PLASMIN REDUCTION BY PHOSPHOGLYCERATE KINASE IS A THIOL-INDEPENDENT PROCESS

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Running Title

Plasmin reductase function of phosphoglycerate kinase
Summary

Phosphoglycerate kinase (PGK) is secreted by tumor cells and facilitates reduction of disulfide bond(s) in plasmin (Lay, A. J., Jiang, X.-M., Kisker, O., Flynn, E., Underwood, A., Condron, R., and Hogg, P. J. (2000) Nature 408, 869-873). The angiogenesis inhibitor, angiostatin, is cleaved from the reduced plasmin by a combination of serine- and metallo-proteinases. The chemistry of protein reductants is typically mediated by a pair of closely-spaced Cys residues. There are 7 Cys in human PGK and mutation of all 7 to Ala did not appreciably affect plasmin reductase activity, although some of the mutations perturbed the tertiary structure of the protein. Cys379 and Cys380 are close to the hinge that links the N- and C-terminal domains of PGK. Alkylation/oxidation of Cys379,380 by four different thiol-reactive compounds reduced plasmin reductase activity to 7-35% of control. Binding of 3-phosphoglycerate and/or MgATP to the N- and C-terminal domains of PGK, respectively, triggers a hinge-bending conformational change in the enzyme. Incubation of PGK with 3-phosphoglycerate and/or MgATP ablated plasmin reductase activity, with half-maximal inhibitory effects at ~1 mM concentration. In summary, reduction of plasmin by PGK is a thiol-independent process although either alkylation/oxidation of the fast-reacting Cys near the hinge or hinge-bending conformational change in PGK perturbs plasmin reduction by PGK, perhaps by obstructing the interaction of plasmin with PGK or perturbing conformational changes in PGK required for plasmin reduction.
Introduction

Disulfide bonds of certain cell surface proteins can interchange between the oxidized and reduced state (1-3). These observations suggest that the function of some secreted proteins may be controlled by interchange of one or more disulfide bonds (1). The reduction of disulfide bonds in plasmin by a tumor cell-derived protein was the first example of disulfide exchange in a secreted soluble protein (4-6). A second example is disulfide exchange in von Willebrand Factor, which is facilitated by thrombospondin-1 (7). Plasmin reduction is the first step in formation of the tumor angiogenesis inhibitor, angiostatin.

Tumor expansion and metastasis is dependent on tumor neovascularization, or angiogenesis (8). Angiogenesis is balanced by several protein activators and inhibitors (9). One such inhibitor is angiostatin (10), which is an internal fragment of the plasma zymogen, plasminogen. Plasminogen contains five consecutive kringle domains followed by a serine proteinase module. Urokinase- or tissue-plasminogen activator convert plasminogen to plasmin by hydrolysis of a single peptide bond in the serine proteinase module. Plasmin is processed in the conditioned medium of tumor cells producing angiostatin fragments consisting of kringle domains 1-4½, 1-4 and 1-3 (6 and references therein).

Plasmin proteolysis occurs in three stages. First, the Cys462-Cys541 and Cys512-Cys536 disulfide bonds in kringle 5 of plasmin are reduced by a plasmin reductase (5). Second, reduction of the kringle 5 disulfide bonds triggers cleavage at Arg530-Lys531 in kringle 5, and also at two other positions C-terminal of Cys462, by a serine proteinase (5). Autoproteolysis can account for the cleavage (5, 11), although another serine proteinase is responsible in human fibrosarcoma cell conditioned medium (5). Third, the kringle 1-4½ fragments are cleaved by matrix metalloproteinases to produce either kringle 1-4 or 1-3 (12-
All three kringle-containing fragments have been shown to inhibit endothelial cell proliferation \textit{in vitro} and angiogenesis \textit{in vivo} (15 and references therein).

The plasmin reductase was recently purified from human fibrosarcoma cell conditioned medium and shown to be the glycolytic enzyme, phosphoglycerate kinase (PGK; ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (6). Plasma of mice bearing fibrosarcoma tumors contained several-fold more PGK than mice without tumors and administration of PGK to tumor-bearing mice caused an increase in plasma levels of angiostatin and decrease in tumor vascularity and rate of tumor growth. Solid tumors employ PGK and other glycolytic enzymes to facilitate anaerobic production of ATP. These findings indicate that PGK plays an additional role in tumorigenesis by initiating extracellular formation of angiostatin from plasmin.

PGK is the sixth enzyme of the glycolytic pathway where it catalyses the high-energy phosphoryl transfer reaction from the acid anhydride bond of 1,3-bisphosphoglycerate to the $\beta$-phosphate of MgADP. PGK also influences DNA replication and repair in mammalian cell nuclei (16, 17), stimulates viral mRNA synthesis in the cytosol (18) and extends through the cell wall of \textit{C. albicans} (19). The human enzyme has a molecular mass of \~45 kDa and consists of a single polypeptide chain of 418 residues. Crystallographic studies of the yeast (20, 21), horse (22) and pig (23-25) enzyme revealed that the molecule is composed of two domains of similar size, which corresponds to the N- and C-terminal halves of the chain, separated by a hinge region.

Mammalian PGK’s contain 7 Cys and only 2 of the 7 are nearby in the primary or tertiary structure (26). Cys379,380 are close to the hinge region and have been referred to as ‘fast-reacting’ as they are amenable to alkylation by several thiol-reactive compounds (27). The role of all 7 Cys, and in particular the two fast-reacting Cys, in reduction of plasmin...
disulfide bonds by PGK has been explored in this study. We show that none of the PGK Cys residues are directly involved in plasmin reduction but that alkylation/oxidation of the fast-reacting Cys or conformational changes in the same region of the protein inhibit reductase activity.
Experimental Procedures

**Chemicals and proteins.** 3-(N-maleimidylpropionyl)biocytin (MPB) and dibromobimane (bBBr) were from Molecular Probes, Eugene, OR. HgCl₂, sodium tetrathionate (TT), reduced (GSH) and oxidised glutathione (GSSG), 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), ATP and 3-phosphoglycerate (3-PG) were from Sigma, St. Louis, MO. Plasminogen was purified from fresh frozen human plasma and separated into its two carbohydrate variants according to Castellino and Powell (28). Glu(1)-plasminogen was used in the experiments described herein. Urokinase plasminogen activator (uPA) was a gift from Serono Australia. Plasmin was generated by incubating plasminogen (20 μM) with uPA (20 nM) for 30 min in 20 mM HEPES, 0.14 M NaCl, pH 7.4 buffer at 37°C.

**Production and purification of wild-type and mutant PGK** - A 1.33 kb hPGK cDNA was isolated by RT-PCR from total RNA extracted from HT1080 cells as described previously (6). The Cys residues at positions 379 and 380 in wild-type PGK were mutated to Ala by replacing the T at position 1214 and 1217 to G and G at position 1215 and 1218 to C using the following primers and the QuikChange Site-Directed Mutagenesis Kit from Stratagene, La Jolla, CA: 5’-GACACTGCCACTGCGGTCAATGGGAACAC-3’ (forward, positions 1202 to 1233) and 5’-GTGGTCTTATCGGCCATGGCA TCGGCGAGCGGTATCTGTC-3’ (reverse, positions 1233 to 1202). The derived mutant was called C379-380A. Subsequently, Cys367, 316, 108, 99, and 50 were cumulatively mutated to Ala using the same approach as for C379-380A. A single Cys-Ala mutation at position 50 was also generated using wt PGK as the template. Integrity of the mutant DNA’s was confirmed by automated DNA sequencing. The PGK DNA’s were sub-cloned into the plasmid vector, pET11a, which was then transfected into *E. coli* strain, BL21 (DE3) (Novagen, Madison, WI). Expression of
recombinant protein was induced by 0.5 mM isopropyl-β-D-thiogalacto-pyranoside for 5 hours at 37°C. The BL21 cells were collected by centrifugation at 5000 g for 15 min and resuspended in B-PER Bacterial Extraction Reagent (Pierce, Rockford, IL) at a ratio of 60 mL.L⁻¹ of culture. The cells were lysed by sonication in the presence of 0.5% Triton X-100 and the lysate was clarified by centrifugation at 14,000 g for 30 min. Solid (NH₄)₂SO₄ was added slowly to the clarified supernatant to a concentration of 70% and stirred for 20 min at 4°C. The pellet was collected by centrifugation at 12,000 g for 20 min and additional (NH₄)₂SO₄ added to the supernatant to a final concentration of 90-100% and stirred for 20 min at 4°C. The pellet was collected by centrifugation at 12,000 g for 20 min, dissolved in 20 mM HEPES, 0.05 M NaCl, 1 mM EDTA, 0.02% NaN₃, pH 7.4 buffer and dialysed extensively against the same buffer. The protein was applied to a 80 mL (2.5 x 17 cm) column of Cibachron Blue-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same HEPES buffer. The column was washed with 3 bed volumes of the HEPES buffer at a flow rate of 1 mL.min⁻¹ to elute unbound proteins and developed with a 240 mL linear NaCl gradient from 0.05 to 2 M in the HEPES buffer. PGK eluted at ~1 M NaCl. The eluate was dialysed against 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, pH 7.9 buffer and applied to a 5 mL column of Heparin-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. The column was washed with 3 bed volumes of the Tris buffer at a flow rate of 0.5 mL.min⁻¹ to elute unbound proteins and developed with a 40 mL linear NaCl gradient from 0.15 to 1 M in the Tris buffer. PGK eluted at ~0.8 M NaCl. The purified PGK was dialysed against 20 mM HEPES, 0.14 M NaCl, 1 mM EDTA, pH 7.4 buffer and stored at -80°C until use.

Plasmin reduction - Plasmin (2 µg.mL⁻¹) was incubated with wt or mutant PGK’s (1 to 40 µg.mL⁻¹) in 20 mM HEPES, 0.14 M NaCl, 0.05% Tween 20, pH 7.4 buffer (HEPES/Tween)
for 30 min at temperatures between 20 and 50°C. On some occasions, the incubation buffer was 50 mM MES, 0.125 M NaCl, 2 mM EDTA, pH 6.0, 50 mM HEPES, 0.125 M NaCl, 2 mM EDTA, pH 7.0 or 50 mM HEPES, 0.125 M NaCl, 2 mM EDTA, pH 8.0. On other occasions, the PGK was incubated with 3-PG (0-13 mM) and/or ATP (0-13 mM) and 1 mM MgCl₂ in HEPES/Tween prior to addition of plasmin. The control reactions were plasmin or PGK incubated alone in HEPES/Tween. Free thiols generated in plasmin/angiostatin were labeled with MPB (100 µM) for 30 min at 37°C, followed by quenching of the unreacted MPB with GSH (200 µM) for 10 min at 37°C. Unreacted GSH, and other free sulfhydryls in the system, were blocked with NEM (400 µM) for 10 min at 37°C. The plasmin kringle products were collected on 50 μl of packed lysine-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) beads by incubation on a rotating wheel for 1 hour at room temperature, washed three times with HEPES/Tween, and eluted with 50 μL of 50 mM ε-amino-caproic acid in HEPES/Tween.

Assays for plasmin reduction – Two assays for plasmin reduction were employed. In one assay, the MPB-labeled plasmin/angiostatin fragments were immobilised on wells coated with the murine anti-angiostatin monoclonal antibody, 8.19, and detected using streptavidin-peroxidase (6). The 8.19 antibody (100 µl of 5 μg.mL⁻¹ in 0.1 M NaHCO₃, 0.02 % NaN₃, pH 8.3 buffer) was adsorbed to PolySorp 96 well plates (Nunc, Roskilde, Denmark) overnight at 4°C in a humid environment. Wells were washed once with HEPES/Tween, non-specific binding sites blocked by adding 200 µl of 5% non-fat milk powder in 20 mM HEPES, 0.14 M NaCl, 0.02 % NaN₃, pH 7.4 buffer and incubating for 90 min at 37°C, and then washed two times with HEPES/Tween. MPB-labeled plasmin/angiostatin fragments were diluted 1:8 in HEPES/Tween and 100 µl aliquots added to antibody coated wells and incubated for 30 min at room temperature with orbital shaking. Wells were washed three times with...
HEPES/Tween and 100 µl of 1:100 dilution of StreptABComplex/HRP (Dako, Carpinteria, CA) in HEPES/Tween added and incubated for 30 min at room temperature with orbital shaking. Wells were washed three times and the bound peroxidase was detected as described previously (5). In the other assay, the MPB-labeled plasmin/angiostatin fragments were resolved on 8-16% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), blotted with a 1:2000 dilution of streptavidin horseradish peroxidase (Molecular Probes, Eugene, OR), then developed and visualised using chemiluminescence (NEN, Boston, MA).

**Alkylation of PGK** – wt PGK (10 µM) was incubated with a 1, 2 or 4-fold molar excess of HgCl₂, bBBr or TT in 0.1 M HEPES, 0.3 M NaCl, 1 mM EDTA, pH 7.0 buffer for 1 h at room temperature. wt PGK (90 µM) was also incubated with a 1,000-fold molar excess of GSSG in the HEPES buffer for 2 h at room temperature and the unreacted GSSG was removed by dialysis against the HEPES buffer. The untreated and alkylated PGK’s (10 µM) were incubated with DTNB (200 µM) in 0.1 M HEPES, 0.3 M NaCl, 1 mM EDTA, pH 7.0 buffer for 30 min at room temperature. The absorbance at 412 nm due to the formation of the TNB dianion was measured using a Molecular Devices Thermomax Plus (Palo Alto, CA) microplate reader. The extinction coefficient for the TNB dianion at pH 7.0 is 14,150 M⁻¹cm⁻¹ at 412 nm (29).
Results

Consequence of mutation of all seven PGK Cys residues to Ala for plasmin reductase activity

The seven Cys in wt PGK were mutated to Ala in a cumulative fashion. The two fast-reacting Cys, Cys379,380, were mutated first and the resulting C379,380A PGK cDNA was then used to mutate Cys367. The corresponding C379,380,367A PGK cDNA was then used to mutate Cys316 and so forth. The Cys were mutated in the order, Cys379, 380, 367, 316, 108, 99 and 50. The C50A PGK mutant was also made. The wt and mutant PGK’s were expressed in E. coli and extracted with lysozyme. The extract was clarified by ammonium sulfate precipitation and the protein purified by affinity chromatography on Cibachron Blue-Sepharose and Heparin-Sepharose. The wt and mutant PGK’s were homogeneous by SDS-PAGE (Fig. 1).

Figure 1

Different concentrations of the wt and mutant PGK’s were incubated with plasmin in HEPES/Tween-buffered saline for 30 min at 37°C and the thiols in the reduced plasmin/angiostatin were labeled with MPB. The plasmin/angiostatin fragments were immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase. The control reactions were incubation of PGK or plasmin alone. These controls tested for any confounding effects of labeling of existing free thiols in plasmin or PGK by MPB. Both reactions resulted in negligible signal. The specific plasmin reductase activity of the wt and mutant PGK’s were similar, with the exception of the Cys-less PGK (C379,380,367,316,108,99,50A PGK) (Fig. 2A). This result suggested that Cys50, which was the last Cys to be mutated, was required for plasmin reductase activity. To test this hypothesis, the C50A PGK mutant was made and assayed for plasmin reductase activity. This C50A mutant had similar specific activity as wt PGK (Fig. 2A). The same qualitative
results were observed for all the PGK mutants when plasmin reduction was measured by resolving the MPB-labeled proteins on SDS-PAGE and blotting with streptavidin-peroxidase to detect the labeled angiostatin fragments (Fig. 2B).

Figure 2

These results indicated that neither Cys50 nor any of the other PGK Cys were required for plasmin reduction. We hypothesised that the Cys-less PGK had lost plasmin reductase activity due to secondary effects of the mutations on the integrity of the PGK tertiary structure. This theory was tested by examining the susceptibility of the mutant PGK’s to proteolysis by plasmin.

Consequence of mutation of PGK Cys residues on the susceptibility of PGK to proteolysis

wt or mutant PGK’s were incubated with plasmin and proteolysis of the PGK’s examined by SDS-PAGE. wt PGK and the C379,380A, C379,380,367,316A, and C50A PGK mutants were resistant to plasmin proteolysis (Fig. 3). In contrast, the C379,380,367A, C379,380,367,316,108A, C379,380,367,316,108,99A and C379,380,367,316,108,99,50A PGK mutants were proteolysed by plasmin to different extents. In particular, the 6 and 7 Cys to Ala mutants were completely degraded by plasmin during the incubation. The proteolysis was plasmin-dependent as all the PGK’s remained intact after incubation with plasmin that had been inactivated with Val-Phe-Lys-chloromethyl ketone (not shown).

Figure 3

Effect of alkylation of the fast-reacting Cys of PGK on plasmin reductase activity

The involvement of the PGK fast-reacting thiols in plasmin reduction was tested by modifying them with various alkylating/oxidising reagents. Four different alkylating/oxidising agents were employed; HgCl₂, bBBr, TT and GSSG. HgCl₂ and bBBr have been used to alkylate the fast-reacting Cys in pig muscle PGK (30).
Reaction of PGK with all four alkylating/oxidising agents reduced the number of reactive thiols per mol of PGK to ~0.2-0.5 (Fig. 4A-D). Equimolar concentrations of HgCl₂, bBBr and TT were maximally effective as four-fold molar excess of these agents did not change the number of residual thiols in PGK. Incubation of PGK with a 1,000-fold molar excess of GSSG reduced the number of reactive thiols per mol of PGK to ~0.4.

The alkylated/oxidised PGK’s were tested for plasmin reductase activity. The PGK’s were incubated with plasmin in HEPES/Tween-buffered saline for 30 min at 37°C and the thiols in the reduced plasmin/angiostatin were labeled with MPB. The plasmin/angiostatin fragments were immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase. The activity of the modified proteins were reduced to 7-35% of control (Fig. 4E).

Figure 4

Effect of pH, ionic strength and temperature on the plasmin reductase activity of PGK

PGK was incubated with plasmin in different pH buffers for discrete times at 37°C (Fig. 5A), with increasing concentrations of NaCl in pH 7.4 buffer for 30 min (Fig. 5B), or at different temperatures in pH 7.4 buffer for 30 min (Fig. 5C). The thiols in the reduced plasmin/angiostatin were labeled with MPB and detected using streptavidin-peroxidase.

Figure 5

The plasmin reductase activity of PGK was optimal at pH 7 at early time points of incubation. There was no obvious effect, however, of pH on plasmin reduction after 60 min incubation. Increasing NaCl concentrations reduced plasmin reduction, although the effects were relatively modest. Reductase activity was reduced by 73% when the NaCl concentration was increased from 0 to 2 M. There was no substantial effect of temperature on plasmin reductase activity of PGK between 20 and 50°C.
Effect of 3-PG and ATP-induced conformational change in PGK on the plasmin reductase activity

The reactivity of the fast-reacting Cys in pig muscle PGK are reduced upon binding of 3-PG and/or MgATP to PGK (31). This is a result of conformational changes in the enzyme induced by substrate binding (21). The consequences of substrate-induced conformational changes in PGK for plasmin reductase activity was tested. PGK was incubated with plasmin in the absence or presence of 3-PG and/or ATP and 1 mM MgCl₂ in HEPES/Tween-buffered saline for 30 min at 37°C. The thiols in the reduced plasmin/angiostatin were labeled with MPB and detected using streptavidin-peroxidase.

Incubation of PGK with 1 mM 3-PG or MgATP reduced plasmin reductase activity by ~60%, while incubation with 1 mM 3-PG and MgATP reduced activity by ~90% (Fig. 6A). Titration of the inhibitory effects of 3-PG and MgATP on plasmin reductase activity is shown in Figs. 6B and C. The half-maximal inhibitory effects of 3-PG and MgATP on plasmin reductase activity were ~1 mM.

Figure 6

Gallic and ellagic acid competitively inhibit PGK kinase activity (32, 33). Gallic acid appears to bind to the same site as MgATP (32). Both gallic and ellagic acid at 100 µM concentration inhibited plasmin reduction by PGK by >80% (not shown). NAD(H), another adenine nucleotide, also inhibited plasmin reduction by >60% at 10 mM concentration, presumably through binding to the same site on PGK as MgATP (not shown).
Discussion

Protein reductant active sites typically contain a redox active dithiol/disulfide with the sequence, CysGlyXCys (34). The Cys thiols cycle between the reduced dithiol and oxidized disulfide bond in coordination with a dithiol or disulfide of a protein substrate. This can result in reduction, formation or interchange of disulfide bonds in the protein substrate. There are seven Cys in PGK, none of which are involved in disulfide bonds, and only two of the seven are nearby in the primary or tertiary structure (22-25). This suggests that the mechanism by which PGK reduces disulfide bonds in plasmin is unconventional. We have reported that the plasmin reductase activity of PGK is inhibited by NEM and iodoacetamide (6), which implies a role for one or more of the PGK Cys residues in plasmin reduction. In this study we have explored the role of all 7 PGK Cys, and in particular the two fast-reacting Cys, in reduction of plasmin disulfide bonds.

The seven Cys in PGK were mutated to Ala in a cumulative fashion in the order, Cys379, 380, 367, 316, 108, 99 and 50. The two fast-reacting Cys, Cys379,380, were mutated first and the resulting cDNA was then used to mutate Cys367, and so forth. The specific plasmin reductase activity of the mutant PGK’s, with the exception of the Cys-less PGK, was similar to that of the wild-type protein. Some mutations were shown to change the tertiary structure of PGK as measured by susceptibility to proteolysis by plasmin. For instance, the Cys-less PGK was rapidly degraded by plasmin which probably accounted for the loss of plasmin reductase activity. These results implied that PGK Cys were not directly involved in plasmin reduction. The question remained, therefore, why alkylation of Cys379,380 inhibited plasmin reduction. This question was explored by reacting the Cys residues with different alkylating/oxidising agents and examining the consequences for plasmin reductase activity.
Carboxymethylation, but not methylation, of the fast-reacting Cys of pig PGK inactivates the kinase activity (35). Reaction of the pig enzyme with DTNB also inactivates the kinase activity (27), while reaction with HgCl₂ or bBBr reduces kinase activity by up to 80% (30). HgCl₂ reacts with free thiols and can facilitate oxidation of a dithiol to a disulfide bond. bBBr is a homobifunctional alkylating agent that can cross-link thiols in close proximity.

The fast-reacting thiols of PGK were also reacted with TT and GSSG. Accessible thiols react with these compounds to form mixed disulfides with thiosulfate and glutathione, respectively. Reaction of PGK with all four alkylating/oxidising agents reduced the number of reactive thiols per mol of PGK to ~0.2-0.5. The plasmin reductase activity of the modified proteins was reduced to 7-35% of control. These results indicate that alkylation of the fast-reacting thiols perturb plasmin reductase activity, which is consistent with our earlier report of inhibition of plasmin reductase activity by NEM and iodoacetamide (6). Neither changes in pH, ionic strength nor temperature markedly affected plasmin reductase activity, which implies that the reaction of PGK and plasmin involved predominantly hydrophobic interactions.

Kinase substrate binding studies have shown that MgATP and MgADP bind to the inner surface of the C-domain (20, 21, 25, 36) while 3-phosphoglycerate binds to the inner surface of the N-domain (23-25). The bound substrates are ≥ 10 Å from each other, too large a distance for direct in-line phosphoryl transfer. The enzyme overcomes this distance by undergoing a ‘hinge-bending’ conformational change that brings the two substrates closer together. The reactivity of both the fast-reacting thiols is reduced by binding of 3-PG and/or MgADP and MgATP to PGK (31). Moreover, carboxamidomethylation of the two fast-reacting Cys in pig PGK blocks the substrate-induced ‘hinge-bending’ conformational change
in the enzyme (37). These observations indicate that alkylation of the fast-reacting thiols in PGK can perturb the conformational changes required for kinase activity.

We tested the effect of 3-PG/MgADP-induced conformational changes in PGK for plasmin reductase activity. Incubation of PGK with 1 mM 3-PG or MgATP reduced plasmin reductase activity by ~60%, while incubation with 1 mM 3-PG and MgATP reduced activity by ~90%. The half-maximal effects of 3-PG and MgATP on plasmin reductase activity were ~1 mM, which is in the range of the Michaelis constants for these substrates in the kinase reaction (38). It is noteworthy that the effects of 3-PG and MgATP were additive, which may reflect cooperativity between 3-PG and MgATP in the conformational change in PGK. These findings indicate that hinge-bending conformational change in PGK negates the plasmin reductase activity of the protein.

A model of the molecular events facilitated by PGK in plasmin kringle 5 is shown in Fig. 7. We have previously proposed that both the Cys462-Cys541 and Cys512-Cys536 disulfide bonds in plasmin kringle 5 are cleaved which renders kringle 5 susceptible to proteolysis at either the Arg530-Lys531 peptide bond or two other unidentified peptide bonds (5). The other peptide bonds are likely to be Arg474-Val475 (5) and Lys467-Gly468 (39).

Figure 7

Plasmin undergoes autoproteolysis in alkaline pH producing a catalytically active microplasmin fragment with a Lys531 N-terminus. Wu et al. (40, 41) noticed that both the Cys462-Cys541 and Cys512-Cys536 disulfide bonds in K5 must have been cleaved to release microplasmin from K1-4 and they proposed that the increased \( \text{OH}^- \) ion concentration at alkaline pH was responsible for cleaving the Cys462-Cys541 disulfide bond. We have suggested that the mechanism of plasmin proteolysis at alkaline pH is the same as the mechanism of proteolysis facilitated by PGK at neutral pH (5).
Calculation of the dihedral strain energies (42, 43) of the kringle 5 disulfide bonds from the crystal structure described by Chang et al. (44) revealed that the left-handed Cys512-Cys536 bond has a high strain energy (2.63 kcal.mol\(^{-1}\)) compared to the right-handed Cys462-Cys541 (1.54 kcal.mol\(^{-1}\)) and left-handed Cys483-Cys524 (0.98 kcal.mol\(^{-1}\)) disulfide bonds. The average dihedral strain energies for left- and right-handed disulfide bonds are 1.68 and 3.19 kcal.mol\(^{-1}\), respectively (42, 43). A high dihedral strain energy correlates with ease of cleavage of the disulfide bond (42).

We propose, therefore, that PGK facilitates cleavage of the Cys512-Cys536 disulfide bond by –OH, which results in formation of a sulfinic acid at position 512 and a free thiol at Cys536. The Cys536 thiol is then available to exchange with the Cys462-Cys541 disulfide bond resulting in formation of a new disulfide at Cys536-Cys541 and a free thiol at Cys462. Kringle 5 is then susceptible to proteolysis at Arg530-Lys531, Arg474-Val475 and/or Lys467-Gly468. This is the simplest sequence of events that can explain all the available data. For instance, cleavage of only the Cys512-Cys536 would not enable release of the kringle 1-4 angiostatin fragments from plasmin. The free thiol that is labeled by MPB in the three angiostatin fragments would be Cys462. We do not exclude cleavage of the Cys483-Cys524 disulfide bond, although this is not required to explain the experimental observations.

PGK presumably binds to plasmin and induces a conformational change in kringle 5 that facilitates \(\text{HO}^\cdot\) attack on the Cys512-Cys536 disulfide bond. This suggests that other molecules that interact with plasmin might also facilitate cleavage of the kringle 5 disulfides. There is recent evidence to support this hypothesis. Interaction of a truncated porcine plasminogen activator inhibitor-1 (residues 80-265), but not full length protein, with plasmin has been shown to result in generation of kringle-containing angiostatin fragments (45). It is
possible that the truncated protein is facilitating the same sequence of events in plasmin that are achieved by PGK.

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Footnotes

Abbreviations: bBBr, dibromobimane; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GSH, reduced glutathione; MPB, 3-(N-maleimidylpropionyl)biocytin; GSSG, oxidised glutathione; 3-PG, 3-phosphoglycerate; PGK, phosphoglycerate kinase; NEM, N-ethylmaleimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TT, tetrathionate;
Figure Legends

Figure 1. SDS-PAGE profile of wt and mutant PGK’s. Five micrograms of wt and mutant PGK’s were resolved on SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue.

Figure 2. Consequence of mutation of all seven PGK Cys residues to Ala on plasmin reductase activity. A wt or mutant PGK’s (1, 10 or 40 µg.mL⁻¹) were incubated with plasmin (2 µg.mL⁻¹) in HEPES/Tween-buffered saline for 30 min. Free thiols in the reduced plasmin/angiostatin were labeled with MPB, the protein immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase. The results have been corrected for the absorbance of the incubation of plasmin alone, which was negligible. Incubation of PGK alone also resulted in negligible absorbance. The bars are the mean and SD of triplicate determinations. B wt or mutant PGK’s (10 µg.mL⁻¹) were incubated with plasmin (2 µg.mL⁻¹) in HEPES/Tween-buffered saline for 30 min and the free thiols in the reduced plasmin/angiostatin were labeled with MPB. The kringle-containing fragments were collected on lysine-Sepharose beads, resolved on SDS-PAGE under non-reducing conditions and blotted with streptavidin-peroxidase. The positions of Mr markers in kDa are shown at left. The arrows indicate the three angiostatin fragments (4, 5).

Figure 3. Consequence of mutation of PGK Cys residues on the susceptibility of PGK to proteolysis. wt or mutant PGK’s (0.5 mg.mL⁻¹) were incubated with plasmin (0.5 mg.mL⁻¹) in HEPES/Tween-buffered saline for 60 min. Samples of the reactions (20 µL) were resolved
on SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue. The positions of Mr markers in kDa are shown at left.

**Figure 4. Effect of alkylation of the fast-reacting Cys of PGK on plasmin reductase activity.**

wt PGK (10 µM) was incubated with a 1, 2 or 4-fold molar excess of HgCl₂ (part A), bBBr (part B) or TT (part C) for 1 h and the moles of thiols per mol of PGK calculated using DTNB. PGK (90 µM) was also incubated with a 1,000-fold molar excess of GSSG (part D) and the moles of thiols per mol of PGK calculated using DTNB after removal of the unreacted GSSG by dialysis. The alkylated PGK’s (1 µg.mL⁻¹) were incubated with plasmin (2 µg.mL⁻¹) in HEPES/Tween-buffered saline for 30 min. Free thiols in the reduced plasmin/angiostatin were labeled with MPB, the protein immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase (part E). The bars are the mean and SD of triplicate determinations.

**Figure 5. Effect of pH, ionic strength and temperature on the plasmin reductase activity of PGK.**

A wt PGK (1 µg.mL⁻¹) was incubated with plasmin (2 µg.mL⁻¹) in pH 6 (solid bars), 7 (open bars) or 8 (hatched bars) buffer for discrete times up to 60 min. Free thiols in the reduced plasmin/angiostatin were labeled with MPB in pH 7.4 buffer, the protein immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase. B wt PGK (1 µg.mL⁻¹) was incubated with plasmin (2 µg.mL⁻¹) in pH 7.4 HEPES/Tween buffer containing the indicated concentrations of NaCl for 30 min. Free thiols in the reduced plasmin/angiostatin were labeled with MPB, the protein immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase. C wt PGK (1 µg.mL⁻¹) was incubated with plasmin (2 µg.mL⁻¹) in HEPES/Tween-buffered saline, pH 7.4, for 30 min at
different temperatures. Free thiols in the reduced plasmin/angiostatin were labeled with MPB, the protein immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase.

Figure 6. Effect of 3-PG and ATP-induced conformational change in PGK on the plasmin reductase activity. PGK (1 µg.mL$^{-1}$) was incubated with plasmin (2 µg.mL$^{-1}$) in the absence or presence of 3-PG (0-13 mM) and/or ATP (0-13 mM) and 1 mM MgCl$_2$ in HEPES/Tween-buffered saline for 30 min. Free thiols in the reduced plasmin/angiostatin were labeled with MPB, the protein immobilised on antibody-coated wells and the MPB detected using streptavidin-peroxidase. Part A shows the effect on plasmin reduction by 1 mM 3-PG and/or ATP/MgCl$_2$ while parts B and C show the concentration dependence of the 3-PG or ATP/MgCl$_2$ effect, respectively. The bars are the mean and SD of triplicate determinations.

Figure 7. Model of the molecular events facilitated by PGK in plasmin kringle 5. Numbering is based on the sequence of human plasminogen (791 residues) beginning at Glu1, excluding the 19 amino acid signal peptide that ends at Met.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
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Angelina J. Lay, Xing-Mai Jiang, Elise Daly, Lisa Sun and Philip J. Hogg

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