

Myosin as cofactor and substrate in fibrinolysis

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Abstract Myosin accelerates plasminogen activation by tissue-type plasminogen activator (tPA), and is degraded extensively by plasmin. Myosin binds both tPA and plasminogen, and enhances activation of des_{1–77}-plasminogen by tPA but not by urokinase-type plasminogen activator (uPA). Myosin decreases K_M and increases k_{cat} for des_{1–77}-plasminogen activation by tPA, to yield catalytic efficiencies in excess of $8000 \text{ M}^{-1} \text{ s}^{-1}$. The effect of myosin is attributed to its C-terminal portion, the myosin rod. With a K_M of $3 \mu\text{M}$, myosin is a high-affinity substrate for plasmin. The findings indicate that myosin is a cofactor for plasminogen activation and a substrate for plasmin.

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Key words: Plasminogen; Plasminogen activator; Myosin

1. Introduction

Homeostatic responses to tissue injury include vasodynamic reactions, hemostasis, inflammation and an extended process of remodeling and healing. The serine protease plasmin participates in bridging these responses by degrading fibrin and possibly other extracellular proteins. Plasmin arises from plasminogen by proteolytic activation which, under physiological conditions, is inefficient in the bulk phase. Activation of plasminogen by tPA or uPA is enhanced by conformational or proteolytic modification of the zymogen [1,2]. Modifiers include some amino acids (conformational), plasmin (proteolysis at Lys-77) or elastase (proteolysis between kringle 4 and 5). All plasminogen activators hydrolyze plasminogen between Arg-561 and Val-562, but tPA activity is uniquely enhanced by cofactors; cofactor activity has been associated with a diverse group of macromolecules that includes fibrin [3], polyamines [4], one or more endothelial cell proteins [5,6], some extracellular matrix components [7] and denatured proteins [8].

When tissue is injured by ischemia, infection or trauma, cytosolic proteins come in contact with the fibrinolytic system. Among these, myosin is abundant and ubiquitous and, owing to its low solubility, has opportune features for participation in the plasminogen system. Recently actin was found to accelerate plasmin generation by tPA [9]. We now report that myosin, via its C-terminal rod domain, enhances plasminogen activation, and that plasmin digests myosin efficiently.

2. Materials and methods

Lysine-Sepharose, 6-aminohexanoate, porcine pancreatic elastase,

bovine and human albumin, porcine cardiac myosin and trypsin were obtained from Sigma Chemical Company, St. Louis, MO. Single-chain tissue-type plasminogen activator (human melanoma), Spectrozyme PL (H-D-norleucylhexahydro-tyrosyllysine-*p*-nitroanilide) and fibrin(ogen) fragments were purchased from American Diagnostica, Hartford, CT. Two-chain uPA was obtained from Abbott Laboratories, Abbot Park, IL. Characterization of porcine plasminogen activation by uPA was described earlier [2].

Published methods were used for preparation of rabbit skeletal muscle myosin, myosin fragments, myosin rod filament and fragment S-1 [10–12]. Human plasminogen, porcine plasminogen, des_{1–77}-plasminogen and des-kringle_{1–4}-plasminogen were prepared as described [2,13,14]. Amino acid sequence analysis (Applied Biosystems gas-phase sequencer) showed the N-terminal amino acid of porcine native plasminogen to be Asp, and that of des_{1–77}-plasminogen an Arg residue. A Pharmacia Phast[®] system was used for polyacrylamide gel electrophoresis.

Plasminogen activation was measured with a two-stage assay that precludes the influence of plasmin substrate (Spectrozyme PL) on activation kinetics [15,16]. For steady-state kinetics, des_{1–77}-plasminogen ($0.1\text{--}8 \mu\text{M}$) was incubated with tPA (60 nM) and myosin ($100\text{--}400 \text{ nM}$) in 50 mM sodium phosphate buffer, pH 7.4, at 22°C . After 20 min, samples ($10 \mu\text{l}$) were diluted into $500 \mu\text{l}$ of 0.2 mM Spectrozyme PL dissolved in 100 mM Tris-HCl, 100 mM NaCl, pH 7.4, containing 2.5 mM 6-aminohexanoate. The change in A_{405} was measured at 22°C with Beckman DU-50 Spectrophotometer. Kinetic parameters were calculated from the Michaelis-Menten equation

$$v = \frac{V_{\max} \cdot S}{K_M + S}$$

with use of non-linear fitting by least-square residues (Sigma Plot 5 software, Jandel Corp., Corte Madera, CA).

Adsorption of tPA and plasminogen to myosin was estimated by densitometry of NaDodSO₄-polyacrylamide gels of supernatant samples taken after ultracentrifugation ($105\,000 \times g$, 30 min, $100 \mu\text{l}$ sample, 100 mM NaCl, 100 mM Hepes, pH 7.4, 22°C).

The K_M for myosin and myosin rod with plasmin and des-kringle_{1–4}-plasmin were measured indirectly from competition with Spectrozyme PL [17]. Plasmin (60 nM) was added to solutions of Spectrozyme PL ($2.5\text{--}320 \mu\text{M}$) containing $0.24\text{--}47 \mu\text{M}$ myosin rod or $0.4\text{--}13 \mu\text{M}$ myosin in 10 mM Hepes, 100 mM NaCl, pH 7.4, and the change of absorbance at 405 nm was measured. The K_M values were calculated from linear curve fits to the equation

$$K_M^{\text{app}} = K_M^{\text{S}} + \text{My} \cdot (K_M^{\text{S}}) / (K_M^{\text{My}})$$

for two substrates competing for the enzyme, where My is the concentration of myosin or myosin rod and S is Spectrozyme PL.

3. Results

The rate of activation of native porcine plasminogen by tPA is increased by rabbit muscle myosin (Fig. 1). The activity is associated with the C-terminal portion (rod), but not with the N-terminal region (S-1 fragment, or head). Addition of 6-aminohexanoate suppresses the rate-enhancement. Treatment of myosin and myosin rod with heat (90°C , 5 min) has no effect on enhancer activities, while S-1 fragment, like some other denatured proteins [8], acquires activity. Contrasting some denatured proteins (Machovich et al., submitted) and

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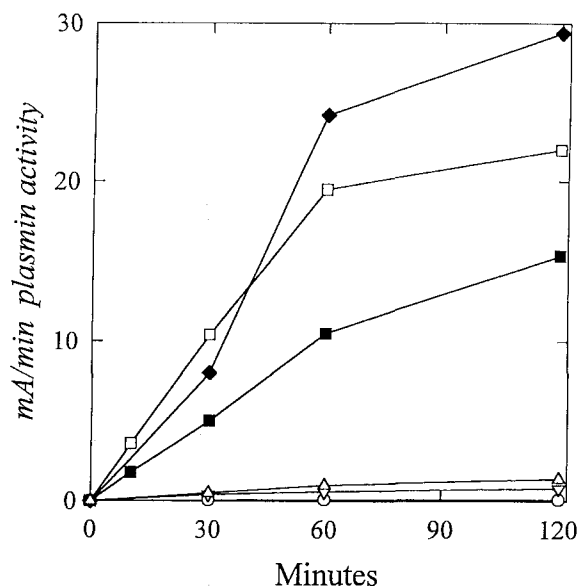


Fig. 1. Effect of myosin and its degradation products on activation of native plasminogen by tPA. Native plasminogen (1.1 μ M) was activated with tPA (0.03 μ M) in 100 mM Hepes, 50 mM NaCl, pH 7.4. Plasmin activity was measured as absorbance at 405 nm arising from hydrolysis of Spectrozyme PL [16]. Additives: none (\circ), 0.2 μ M rabbit skeletal muscle myosin (\blacklozenge), 0.1 μ M myosin rod (\square), 0.1 μ M myosin rod plus 250 μ M 6-aminohexanoate (\blacksquare), 1 μ M myosin S-1 (\triangle) and 1.4 μ M native albumin (∇).

actin [9], myosin does not require plasmin digestion to develop cofactor activity (not shown).

Activation of native plasminogen by uPA is accelerated by both native myosin and myosin rod (Fig. 2). Relative to the native zymogen, des₁₋₇₇-plasminogen is a more sensitive substrate for both uPA and tPA, so enhancement of the plasmin-catalyzed conversion would enhance plasmin generation in turn [1,2]. However, the impact of myosin rod on plasmin-

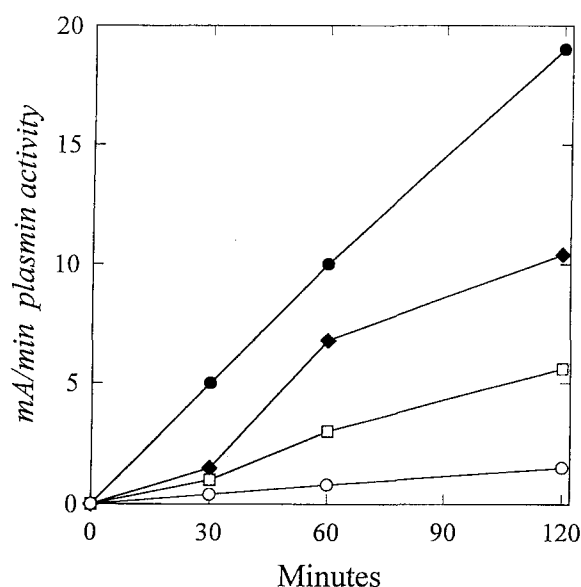


Fig. 2. Effect of myosin on native plasminogen activation by uPA. Native plasminogen (1.1 μ M) was activated with uPA (0.04 μ M) in 100 mM Hepes, 50 mM NaCl pH 7.4. Additives: none (\circ), 0.2 μ M rabbit skeletal muscle myosin (\blacklozenge), 0.06 μ M myosin rod (\square) and 0.2 μ M myosin rod (\bullet).

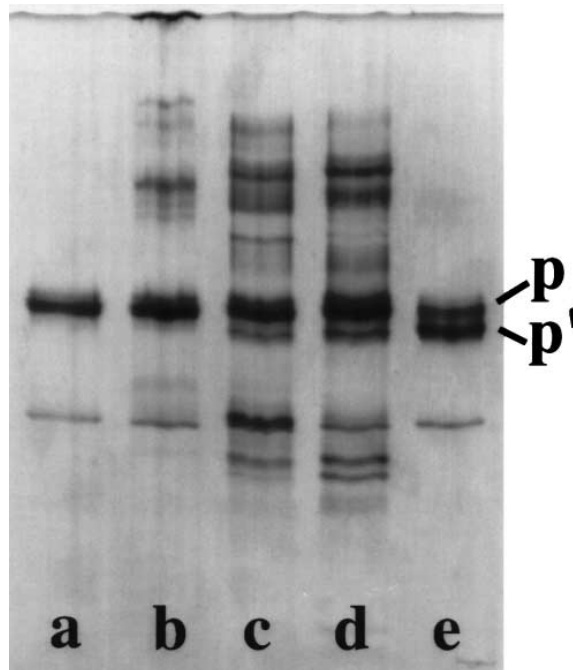


Fig. 3. Effect of myosin rod on the proteolysis of plasminogen with plasmin. Native plasminogen (2.2 μ M) was incubated with plasmin (250 nM) and either 660 nM rabbit skeletal muscle myosin rod or 5 mM 6-aminohexanoate. At intervals, samples were taken for SDS-gel (10–15%) electrophoresis without reduction. a: No additives (control), 60 min; b–d: myosin rod, 0, 30 and 60 min; e: 6-aminohexanoate, 60 min. p, Native plasminogen; p', des₁₋₇₇-plasminogen. Digestion of myosin rod by plasmin at concentrations used for cofactor assays yields no degradation products between M_r 80 000 and 90 000 (not shown).

catalyzed conversion of native plasminogen to des₁₋₇₇-plasminogen (Fig. 3e) is too small to account for its full effect on uPA activation (Fig. 2).

Native myosin and myosin rod enhance activation of des₁₋₇₇-plasminogen by tPA, while their impact on activation with uPA is negligible (Table 1). Steady-state kinetics of des₁₋₇₇-plasminogen activation by tPA indicate that both rabbit skeletal muscle myosin and porcine cardiac myosin decrease K_M and increase V_{max} (Table 2). In the absence of myosin the K_M of tPA for porcine des₁₋₇₇-plasminogen is higher than 8 μ M (for human plasminogen values of 19 μ M or >100 μ M are published in [3] and [9], respectively). The variations of the K_M value at various myosin concentrations are not significant. Ultracentrifugation (105 000 $\times g$, 30 min) removes cofactor activity of myosin. When added prior to

Table 1
Effect of myosin preparations on des₁₋₇₇-plasminogen activation

Additives	Rate-enhancements (fold)	
	(uPA)	(tPA)
Albumin	1.3	1.7
Myosin	1.8	7
Rod	2.2	9
S-1	1.7	2
Ff	-	10

Des₁₋₇₇-plasminogen (1.1 μ M) was activated with tPA (0.03 μ M) or uPA (0.01 μ M) in the presence of the indicated proteins (100 μ g/ml each) for 30 min.

Enhancements are relative to the rate without additives.

Ff = fibrin(ogen) fragments.

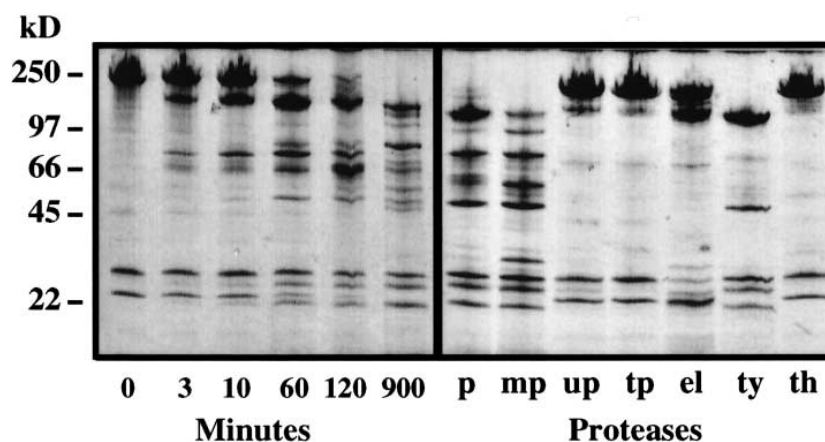


Fig. 4. Degradation of myosin with proteases. Left panel: Porcine cardiac myosin (1.6 μ M) was digested with human plasmin (120 nM) in 100 mM NaOH–Hepes, 100 mM NaCl, pH 7.4. At the indicated intervals, samples were removed for analysis (Coomassie Blue staining) by polyacrylamide (4–15%) gel electrophoresis in NaDodSO₄ with reduction. Right panel: Porcine cardiac myosin (1.6 μ M) was digested with proteases (200 nM, each) for 180 min under the same conditions as indicated in the left panel. Legend: p, porcine plasmin; mp, porcine des-kringle_{1–4}-plasmin; up, uPA; tp, tPA; el, porcine pancreatic elastase; ty, trypsin; th, porcine thrombin.

centrifugation, des_{1–77}-plasminogen (1 μ M) was quantitatively removed with cardiac myosin (2 μ M), while tPA (1 μ M) was partially cleared (\approx 50%).

It is also noteworthy that, for a native protein, myosin appears exceptionally sensitive to degradation by plasmin (Fig. 4). After 60 min at 22°C, digests of myosin with plasmin (13:1 mol/mol), contain at least seven discrete products and little residual myosin (Fig. 4, left panel). Among other proteases at equimolar concentrations, only trypsin catalyzes degradation at a rate comparable to plasmin or des-kringle_{1–4}-plasmin (Fig. 4, right panel).

By steady-state kinetic analysis, myosin is a high-affinity substrate for plasmin; K_M values are 17 μ M for Spectrozyme PL, 31 μ M for myosin rod, and 3.2 μ M for myosin. Des-kringle_{1–4}-plasmin (miniplasmin), having a K_M of 3.9 μ M with myosin, degrades myosin somewhat more extensively than plasmin (Fig. 4, right panel).

4. Discussion

Complex and protracted, the healing process that follows injury requires catabolism of proteins deposited both from plasma and from necrotic cells. Like fibrin, the contractile proteins are by nature insoluble and so would be expected

to comprise a major portion of the detritus arising from cellular injury. Myosin is known to be sensitive to proteolytic degradation [18,19]; plasmin, like seven other proteases, yields three fragments from S-1 and we now find that the rod like-wise efficiently becomes highly fragmented (Fig. 4). Because myosin is a substrate for plasmin, the enzyme bound to myosin may be protected against the inhibitor system of fibrinolysis like fibrin-bound plasmin [20]. Thus, the proteolytic activities of plasmin may be very efficient under in vivo conditions. By simultaneously enhancing tPA activity while serving as a sensitive plasmin substrate, myosin shares with fibrin the dual properties of cofactor and substrate. Myosin also stimulates plasminogen activation with uPA via feedback proteolysis by plasmin (Table 1, Figs. 2 and 3). The efficiency of myosin in the facilitation of plasminogen activation by tPA is comparable to that of actin; in the presence of 5.7 μ M actin, the k_{cat}/K_M value is 9900 M^{–1} s^{–1} [9], and in our experimental system, 0.3 μ M myosin yields a value of 8600 M^{–1} s^{–1} (Table 2).

Millimolar-range concentrations of 6-aminohexanoate facilitate native plasminogen activation via conformational modification of zymogen [2], whereas in micromolar concentrations this amino acid and other amines inhibit the cofactor activity of fibrin [3], endothelial extracts [6] and denatured proteins [8] in the plasminogen-tPA reaction. Thus, all proteins known to have a capacity to accelerate plasminogen activation by tPA, now including myosin, lose this capacity in the presence of 6-aminohexanoate. As a cofactor for plasminogen activation, myosin sediments with the aggregate fractions and binds both plasminogen and tPA. These findings provide a plausible function for plasmin, tPA and possibly uPA in regulated general proteolysis at sites of injury. Myosin and actin occur in most cells, but are especially abundant in smooth muscle, skeletal muscle, and cardiac muscle cells and in platelets, all prevalent participants sites of trauma, infarction and inflammation. If means for activating plasminogen exists in infarcted tissue, then plasmin would provide a mechanism for solubilizing the major contractile proteins. If such a pathway does occur, then the difference in the affinity (lower K_M) of myosin relative to myosin rod is significant, inasmuch as degradation of intact myosin takes precedence over later

Table 2
Impact of myosin on steady-state kinetics of des_{1–77}-plasminogen activation by tPA

Myosin (μ M)	K_M (M $\times 10^6$)	k_{cat} (s ^{–1} $\times 10^3$)	k_{cat}/K_M (M ^{–1} s ^{–1})
None	> 8		
Heart			
0.08	0.41 \pm 0.09	3.2 \pm 0.3	7800
0.16	0.77 \pm 0.25	5.3 \pm 0.6	6900
0.33	1.06 \pm 0.43	9.2 \pm 2.3	8600
Skeletal muscle			
0.1	1.04 \pm 0.32	3.2 \pm 0.5	3100
0.4	0.85 \pm 0.17	7.5 \pm 1.8	8800

Steady state kinetics of des_{1–77}-plasminogen activation were measured and calculated as detailed in the Materials and Methods. In all assays, the activation rate was linear for 30 min. Without myosin, there is no saturation of tPA (60nM) up to 8 μ M des_{1–77}-plasminogen.

fragments, while the fragments maintain constant tPA cofactor activity.

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