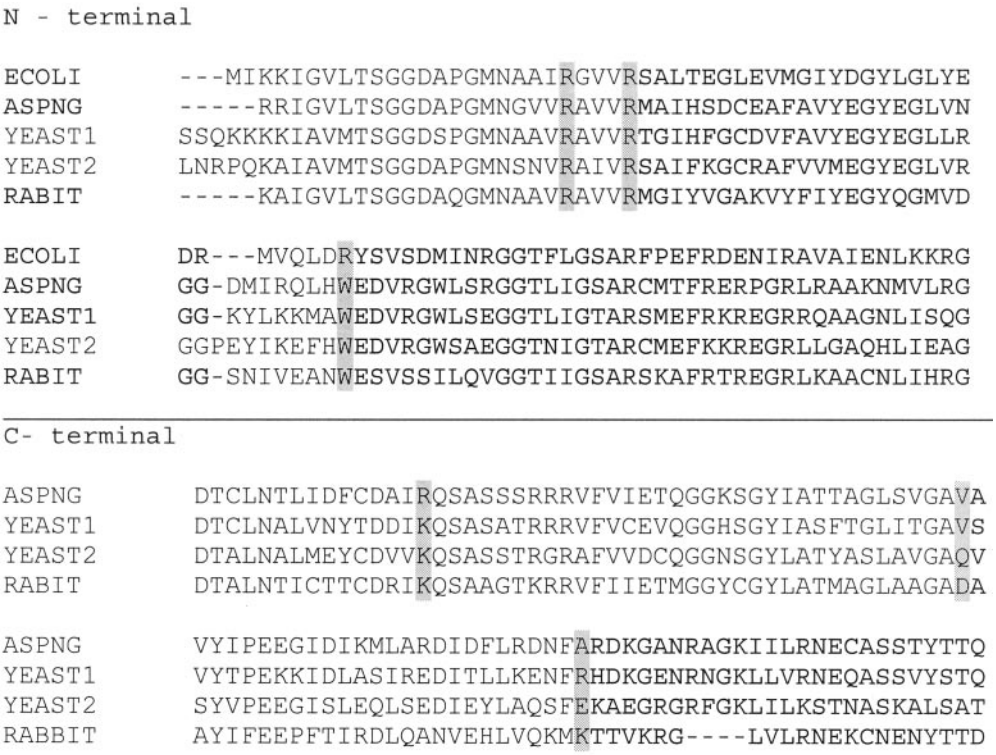

FIG. 9. Multiple sequence alignment of deduced amino acid residues of N-terminal and C-terminal parts of PFK1 proteins, where binding sites for citrate (gray) are located. ECOLI, *E. coli* (P0A796); ASPNG, *A. niger* (P78985), YEAST1, *S. cerevisiae* (P16861); YEAST2, *S. cerevisiae* (P16862); RABBIT, *Oryctolagus cuniculus* (P00511). Dashes represent gaps introduced into a sequence for alignment.

the remaining protein molecule that could result in resistance to citrate-negative effects.

The shorter form of *A. niger* PFK1 resembled more prokaryotic enzymes in respect to its molecular weight; however, no inhibition by PEP, a characteristic inhibitor of bacterial enzymes (15), could be detected. By comparing ligand binding sites for PEP (citrate) at the N-terminal part of the aligned molecules (Fig. 9), tryptophan (W-79; rabbit enzyme) was observed in all eukaryotic enzymes, while arginine (R) was found only in *E. coli* protein (Fig. 9). Evolutionary change from arginine to tryptophan was important for the development of citrate binding sites in eukaryotes, since both amino acid residues differ significantly in their electrochemical properties. However, citrate binding sites located also at the C-terminal part seemed to be essential for efficient deactivation of the enzyme. Cleavage of the C-terminal part of the *A. niger* PFK1 enzyme, therefore, rendered the shorter fragment resistant to both citrate and PEP inhibition.

Although the inhibition of PFK1 enzyme by ATP seemed to be equally relevant for regulating the metabolic flux through the glycolysis in eukaryotic organisms, there are two features that cause *A. niger* enzyme to be more dependent on citrate inhibition. First, the ATP inhibition of the shorter fragment was reported to be largely revealed in the presence of fructose-2,6-bisphosphate (20), while the overall ATP concentration might be reduced in *A. niger* cells due to the activity of an alternative oxidase that uncouples NADH reoxidation from ATP formation (14).

It is therefore important to realize that after a posttranslational modification of the native PFK1 protein in *A. niger* cells, the negative regulation of the allosteric enzyme turned out to be significantly reduced, while AMP and fructose-2,6-bisphosphate (20) manifested more prominent positive effects on enzyme activity.

Although the native PFK1 enzyme from *A. niger* cells does not differ significantly from the proteins of other fungal enzymes in its kinetic specifications, it seems to be the posttranslational modification of the native PFK1 protein that makes the *A. niger* enzyme the key element in reducing metabolic control over the flux through the glycolytic pathway, which ranks *A. niger* among the most productive commercial microorganisms. Therefore, the shorter fragment of *A. niger* PFK1 could, if synthesized from a truncated gene, offer a useful tool in engineering primary metabolism.

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