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Structure and function of microplasminogen: II. Determinants of activation by urokinase and by the bacterial activator streptokinase

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Abstract

We have used a group of human microplasminogens (*mPlg*), modified by residue substitutions, insertions, deletions, and chain breaks (1) to study the determinants of productive interactions with two plasminogen activators, urokinase (*uPA*), and streptokinase (*SK*); (2) to explore the basis of species specificity in the *zymogen-SK* complex activity; and (3) to compare active *SK* complex formation in *mPlg* and microplasmin (*mPlm*). Modifications within the disulfide-bonded loop containing the activation site and the adjacent hexadecapeptide upstream sequence showed that *uPA* recognition elements encompassed R_{29} at the activation site and multiple elements extending upstream to perhaps 13 residues, all maintained in specific conformational register by the surrounding pairs of disulfide bonds. A generally parallel pattern of structural requirements was observed for active *zymogen-SK* complex formation. Changes within the loop downstream of the activation site were tolerated well by *uPA* and poorly by *SK*. The introduction of selected short bovine (*Plg*) sequences in human *mPlg* reduced the activity of the resulting SK complexes. The requirements for active SK complex formation are different for *mPlg* and *mPlm*.

Keywords: microplasminogen; microplasmin; plasminogen activator; streptokinase; urokinase

Plasminogen is the principal serine protease zymogen in the extracellular fluids of vertebrates, and its active form – plasmin – is implicated in pericellular proteolysis associated with a wide range of physiological and pathological processes. Plasminogen is normally recruited for extracellular proteolysis by regulated cellular secretion of plasminogen activators. Two *PA*s are known in vertebrate organisms, urokinase, and tissue plasminogen activator, the latter, on present knowledge, restricted to placental mammals. Both *uPA* and *tPA* are serine proteases belonging to the chymotrypsinogen family of enzymes; both activate *Plg* by proteolysis at a single site within the polypeptide chain, and both are among the most specific and limited proteases known.

Excepting several specific macromolecular inhibitors, *Plg* is the only known efficient protein substrate for *uPA*. Detailed knowledge of the determinants of *uPA* substrate specificity would contribute to understanding the basis of specificity in limited proteolysis and might be of value in several other contexts as well.

Plg can also be activated by streptokinase (Tillett & Garner, 1933; Milestone, 1941), a bacterial protein of $M_r = 47$ kDa (De Renzo et al., 1967; Jackson & Tang, 1982). The overall mechanism by which it activates fibrinolysis is a two-stage process (Reddy & Markus, 1972): (1) *SK* first combines reversibly with a compatible *Plg* or *Plm* to form a strongly associated 1:1 complex; (2) these complexes then act as *PA*s and convert the zymogen *Plg* to the active protease *Plm* by limited proteolysis of the same R–V bond that is cleaved during activation[2] by *uPA* or *tPA*. Both *Plg* and *Plm* can form active *SK* complexes: the *Plm-SK* complex is an efficient *PA*, although *Plm* itself displays no such activity; here binding to *SK* simply modifies the substrate specificity of the preformed enzyme, *Plm*. The *Plg-SK* complex is more remarkable: in this case, *SK* reversibly induces

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Abbreviations: CNBr/HFo, cyanogen bromide in 73.5% formic acid; *PA*, plasminogen activator; *tPA*, tissue plasminogen activator; *uPA*, urokinase; *Plg*, plasminogen; *Plm*, plasmin; *mPlg*, microplasminogen; *mPlm*, microplasmin; *mPlgM*$^-$, methionineless microplasminogen; *mPlmM*$^-$, methionineless microplasmin; *mPlg-SK mPlm-SK*, the streptokinase complexes of microplasminogen and microplasmin, respectively; *SK*, streptokinase; *mPlg-B*$_1$, *mPlg-B*$_2$, *mPlg-B*$_3$, human-bovine hybrid *mPlg*s; *mPlg-M*, human-murine hybrid *mPlg*; PBS, phosphate-buffered saline; SBTI, soybean trypsin inhibitor. Amino acids are represented by the single-letter code.

[2] In this paper the term "activation" carries two connotations: it means proteolytic cleavage of *mPlg* when mediated by urokinase; with streptokinase it signifies the appearance of an active enzymatic site, without chain cleavage, in the *zymogen-SK* complex.

in the zymogen a typical serine protease active site that expresses characteristic amidolytic and *PA* activity (McClintock & Bell, 1971; Reddy & Markus, 1972). Active site formation is achieved without covalent modification of either *Plg* or *SK* and, hence, in the absence of any of the structural changes, such as the appearance of a new N-terminus and formation of a salt bridge, that ordinarily accompany proenzyme activation in the chymotrypsinogen family of proteases. Another property of active complex formation is species specificity. For example, *SK* preparations that efficiently activate human, monkey, and cat plasminogens are inert with bovine, sheep, swine, mouse, or rat plasminogens (Marcum & Kline, 1983; Schaller & Rickli, 1988), and it is clear that species specificity is a function of the *Plg* in the *Plg-SK* complexes rather than the *Plg* substrate undergoing cleavage by the complex. Because all of the mammalian *Plgs* show a high degree of sequence homology, elucidation of the basis of species specificity might help to clarify the mechanism of active complex formation.

Having initially established that microplasminogen apparently interacted with *SK* in a manner similar to the full-length parent *Plg* (Wang et al., 1995), we wished to compare the structural determinants of *mPlg* activation by *uPA* and *SK*, and to address a number of questions concerning the *mPlg-SK* interaction, such as: (1) Do the *mPlg* and *mPlm-SK* complexes differ, for example, in their catalytic parameters, or in structural factors required for their formation? If so, can we identify significant structural determinants of active complex formation in each case? (2) Can catalytic deficiencies resulting from modifications engineered into *mPlg* or *mPlm* be complemented or repaired by complex formation with *SK*? (3) Can we identify determinants of species specificity in the *mPlg(mPlm)-SK* interaction?

We have prepared and studied a series of mutant *mPlgs* with the above questions in mind and were attentive to the unusual disulfide structures in the vicinity of the activation site (Summaria et al., 1967; Sottrup-Jensen et al., 1975) (Fig. 1).

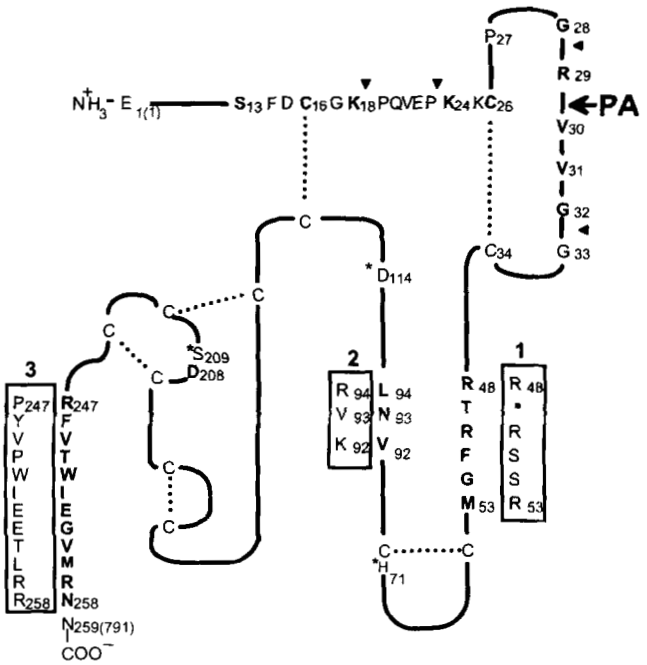


Fig. 1. Diagram of *mPlg* showing the structural modifications considered in this paper; the activation site cleaved by plasminogen activators is indicated by the arrow. Residues in bold type identify sites of modification. Solid triangles designate sites of residue insertion. The three boxed sequences shown alongside their human counterparts are from bovine plasminogen. $H_{71}D_{114}S_{209}$ are the catalytic triad.

Results

Figure 1 is a drawing of human *mPlg* that highlights the altered sites pertaining to the present work; the mutational changes are specified in the individual tables. The modifications included residue substitutions, insertions, deletions, selected chain breaks, as well some combinations of these. Two variants—1 and 4—listed in Table 1, and their substrate properties for *uPA*, have been described in a previous report (Wang et al., 1995).

Constructs in which M was substituted for other residues, or that were intended for M shuffling and site-directed proteolysis, were prepared in the methionineless background of $mPlgM^-$ (variant 4). As a substrate for *uPA*, this protein was indistinguishable from wild-type *mPlg*, which provided the genetic background for all other constructs.

Activation by uPA

The results of a limited number of preliminary observations had suggested that *uPA* tolerated changes downstream of the activation site without large perturbations of its catalytic parameters but was less accommodating with respect to upstream changes. A substantial fraction of the variants considered here were therefore designed to alter either the relative position or the conformation of residues in the C_{16}–C_{34} interval and, thereby, either the distance or the stereochemical relationship between R_{29} at the activation site and other elements in the surrounding sequence. Each construct was cloned, purified, tested as substrate for *uPA*, and assayed, using quantitative chromogenic assays of the *mPlm* product as described elsewhere (Wang et al., 1995). This procedure became unsatisfactory where the *mPlm* activity generated was less than 1% of that found with wild-type *mPlg* (variant 1, Table 1). In several cases (see below), the *mPlm* activity fell below this threshold either because the *uPA* cleavage products were intrinsically poor catalysts or because the particular *mPlgs* in question, being poor *uPA* substrates, were insufficiently cleaved. Acceptable quantitative data being unobtainable in these instances, *uPA*-catalyzed cleavage was monitored by observing the conversion of the single-chain *mPlg* to the two-chain *mPlm* form in SDS-PAGE; here, qualitative and/or semiquantitative comparisons to the reference wild-type *mPlg* control could be based on the visually estimated intensity of Coomassie blue-stained bands.

Modification of disulfide bonds

Three C residues participating in two disulfide bonds are located in the C_{16}–C_{34} interval and two of these, C_{16} and C_{26}, were replaced by A in stepwise fashion to yield three mutants (variants 21–23), whose polypeptide structure upstream of the activation site was accordingly disordered to various extents.

All three C mutants presented a problem in isolation that was not encountered with any other of the more than 40 *mPlg* mutants isolated so far, in that the usual purification procedure yielded a population of catalytically inert proteins that appeared in SDS-PAGE as a single band of lower than expected molecu-

Table 1. *uPA activation and microplasmin amidolysis parameters of variant proteins*[a]

Variant no.	Kinetics of: Activation by *uPA* K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	Amidolysis by *mPlm* K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
I. Controls						
1. $M_{53,256}$	17.1 ± 3.4	9.5 ± 1.6	0.56	0.34 ± 0.03	42.7 ± 1.2	127.1
4. Q_{53},L_{256}[a]	17.4 ± 2.8	9.3 ± 1.8	0.53	0.41 ± 0.03	47.3 ± 2.6	115.4
4. p	18.9 ± 3.1	9.6 ± 2.2	0.51	0.45 ± 0.04	18.3 ± 1.9	40.7
II. Modification upstream of the activation site						
A. Replacement of cysteine residues						
21. $A_{16},M_{53,256}$	36.4 ± 7.1	4.8 ± 1.1	0.13	2.51 ± 0.2	10.8 ± 0.4	4.29
22. $A_{26},M_{53,256}$	Very low cleavage, see Figure 2			1.71 ± 0.1	1.73 ± 0.1	1.01
23. $A_{16,26},M_{53,256}$	Very low cleavage, see Figure 2			2.70 ± 0.7	0.79 ± 0.1	0.29
B. Residue substitutions upstream of activation site before (a) and after (p) chemical cleavage						
24. M_{13},Q_{53},L_{256}[a]	17.9 ± 0.4	10.1 ± 0.2	0.56	0.53 ± 0.03	40.1 ± 0.9	75.7
* p	20.2 ± 3.5	9.7 ± 1.1	0.48	0.52 ± 0.09	17.4 ± 1.8	33.2
25. M_{18},Q_{53},L_{256}[a]	23.5 ± 3.6	11.7 ± 1.4	0.50	0.48 ± 0.05	41.7 ± 1.8	87.8
* p	21.4 ± 2.3	9.1 ± 1.2	0.43	0.91 ± 0.07	22.9 ± 0.8	25.1
26. M_{24},Q_{53},L_{256}[a]	72.1 ± 11.5	1.1 ± 0.25	0.015	0.68 ± 0.02	55.0 ± 0.4	81.1
* p	No detectable activation					
6. M_{28},Q_{53},L_{256}[a]	215.9 ± 36.4	4.1 ± 1.0	0.019	0.44 ± 0.06	49.3 ± 2.1	111.8
* p	No detectable activation					
C. Insertions upstream of activation site						
27. $G_{18a},M_{53,256}$	93.9 ± 24.9	3.1 ± 0.7	0.032	2.08 ± 0.16	35.4 ± 1.3	17.2
28. $G_{23a},M_{53,256}$	446.6 ± 24.0	0.29 ± 0.02	0.0006	0.61 ± 0.15	38.9 ± 3.7	63.6
29. $G_{28a},M_{53,256}$	314.0 ± 131.7	1.1 ± 0.4	0.0034	0.46 ± 0.04	37.6 ± 1.3	81.2
30. $G_{28a,28b},M_{53,256}$	720.6 ± 92.2	0.1 ± 0.02	0.00014	0.49 ± 0.07	36.7 ± 1.9	74.1
III. Changes downstream of activation site						
A. Loop contractions						
16. $\Delta V_{30},M_{53,256}$	Very low cleavage, see Figure 3			No detectable activity		
17. $\Delta G_{32},M_{53,356}$	Very low cleavage, see Figure 3			No detectable activity		
18. $\Delta V_{30},\Delta G_{32},M_{53,256}$	Barely detectable cleavage			No detectable activity		
B. Loop expansions						
15. $G_{32a},M_{53,256}$	Very low *mPlm* activity, see Figure 3 and Wang et al. (1995)					
9. $M_{30},G_{32a},Q_{53},L_{256}$	Very low *mPlm* activity, see Figure 3 and Wang et al. (1995)					
11. $M_{31},G_{32a,32b},Q_{53},L_{256}$	Very low *mPlm* activity, see Figure 3 and Wang et al. (1995)					
C. Residue substitutions						
7. M_{30},Q_{53},L_{256}	15.6 ± 1.8	5.9 ± 0.4	0.38	4.2 ± 0.06	16.6 ± 0.1	4.0
8. M_{31},Q_{53},L_{256}	32.7 ± 8.1	6.5 ± 1.1	0.2	1.45 ± 0.02	1.9 ± 0.02	1.31
10. $G_{30},M_{53,256}$	Very low *mPlm* activity, see Figure 3 and Wang et al. (1995)					
12. $P_{30},M_{53,256}$	No detectable cleavage, see Figure 3 and variant 13, Wang et al. (1995)					
14. $M_{30},K_{31},Q_{53},L_{256}$	Very low cleavage, see Figure 3					

[a] The single-letter code for amino acids is used to indicate both the position of all methionine residues in each variant and the nature and location of other residue substitutions. For variant 4, a and p designate the values for *uPA* activation and *mPlm* amidolysis, before and after exposure to the conditions used for chemical cleavage of other variants, respectively; the more rigorous conditions used in these experiments reduced *mPlm* k_{cat} somewhat. * Denotes a variant designed for chemical cleavage in CNBr/HFo. Subscripts a and b designate insertions at the indicated sites. Δ identifies a deleted residue.

lar weight (23 kDa). This was traced to the presence of a protease in High Five culture supernatants that attacked the *mPlg* mutants during purification (the zymogens in the crude culture supernatants were undegraded) and was sensitive only to iodoacetamide among the many protease inhibitors tested. Accordingly, for these three mutants the regular protocol was modified, as outlined in the Materials and methods, by the inclusion of iodoacetamide throughout and by changing the sequence of column procedures in order to remove the offending protease. These measures did not completely eliminate the problem of mutant protein destruction, because the final product consisted of approximately equal proportions of native and partially degraded species; nonetheless, sufficient native proenzymes were obtained for catalytic characterization.

In all three of these mutants the C → A substitutions destroyed one or two disulfide bonds and generated a corresponding num-

ber of free sulfhydryl groups that were capable of reacting with the iodoacetamide used to suppress the contaminating protease. Assays of enzyme activity in the unpurified starting material showed that iodoacetamide treatment did not affect the catalytic parameters either of the *mPlg*s or *mPlm*s derived from them (data not shown).

The characteristics of the three C mutants are presented in Table 1-IIA, where the data are shown in two parts: the first corresponds to kinetic properties of the *mPlg*s as substrates for *uPA*, and the second to the catalytic parameters of the respective *mPlm*s with S2251 as substrate. Of the three C mutant proteins, only one, variant 21 ($C_{16} \rightarrow A_{16}$), showed a cleavage rate permitting assessment as a *uPA* substrate; the other two, (variants 22 and 23) were characterized by SDS-PAGE (Fig. 2). As substrates for *uPA*, all three mutants were inferior to the control *mPlg*, A_{16} (#21) only slightly, and the other two profoundly. As shown by the data for the *mPlm* forms, the disulfides were important also for the catalytic activity of the enzyme itself; here the effects of removing either disulfide were nearly equal, and roughly additive in the double mutant.

Residue substitutions and chemical cleavage upstream of the activation site (Table 1-IIB)

To explore the consequences for *uPA* catalysis of truncating the upstream sequence, M was substituted in *mPlgM*$^-$ at four positions intended as sites of subsequent chemical proteolysis; the mutant proteins were, in each case, assessed before and after chemical proteolysis, both as *uPA* substrates, and in amidolysis as the *mPlm* forms. With this group of variants, the conditions were designed to achieve complete chemical cleavage and thereby minimize any risk of heterogeneity in the substrates for, and products of, the *uPA* reaction. The more stringent than usual exposure to CNBr/HFo (4 × 1 hourly additions of 40 mg/mL CNBr) did not affect subsequent *uPA* catalysis but impaired somewhat the catalytic parameters of the resulting *mPlm* form (e.g., control *mPlmM*$^-$, variant 4, Table 1-IIA). The catalytic data for these *mPlm*s should therefore not be compared to those obtained in other experiments under different conditions of CNBr/HFo incubation.

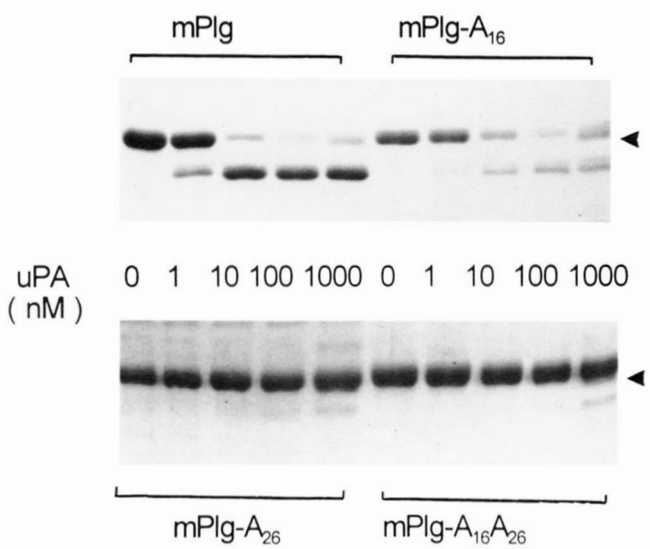


Fig. 2. Urokinase-catalyzed cleavage of *mPlg*s containing modified disulfide bonds: SDS-PAGE analysis. Each variant (final concentration 10 μM) was incubated for 15 min at 37 °C with the indicated concentration of *uPA*. The reaction was terminated by adding an equal volume of 2× sample buffer for reducing SDS-PAGE and an aliquot applied to the gel. Arrowheads identify the Coomassie blue-stained band representing *uPA* itself, which is visible only at the highest concentration of 1,000 nM. *mPlg*, variant 1; *mPlg*-A_{16}, variant 21; *mPlg*-A_{26}, variant 22; *mPlg*-$A_{16}A_{26}$, variant 23. The appearance of the *mPlm* B chain migrating ahead of the *mPlg* starting material demonstrates cleavage; the short A chain is not retained by the gel.

Variant 24. This M substitution, the furthest upstream, was neutral as regards activation by *uPA* both before and after chemical cleavage. Likewise, the amidolytic activities of the respective *mPlm*s did not differ significantly from that of the reference protein *mPlgM*$^-$ (variant 4) subjected to the same treatment.

Variant 25. This M substitution, between the two disulfide bonds, did not impair *uPA* function before chemical scission, and the effect of chemical cleavage was minor: the product was not significantly altered either as a substrate for *uPA*, or in amidolysis by the *mPlm* form.

Variant 26. This M substitution, for K_{24}, strongly reduced the substrate efficiency with *uPA*, both K_m and k_{cat} being appreciably affected, implying that K_{24} may be an important substrate recognition element for *uPA*. Amidolysis by the resulting *mPlm* was barely changed from the reference enzyme, an expected result, the entire catalytic domain remaining unmodified. Chemical cleavage eliminated the small residual substrate function for *uPA*, the cleaved protein having apparently lost the potential for activation by *uPA*.

Variant 6. The $G_{28} \rightarrow M_{28}$ change made this variant a poor substrate for *uPA*. The resulting *mPlm*, having retained a normal enzyme domain, was unchanged from the reference structure in amidolysis. Here, too, chemical cleavage yielded a protein that was inactive both as an enzyme and as a substrate for *uPA* (Wang et al., 1995).

Insertions upstream of the activation site (Table 1-IIC)

The preceding results supported the premise that *uPA* substrate character depends upon a combination of structural features, e.g., the R_{29} and one or more upstream side chains, all in proper conformational "register." As a further test of this assumption, G residues were inserted at several positions to alter the relative spacing and to perturb the conformation in different parts of the upstream sequence. Four constructs comprise this group.

Variant 27. This G insertion produced a significant drop both in substrate affinity for *uPA* and in k_{cat} of the activation reaction. It is of interest that the amidolysis parameters of the *mPlm* form were also somewhat altered, likewise in the direction of decreasing enzyme efficiency.

Variant 28. Further downstream, the insertion of G_{23a} impaired *uPA*-catalyzed activation much more profoundly, although the effect on amidolysis by the corresponding *mPlm* was only modest.

Variants 29 and 30. These two mutations drastically reduced both substrate affinity for *uPA* and k_{cat}, but the amidolysis ki-

netics of the respective *mPlm*s were not significantly affected. The latter result is not surprising because, following cleavage at the activation site, the inserted residues would be located in the free carboxyl end of the *mPlm* A chain and hence would not perturb the crosslinking function of the interdisulfide sequence.

Alterations within the activation loop downstream of the uPA cleavage site (Table 1-III; Fig. 3)

Loop contractions. Three variants (16–18) comprise this group. Loop contraction was achieved by deleting either one valine (V_{30}) or glycine residue (G_{32}), or both. Cleavage of any of these mutants at the usual site (R_{29}) would yield *mPlm*s with new N-terminal sequences shorter by one or two residues than the normal enzyme and, hence, expected to be catalytically inert (Wang et al., 1995). This expectation was confirmed experimentally and *mPlm* activity was therefore not available as a quantitative measure of *uPA* action on these proteins; their cleavage rates were monitored qualitatively by observing the products appearing on SDS-PAGE. As seen in Figure 3, deletion of a single residue within the loop greatly reduced the rate of cleavage by *uPA*; with two residues deleted, cleavage was barely detectable, even in the presence of large amounts of enzyme and after prolonged periods of incubation.

Loop expansions. We have previously (Wang et al., 1995) described two examples (variants 9 and 11) of loop expansions based on the insertion of one or two residues downstream of the activation site. Given the low catalytic activity of the corresponding *mPlm*s, these variants, whose sequence was further complicated by the presence of a residue substitution, could not be quantitatively characterized as *uPA* substrates. When examined using SDS-PAGE, these mutants, and a third loop expansion (#15) free of residue substitution, were cleaved by *uPA* at or close to control rates (Fig. 3).

Residue substitutions (Table 1-IIIC; Fig. 3). Residue substitutions downstream of the activation site have so far been limited to the new N-terminal dipeptide because the high degree of conservation of the G residues in the third (dominant) and fourth (invariant) positions suggested that they were likely to be essential for proenzyme activation. Of the five variants comprising this group (7, 8, 10, 12, 14), only two (7, 8) yielded *mPlm*s of sufficient catalytic activity to allow quantitative determination of *uPA* cleavage kinetics for their parent zymogens. The remainder were characterized as *uPA* substrates semiquantitatively by monitoring cleavage in reducing SDS-PAGE (Fig. 3).

The substitution of M for V_{30} or V_{31}, respectively, did not substantially affect *uPA* cleavage kinetics, although both of the resulting *mPlm*s were less effective enzymes than the reference protein. Of the remaining three zymogens, two (variants 10 and 14) were cleaved at rates comparable to wild-type *mPlg*, and variant 12, where P_{30} replaced V_{30}, was totally resistant to cleavage by *uPA*.

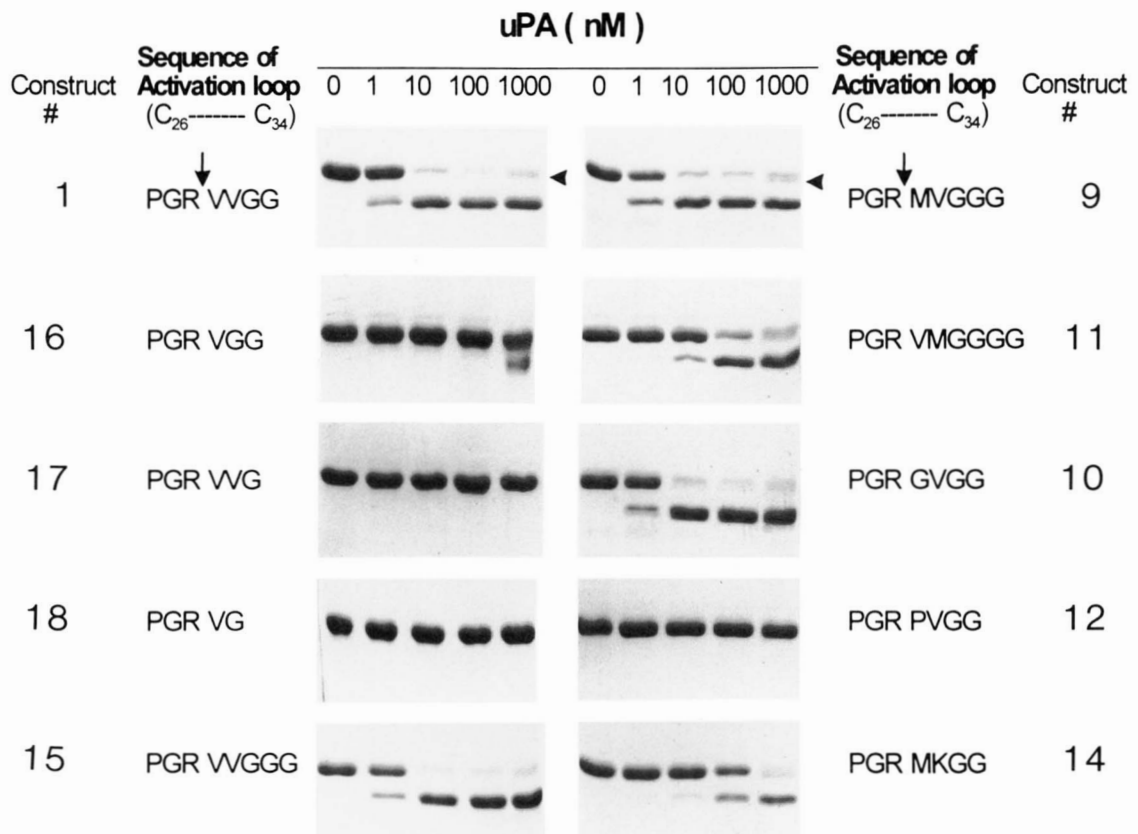


Fig. 3. Urokinase-catalyzed cleavage of selected variants modified in the activation loop: SDS-PAGE analysis. Reaction conditions and SDS-PAGE as in Figure 2. The amino acid sequence surrounding the presumptive activation site is shown alongside the stained gel pattern for each variant. Arrowheads identify the band due to *uPA* itself.

Interaction with SK

Comparison of mPlg-SK and mPlm-SK complexes

Under standard conditions of amidolysis assays, our initial comparisons of the *mPlg-SK* and *mPlm-SK* complexes for the reference variants 1 and 4 appeared unexpectedly at first not to confirm previous reports (Reddy & Markus, 1974; Robbins et al., 1981), showing amidolytic equality between the two forms; at equimolar concentrations of the respective *mPlg*s and *mPlm*s, the *mPlm-SK* complexes consistently expressed approximately twofold higher activity than their counterpart *mPlg-SK* complexes. Systematic analysis revealed that this was a fortuitous consequence of the *SK* concentration (3,000 μ/mL) used in the standard assay, because the difference disappeared when the *SK* concentration was raised, e.g., to the 10-fold higher concentration of 30,000 μ/mL. Because the two forms differ chiefly in the activation loop, this observation suggested that the loop structure might influence some properties of the *SK* complexes.

The properties of variant 5 (*mPlgM*$^{-}$-M_{29}) supported the involvement of the activation loop because the amidolytic activity associated with the $R_{29} \rightarrow M_{29}$ change (Table 2-IIIC) was significantly reduced compared to that of the complexes formed

Table 2. *Amidolysis parameters of zymogen-SK complexes*[a]

Variant no.	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
I. Controls			
1. $M_{53,256}$	0.41 ± 0.04	50.2 ± 2.0	123.3
4. Q_{53},L_{256}	0.41 ± 0.03	44.2 ± 1.1	107.8
4. * Q_{53},L_{256}	0.388 ± 0.04	25.2 ± 0.8	64.9
II. Modifications upstream of the activation site			
A. Replacement of cysteine residues			
21. $A_{16},M_{53,256}$	1.04 ± 0.13	6.67 ± 0.41	6.41
22. $A_{26},M_{53,256}$	2.06 ± 0.62	0.04 ± 0.007	0.019
23. $A_{16,26},M_{53,256}$	2.52 ± 0.15	0.008 ± 0.001	0.003
B. Residue substitutions upstream of activation site before (a) and after (p) chemical cleavage			
24. M_{13},Q_{53},L_{256}[a]	0.436 ± 0.034	65.4 ± 1.9	150.1
* p	0.464 ± 0.044	27.1 ± 1.0	58.4
25. M_{18},Q_{53},L_{256}[a]	0.520 ± 0.054	78.8 ± 3.2	151.5
* p	0.543 ± 0.024	24.6 ± 0.41	45.3
26. M_{24},Q_{53},L_{256}[a]	0.420 ± 0.04	66.2 ± 2.2	157.6
* p	Activity undetectable		
6. M_{28},Q_{53},L_{256}[a]	0.421 ± 0.05	51.2 ± 2.3	122.3
* p	Activity undetectable		
C. Insertions upstream of activation site			
27. $G_{18a},M_{53,256}$	0.894 ± 0.12	27.5 ± 1.4	30.8
28. $G_{23a},M_{53,256}$	0.703 ± 0.06	40.9 ± 1.1	58.2
29. $G_{28a},M_{53,256}$	0.873 ± 0.07	1.7 ± 0.05	1.9
30. $G_{28a,28b},M_{53,256}$	0.708 ± 0.08	33.6 ± 1.2	47.5
III. Changes downstream of activation site			
A. Loop contractions			
16. $\Delta V_{30},M_{53,256}$	1.71 ± 0.30	0.98 ± 0.11	0.57
17. $\Delta G_{32},M_{53,356}$	2.87 ± 0.65	0.13 ± 0.014	0.045
18. $\Delta V_{30},\Delta G_{32},M_{53,256}$	3.33 ± 0.48	0.04 ± 0.003	0.013
B. Loop expansions			
15. $G_{32a},M_{53,256}$	1.57 ± 0.52	0.62 ± 0.08	0.39
9. $M_{30},G_{32a},Q_{53},L_{256}$	2.17 ± 0.38	0.74 ± 0.07	0.34
11. $M_{31},G_{32a,32b},Q_{53},L_{256}$	0.98 ± 0.17	0.29 ± 0.03	0.29
C. Residue substitutions			
5. M_{29},Q_{53},L_{256}	6.86 ± 1.3	21.2 ± 2.4	3.1
7. M_{30},Q_{53},L_{256}	0.834 ± 0.12	44.0 ± 2.	52.8
8. M_{31},Q_{53},L_{256}	0.964 ± 0.13	10.3 ± 1.2	10.7
10. $G_{30},M_{53,256}$	3.18 ± 1.4	2.04 ± 0.6	0.64
12. $P_{30},M_{53,256}$	9.08 ± 2.4	4.71 ± 0.9	0.52
14. $M_{30},K_{31},Q_{53},L_{256}$	19.28 ± 4.2	17.1 ± 2.3	0.89

[a] The single-letter code for amino acids is used to indicate both the position of all methionine residues in each variant and the nature and location of other residue substitutions. For variant 4, a and p designate the values for *uPA* activation and *mPlm* amidolysis, before and after exposure to the conditions used for chemical cleavage of other variants, respectively; the more rigorous conditions used in these experiments reduced *mPlg-sk* k_{cat} somewhat. * Denotes a variant designed for chemical cleavage in CNBr/HFo. Subscripts a and b designate insertions at the indicated sites. Δ identifies a deleted residue.

by variants 1 and 4 (Table 1-I, controls). Variant 5 was convenient because, lacking R_{29} at the activation site, its *zymogen-SK* complex could be assayed in the absence of any complicating enzymatic cleavage of *mPlg*, and controlled generation of *mPlm* could be accomplished instead by chemical proteolysis in CNBr/HFo. When this was performed, as seen in Figure 4, *mPlm* (i.e., amidolysis in absence of *SK*) accumulated progressively with increasing incubation time; the activity in the presence of *SK* also increased in parallel until both reached a common plateau level, corresponding to complete cleavage of the zymogen, close to sixfold higher than the initial *SK* value. Clearly, for variant 5, the catalytic activity of the *mPlm-SK* complex was considerably higher than that of the *mPlg-SK* complex; the *mPlm-SK* value equalled that of the *mPlm* form, itself essentially identical to the reference *mPlm*s of variants 1 and 4 (Wang et al., 1995). An analogous result was obtained with another variant (Fig. 5). These findings led us to survey the *SK* interaction of additional variants that were modified either within or upstream of the activation loop.

Variants modified upstream of the activation site

Modification of disulfide bonds. The amidolysis pattern obtained from the three C variants (Table 2-IIA) resembled that found with *uPA* because: (1) the removal of either disulfide reduced both the affinity for substrate and the catalytic rate of the resulting *zymogen-SK* complex; (2) the adverse consequences of mutating the downstream disulfide ($C_{26} \rightarrow A_{26}$) were much greater than those resulting from elimination of its upstream counterpart; and (3) the double $C_{16,26} \rightarrow A_{16,26}$ replacement suppressed amidolytic activity almost completely. It seems clear that the formation of a catalytically effective *zymogen-SK* complex depends on the maintenance of specific structure for some distance upstream of the normal activation site.

Upstream residue substitution and chain scission (Table 2-IIB). As noted with activation by *uPA*, the more rigorous conditions used for chemical cleavage of this group of variants were reflected in some catalytic impairment of the control (Table 1-I, controls). The amidolysis results for this series (Table 2-IIB) were:

1. M substitution for S_{13}, K_{18}, K_{24}, or G_{28}, respectively, did not materially affect enzymatic activity; if anything, there was a modest enhancement of catalytic efficiency resulting from a slight improvement in k_{cat} for the *SK* complexes of the uncleaved zymogen forms.

2. Chemical proteolysis at positions 13 or 18 yielded truncated zymogen variants whose *SK* complexes showed only minimal decreases in K_m compared with the chemically treated control.

3. Truncation by chain cleavage at either of positions 24 and 28 abolished formation of catalytically active *SK* complexes.

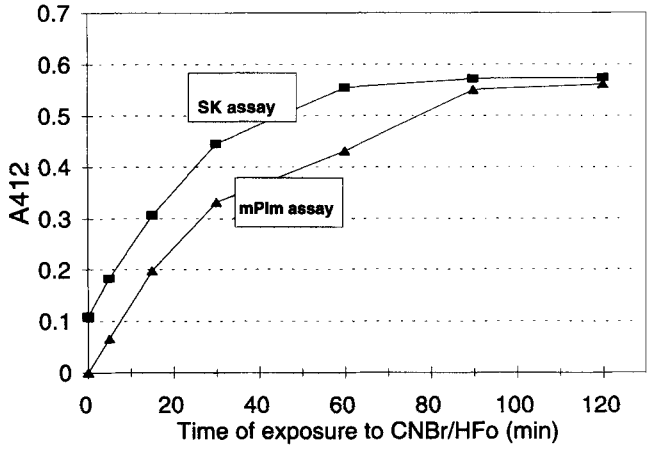


Fig. 4. Cleavage of variant 5 ($mPlgM^{-}$-M_{29}): effect on activity of *SK* complex and activation to *mPlm*.

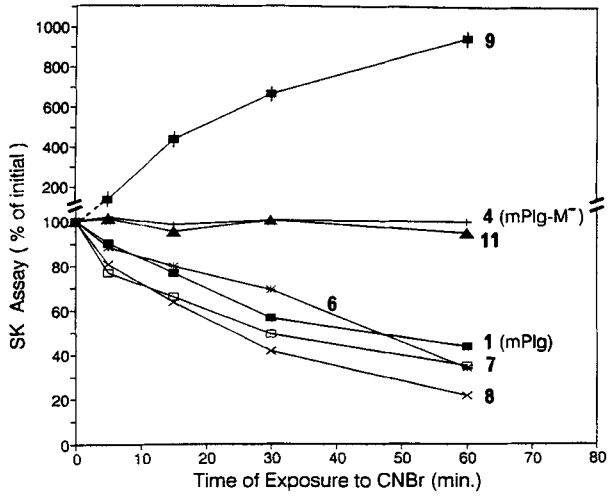


Fig. 5. Cleavage of different *mPlg* variants: effect on activity of *SK* complexes.

Upstream residue insertions (Table 2-IIC). Of the four single upstream glycine insertions only one—the G_{28a} insertion—seriously reduced the activity of the *zymogen-SK* complex, and this effect was substantially relieved upon the insertion of a second G. The inhibitory effect of the further upstream insertions was relatively modest.

Changes within the loop downstream of the activation site (Table 2-IIIA, B, C)

Loop contractions. Deletion of one or more downstream residues to shorten the activation loop profoundly depressed the amidolytic activity of the resulting *zymogen-SK* complexes. Deletion of V_{30} or G_{32} reduced activity some 200-fold and 2,500-fold, respectively; with both residues removed activity declined further. Even so, it is significant that some residual activity remained; this stands in contrast to the respective *mPlm* forms, which were completely inert (Wang et al., 1995).

Loop expansions. For all three variants of this group, the amidolytic activity of the *zymogen-SK* complexes was depressed approximately 300-fold and it is of interest that the insertion of one or two G residues gave essentially identical results. The effect of the M substitutions in variants 9 and 11 was minor; this is seen by comparing their activity levels with those of variants 7 and 8 immediately following, and with variant 15.

Residue substitutions. Residue substitutions at one or more of positions 29, 30, and 31 reduced amidolytic activity of the *zymogen-SK* complexes in all cases; the effects were mild in one case (variant 7), moderate in two cases (variants 5 and 8), and marked in the remaining three (variants 10, 12, 14). The conse-

quences of M substitution at any of the positions were relatively benign, but the presence of P_{30}, G_{30}, or K_{31} inhibited strongly.

Further comparison of various mPlg and mPlm forms

The finding of substantial catalytic differences between the *mPlg* and *mPlm-SK* complexes of variant 5 (see above, Fig. 4) prompted a comparison of the zymogen and cleaved forms of two further groups of variants (Fig. 5; Table 3). The first group consisted of proteins in which M residues were positioned to yield chemically cleaved products whose new N-termini would be modified in length, amino acid sequence, or both. The *mPlm* forms with shortened N-termini (Fig. 5, curves 6, 7, 8), resulting from cleavage of M-containing proteins to yield either shortened N-termini or N-terminal R_{29}, were all enzymatically inactive in the presence of *SK*, just as they were in its absence (Wang et al., 1995). When monitored by SDS-PAGE, the activity remaining at 60 min in Figure 5 was seen to correspond to residual uncleaved material, and it fell to zero upon completion of the reaction. For variant 9 (Fig. 5), chemical cleavage generated a new N-terminus of normal length with a V → G change yielding the sequence VGGG. Here the activity of the product *mPlm-SK* was much greater than those of the starting *zymogen-SK* complex or the free *mPlm* itself (Wang et al., 1995).

The behavior of variant 11 was of particular interest. The activity of this *zymogen-SK* was 400-fold below the control and showed no change when cleaved to the *mPlm* form (Fig. 5). Chemical cleavage generated an N-terminal tetrapeptide of normal length, but with the terminal divaline replaced by a diglycine to yield a tetraglycine sequence. The resulting free *mPlm* was enzymatically inactive (Wang et al., 1995), but a low level of amidolytic activity appeared upon addition of *SK*.

Three additional variants, designed to test the postulated role of the salt bridge in proenzyme activation (Wang et al., 1995), were assayed as *SK* complexes in both zymogen and *mPlm* forms; all three *mPlm*s expressed very low catalytic activity. In variant 13, where $R_{29}V_{30} \rightarrow M_{29}P_{30}$, the introduction of P_{30} might alter the conformation of the activation loop in the zymogen, whereas chemical cleavage, by generating new N-terminal P_{30}, would substantially modify the terminal structure in the enzyme form. As seen in Table 3, the impairment due to the mutated sequence was severe in both forms, though more pronounced in the zymogen. In the two remaining variants the other component of the salt-bridge, D_{208}, the upstream nearest neighbor of the active site serine, was mutated to N_{208} (variant 19) and A_{208} (variant 20). The activity of the respective *mPlm*s, reproduced here for purposes of comparison, was known to be very low (Wang et al., 1995), and that of the *zymogen-SK* complexes was, surprisingly, of the same order of magnitude. This might imply that D_{208} participates in some way in the process of de novo active site formation within the *zymogen-SK* complex. In contrast, the values for the *mPlm-SK* complexes were considerably higher, nearly 1,000-fold and 100-fold for variants 19 and 20, respectively, with the former reaching a level about one-seventh of the reference *enzyme-SK* complex. Here association with *SK* greatly enhanced the catalytic activity of the enzyme.

mPlg sequences contributing to species specificity in interaction with SK

Bovine *Plg* is completely inert with respect to *SK*s capable of activating human *Plg*. Thus, five regions of bovine *Plg* were selected for study, of which the three most promising have so far been substituted individually for the corresponding sequences in human *mPlg* (Fig. 1, boxes 1-3; Fig. 6). A variant containing the mouse sequence in region 1 was prepared in parallel.

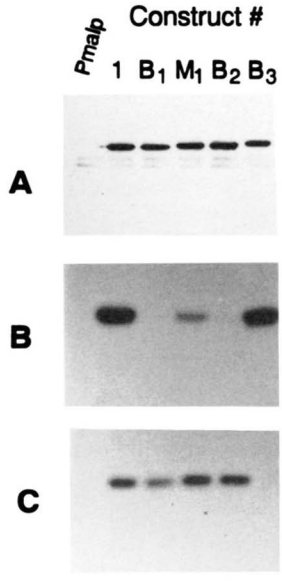
The constructs were initially cloned in *pMal-p*, expressed in *Escherichia coli* as fusion proteins, the level of expression determined by immunoblotting (Fig. 6A), and the preparations then assayed zymographically for *SK* and *uPA* interaction (Fig. 6B,C). The fusion proteins were all expressed to approximately the same level; all but variant B_3 were activated by *uPA*; with *SK*, both B_1 and B_2 were apparently inactive, and the activity of *mPlg-M* (the murine variant) was reduced. These results suggested that the sequences in regions B_1 and B_2 were not important either for recognition by *uPA*, or for active site formation in *mPlm*. The apparent impairments of *SK* complex activity were analyzed further.

To establish whether a single sequence alteration was responsible for the loss of activity with *SK*, the bovine B_1 sequence

Table 3. *Amidolysis by SK complexes: Comparison of zymogen and enzyme forms of variants mutated in salt bridge-forming residues*

	Variant 4 Q_{53},L_{256}	Variant 13 $*M_{29},P_{30},Q_{53},L_{256}$	Variant 19 M_{53},N_{208},M_{256}	Variant 20 M_{53},A_{208},M_{256}
Amidolysis by *mPlm* form				
K_m (mM)	0.41 ± 0.07	11.3 ± 3.5	3.1 ± 0.10	6.4 ± 0.41
k_{cat} (s^{-1})	47.3 ± 2.6	0.17 ± 0.04	0.19 ± 0.03	0.025 ± 0.001
k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	115.4	0.015	0.062	0.0039
Amidolysis by *zymogen-SK* complex				
K_m (mM)	0.41 ± 0.0	2.1 ± 0.25	3.2 ± 0.15	3.4 ± 0.18
k_{cat} (s^{-1})	44.2 ± 1.1	0.69 ± 0.05	0.06 ± 0.002	0.057 ± 0.002
k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	107.8	0.336	0.019	0.017
Amidolysis by *mPlm-SK* complex				
K_m (mM)	0.41 ± 0.03	0.41 ± 0.09	1.34 ± 0.09	3.39 ± 0.11
k_{cat} (s^{-1})	50.9 ± 1.2	0.80 ± 0.04	23.9 ± 0.83	6.71 ± 0.34
k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	124.1	1.95	17.82	1.98

**mPlm* generated by chemical cleavage.



Construct #	Region	Amino acid sequence
1	1 (human)	R_{48} T R F . G M H_{54}
B_1	1 (bovine)	R . R S . S R H
M_1	1 (mouse)	R T R F TG Q H
1	2 (human)	E_{91} V N L E_{95}
B_2	2 (bovine)	E K V R E
1	3 (human)	S_{246} RFVTWIEGVMRNN $_{259}$
B_3	3 (bovine)	S PYVPWIEETLRRN

. *represents a vacant position*

Fig. 6. Comparison of human, human-bovine and human-mouse hybrid *mPlg*s. A, immunoblot; B, zymogram of *SK* complexes; C, zymogram of *uPA* activation products.

was progressively substituted by the stepwise reintroduction of human elements; a comparable series of variants of the mouse sequence was also prepared, and all of the constructs reassayed. With respect to the B_1 sequence, two of the four residue differences to the human sequence, namely $F_{51} \rightarrow S_{51}$, and $G_{52} \rightarrow S_{52}$, contributed nothing detectable to the loss of *SK* reactivity; the two remaining changes (ΔT_{48}, $M_{53} \rightarrow R_{53}$) both contributed to the activity reduction, the T_{48} deletion being the more significant (data not shown). In the mouse sequence, the residue inserted at position 51a was responsible for the lowered *SK* reactivity; this was consistent with previous data showing that no significant activity changes were associated with the $M_{53} \rightarrow Q_{53}$ substitution (data not shown).

In view of the limited sensitivity of the zymographic assay, the three bovine sequence-containing variants were expressed in the baculovirus system and purified in the usual way to obtain a more quantitative description of their interactions with *SK* and *uPA*. Amidolytic activity was first measured over a tenfold range of *SK* concentration (Fig. 7A). To block amidolysis resulting from *mPlm* generated during the reaction, the assays were repeated in the presence of soybean trypsin inhibitor (Fig. 7B), which does not affect the activity of the *zymogen-SK* complex (Reddy & Markus, 1974; Friberger, 1982). For one of these bovine variants, *mPlg-*B_3, *SK*-activated amidolysis was identical to that of the control, at least at 20 μM *SK*, although the activity profile at lower *SK* values might suggest some decreased affinity of this variant for *SK*. For the other two variants, *mPlg-*B_1 and *mPlg-*B_2, amidolytic activity was much reduced throughout the *SK* concentration range, to about 15–20% of the control level.

The bovine variants were characterized further to identify the basis of the reduced amidolytic activity. Active site titration at saturating *SK* (20 μM) gave 15%, 20%, and 100% active sites, respectively, for *mPlg-*B_1, *mPlg-*B_2, and *mPlg-*B_3; the corresponding kinetic parameters for the *zymogen-SK* complexes and for the *mPlm* forms, as well as the substrate parameters for activation by *uPA* are given in Table 4. Taken together with the results illustrated in Figure 7, these findings showed the following. (1) The reduced *zymogen-SK* amidolytic activity of *mPlg-*B_1 and *mPlg-*B_2 reflected chiefly a proportional reduction in active site formation, although a slight decrease in catalytic efficiency was also noted. (2) All three bovine variants were essentially equal to the reference *mPlg* as substrates for *uPA*, but the result-

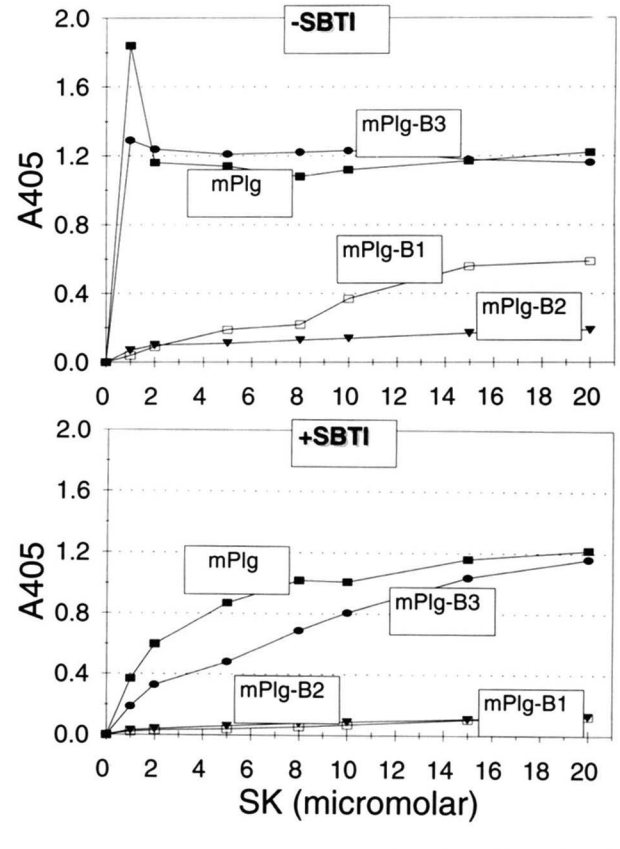


Fig. 7. Human-bovine hybrid *mPlg*s. Amidolytic activity as function of *SK* concentration, in presence and absence of SBTI.

Table 4. *uPA activation and amidolysis parameters of human-bovine hybrid mPlgs*

	Variant 1 *mPlg*	Variant 31 *mPlg-B*$_1$	Variant 32 *mPlg-B*$_2$	Variant 33 *mPlg-B*$_3$
Activation by *uPA*				
K_m (μM)	17.1 ± 3.4	12.1 ± 1.5	11.3 ± 0.8	11.9 ± 1.5
k_{cat} (s^{-1})	9.5 ± 1.6	6.1 ± 0.3	6.1 ± 0.2	8.7 ± 0.61
k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	0.56	0.50	0.54	0.73
Amidolysis by *mPlm* form				
K_m (mM)	0.34 ± 0.03	0.49 ± 0.03	0.56 ± 0.1	18.0 ± 4.1
k_{cat} (s^{-1})	42.7 ± 1.2	59.7 ± 1.2	50.2 ± 1.1	32.9 ± 6.8
k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	127.1	122.8	89.0	1.82
Amidolysis by *zymogen-SK* complex				
K_m (mM)	0.41 ± 0.04	1.8 ± 0.2	1.1 ± 0.1	0.45 ± 0.07
k_{cat} (s^{-1})	50.2 ± 2.0	79.8 ± 5.9	57.0 ± 3.1	50.2 ± 2.9
k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	123.3	45.3	52.8	112.6

ing *mPlm* forms differed, *mPlg-B*$_3$ showing some 50–70-fold lower amidolytic activity compared to the others, a difference mainly accounted for by a large change in K_m. These data confirmed the qualitative zymographic results obtained with fusion proteins expressed in bacteria (Fig. 6).

Discussion

uPA

Our results suggest that the ability of *mPlg* to serve as a substrate for *uPA* is determined by a multipoint interaction involving a combination of R_{29} and multiple upstream structures extending perhaps as far as C_{16}, all maintained in proper stereochemical register by the disulfide bonds and, perhaps, by interactions with other regions of the protein: nearly every structural modification upstream of the activation site and within the C_{16}–R_{29} interval adversely influenced the substrate behavior of *mPlg* for *uPA*. In some cases (e.g., variant 25) the effect, if any, was borderline or insignificant; in another (variant 21, $C_{16} \rightarrow A_{16}$) it was small, though real. But the remaining upstream changes, whatever their nature, affected *uPA* cleavage rates more or less strongly, some even profoundly. Further, the adverse effect of every class of modification increased, in general, as a function of proximity to the activation site.

In their investigation of *uPA* substrate requirements, Ganu and Shaw (1982) established that the isolated, synthetically prepared activation loop was not cleaved and was insufficient by itself for productive interaction with *uPA*. Our results are in accord with theirs: thus, residue substitution (variant 26), chain scission (variant 26), and G insertion (variant 14) upstream of the loop all strongly reduced cleavage by *uPA*, demonstrating the existence of essential upstream substrate recognition elements.

Although the activation loop sequence downstream of the activation site is of central importance for proenzyme activation (Wang et al., 1995) and of considerable significance for *SK* interaction, it appears at best to be a marginal determinant of *uPA* cleavage. The effects of downstream changes seemed, at first sight, to present no consistent pattern. Thus, residue substitutions (by G, M, or K) within the divaline dipeptide (positions 30 and 31), or loop expansion by elongation of the diglycine sequence due to insertion of one to two G residues, were all tolerated without significant impairment of the cleavage rate. In contrast, both the $V_{30} \rightarrow P_{30}$ substitution (variant 12) and the three examples of loop contraction (variants 16–18) all strongly reduced or even abolished cleavage. Nonetheless, in the context of *uPA* substrate specificity, we believe that the latter results should not be attributed to the results of sequence changes per se: firstly, because the effect of $V_{30} \rightarrow P_{30}$ substitution is best explained on the basis of the well-known unique structural and conformational properties of proline-containing peptides, and, secondly, because the effects of loop contraction are better understood as the indirect result of their conformational consequences for the R_{29} residue at the cleavage site. The predominant importance of upstream structure for *uPA* action is also persuasively illustrated by the contrasting consequences of two examples of loop expansion based on G insertion: insertions upstream of R_{29} drastically inhibited *uPA* cleavage, whereas the equivalent downstream insertions did not. We therefore tentatively conclude that, given a permissive (i.e., non-proline-containing) peptide bond at the activation site, substrate character for *uPA* would normally be determined solely by the combination of R_{29} and upstream structure.

Finally, we note that the disulfide bonds, which allow the polypeptide in the C_{16}–C_{26} interval to crosslink widely separated parts of the protein sequence, are probably important for stabilizing *mPlm* after normal activation by chain cleavage; fracturing the crosslink by means of disulfide disruption, whether mediated by C substitution (variants 21–23), by reduction (as in isolation of plasmin B chain [Summaria & Robbins, 1976]), or by chain scission (variant 25) substantially reduced enzyme activity. Insertion of G residues at different sites in the interdisulfide sequence produced similar effects.

SK

Most of our observations pertaining to *SK* concern the *mPlg-SK* interaction; we do not know how many will ultimately apply to *mPlm-SK*, *Plg-SK*, or *Plm-SK*. Even so, the results are of interest and can be considered from several points of view; some

of the findings were unpredictable, and a number of structural determinants of the interaction have been identified.

Structure within and upstream of the activation loop

In view of the report (Summaria & Robbins, 1976) that a functionally active complex was formed between *SK* and the isolated plasmin B-chain (which contains no structure upstream of the *Plg* activation site) it was somewhat surprising to find that the activity of the *mPlg-SK* complex was very sensitive to a variety of sequence and/or structural changes either within or upstream of the activation loop.

Concerning upstream perturbations, three points stand out. Firstly, in general, the adverse consequences resulting from any modification were greater the nearer the change was to the activation site. This applied to disulfide disruptions, chain breaks, and residue insertions. In this respect the requirements of the *zymogen-SK* interaction appeared to parallel those governing *mPlg* substrate activity for *uPA*, but the *SK* interaction was less exacting. Secondly, the profound activity reduction accompanying the $C_{26} \rightarrow A_{26}$ change and consequent disruption of the second disulfide bond and the abolition of activity following chain cleavage at either of positions 24 or 28 clearly established that some upstream structure is indispensable for active *zymogen-SK* complex formation. Thirdly, most of the changes affected amidolysis adversely.

The *SK* activity pattern differed from *uPA* chiefly in relation to changes downstream of G_{28}; here the responses spanned a wide range, varying from minor ($V_{30} \rightarrow M_{30}$) at one extreme, to significant ($R_{29} \rightarrow M_{29}$; $V_{31} \rightarrow M_{31}$), to profound at the other extreme (the remaining substitutions, loop expansions, and contractions). Evidently, the *zymogen-SK* complex tolerates changes in the activation loop poorly, because even some ostensibly minor deviation from the reference structure impaired activity of the complex. In its sensitivity to changes downstream of the activation site, e.g., variants 9, 10, 11, 15, the *zymogen-SK* complex differed clearly from *uPA*, for which these variants were excellent substrates.

We conclude that a particular conformation of the activation loop and the individual residues within it, as well as upstream protein sequence including at least position 24, are required for the formation of an active *zymogen-SK* complex.

Comparison of mPlg-SK versus mPlm-SK complex

It has long been known that active *SK* complexes are formed both by the zymogen *Plg* and the active enzyme *Plm*, and that the kinetic properties of the two are very similar (Reddy & Markus, 1974; Robbins et al., 1981). Several lines of evidence now indicate that, at least with *mPlg/mPlm*, the structural requirements for active *SK* complex formation differ, both qualitatively and quantitatively.

1. As seen above, active complex formation with *mPlg* required upstream sequence at least up to and including K_{24}, yet the free *Plm* B chain, which lacks any peptide structure preceding V_{30}, forms an active complex (Summaria & Robbins, 1976).

2. The $R_{29} \rightarrow M_{29}$ change yielded a variant *mPlg-SK* complex whose amidolytic efficiency was some 40-fold below that of the reference protein, but the values for the respective *mPlm-SK*s were essentially identical and equal to the wild-type *mPlm-SK*.

3. The zymogen forms of variants 6, 7, 8, and 14 all yielded active SK complexes, but their chemical cleavage products, with new N-termini abbreviated by one or two residues (variants 7, 8, 14) or elongated (variant 6) were enzymatically inactive both as free "*mPlm*s" and as "*mPlm*"-*SK* complexes.

We suggest that, although one distinctive catalytic specificity—plasminogen activation—is conferred on both *mPlg* and *mPlm* by *SK*, the structures needed to form active *SK* complexes are different in the two cases. (1) With the intact zymogen, *SK* directs the appearance of an active site de novo; in so doing, it compensates for the absence of the elements ordinarily associated with proenzyme activation (e.g., new N-terminus of appropriate structure and composition), but it requires particular sequence and conformation both within and upstream of the activation loop (i.e., in the C_{16}–C_{34} interval) in addition to still undefined elements elsewhere. (2) Once the zymogen has been cleaved (at or close to the activation site), upstream structure becomes irrelevant to the formation of an active site, either in the presence or absence of *SK*. Now *SK* can no longer direct the formation of an active site; rather, the emergence of an active site depends on the nature of the new N-terminus. If the preformed active site is catalytically functional, or at least potentially so (as in variant 11 following chemical cleavage), *SK* may enhance its activity and/or modulate its substrate specificity, but *SK* cannot compensate for structural restrictions at the N-terminus, (e.g., variants 6, 7, 8, 14 after chemical cleavage) that exclude the independent, spontaneous organization of an active site. The activity enhancements of several feebly active enzymes (Table 3) and of one otherwise inactive *mPlm* (variant 11, chemically cleaved, see Wang et al. [1995]) may be impressively large, but we propose that these effects are best regarded as a stabilization of marginally active enzyme structures accompanying their incorporation into an *SK* complex. For chemically cleaved variant 11 in particular, which retains the potential for salt bridge formation, *SK* complexing appeared to compensate, in part, for loss of important hydrophobic interactions normally contributed by the terminal divaline side chains (Wang et al., 1995).

Species specificity of SK interaction

This aspect of the *mPlg-SK* interaction has so far been explored by substituting three bovine *Plg* sequences consisting of 5 (B_1), 3 (B_2), and 12 (B_3) residues, respectively, for their counterparts in human *mPlg*. Of these, only the first two significantly impaired (by ~80%) the catalytic activity of the complex. We therefore presume that each of the relevant human sequences determines, to some degree, the ability of human *mPlg* to form an active *SK* complex, and that the cognate bovine sequences contribute to the inertness of bovine *Plg* toward *SK*: this suggests that structures in the enzyme domain account for most and perhaps all of the species specificity in the *Plg-SK* interaction. However, neither B_1 nor B_2 alone accounts for the total inactivity of the bovine zymogen with *SK*, and the effect of a double replacement remains to be assessed. The influence of these changes in the context of *mPlm-SK* likewise remain to be determined.

It is of interest that the reduced activity of *mPlg-*B_1 and *mPlg-*B_2 complexes at saturating *SK* concentrations can be accounted for almost entirely by a deficiency in the number of active catalytic centers. The simplest interpretation of this result is that the *mPlg-SK* complex, once formed, can exist in either of two readily interconvertible states, only one of which would be enzymatically active; in each of these cases, the fraction of active complexes at equilibrium would amount to 15–20% of the

total. If this view is correct, bovine *Plg* might actually form an *SK* complex as avidly as human *Plg* but then simply fail to express a catalytic center. Direct binding measurements can clarify this point.

Materials and methods

Materials and methods were exactly as described previously (Wang et al., 1995) except for the modifications outlined below.

Production of variant genomes

All variant sequences were produced in appropriately primed PCR reactions and with a suitable *mPlg* fragment as template. Each primer was designed to be compatible with one of the unique restriction sites in *mPlg*. The primer sequences (shown 5′ → 3′, left to right) employed for the variants not described elsewhere were as follows:

9. att aat cct gag gct tcc cag cat caa atg agg gc
10. att aat cct cag gtt gaa cct aag aaa gct ccc g
11. att aat cct gag gct tcc cac aat caa aca tgg gcc c
12. att aat cct gag cat ccc aca atc aaa tga
13. att aat cct cag gtt gaa cct atg aaa tgt cc
16. att aat ccc ggg aca ttt ctt agg ttc aac ctg agg tcc ctt ccc aca
17. att aat ccc ggg aca ttt ctt tcc agg ttc aac ctg
18. att aat ccc ggg gga agg gtt gtg ggg ggg tgt
19. att aat ccc ggg gga ggt agg gtt gtg ggg ggg tgt
37. att aat ggt gcc tcc aca gaa gtg tcg cga aga cct tct aag act gac
38. att aat aac atg ctg ttc gcg aac ctt ttc ttg gtg tgc acc
39. att aat tct aga gct agc tta att acg tct cag cac ttc ctc aat cca agg aac ata cgg tga aac
40. att aat ggt gcc tcc aca gaa gtg ctg acc ggt aaa c

Primers 9 and 10 were used for variants 21, 22, 23; primers 11, 12, 13 for variants 24, 25, and 26, respectively; 16, 17, 18, 19 for variants 27, 28, 29, and 30, respectively. Primers 37 and 40 were for variants B_1 and M, respectively; 38 was for variant B_2; 39 for variant B_3. All variant sequences were verified by DNA sequencing of the recombinant vector pJVP10Z-*mPlg* and the relevant region of recombinant baculoviral DNA.

Purification of variants containing modified disulfide bonds

The regular isolation procedure was modified as follows: High Five cell expression medium was made 100 mM in iodoacetamide, and all the subsequent steps for purification were performed in the presence of 10 mM iodoacetamide. The sequence of column procedures was changed, all the buffer conditions remaining the same except for the presence of 10 mM iodoacetamide. The heparin-agarose column was used as the first step, and the eluted protein was dialyzed against 10 mM cacodylate, passed through the DEAE column before adsorbing to Toyopearl-CM. The C mutants were eluted from the CM column, dialyzed, and lyophilized as in the regular procedure.

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References

Christensen LR. 1945. Streptococcal fibrinolysis: A proteolytic reaction due to a serum enzyme activated by streptococcal fibrinolysin. *J Gen Physiol 28*:363–383.

De Renzo EC, Siiteri PK, Hutchings BL, Bell PH. 1967. Preparation and certain properties of highly purified streptokinase. *J Biol Chem 242*(3): 533–542.

Friberger P. 1982. Chromogenic peptide substrates: Their use for the assay of factors in the fibrinolytic and plasma kallikrein-kinin system. *Scan J Clin Lab Invest 42*(suppl 162):15–23

Ganu VS, Shaw E. 1982. Synthesis and properties of cyclic peptides containing the activation site of plasminogen. *Int J Pept Protein Res 20*:421–428.

Jackson KW, Tang J. 1982. Complete amino acid sequence of streptokinase and its homology with serine proteases. *Biochemistry 21*(26):6620–6625.

Kline DL. 1953. The purification and crystallization of plasminogen (profibrinolysin). *J Biol Chem 204*:949–955.

Marcum JA, Kline DL. 1983. Species specificity of streptokinase. [Review]. *Comparative biochemistry & physiology 75B*(3):389–394.

McClintock DK, Bell PH. 1971. The mechanism of activation of human plasminogen by streptokinase. *Biochem Biophys Res Commun 43*(3):694–702.

Milestone H. 1941. A factor in normal human blood which participates in streptococcal fibrinolysis. *J Immunol 42*:109–116.

Reddy KNN, Markus G. 1972. Mechanism of activation of human plasminogen by streptokinase. Presence of active center in streptokinase-plasminogen complex. *J Biol Chem 247*(6):1683–1691.

Reddy KNN, Markus G. 1974. Esterase activities in the zymogen moiety of the streptokinase-plasminogen complex. *J Biol Chem 249*(15):4851–4857.

Robbins KC, Summaria L, Wohl RC. 1981. Human plasmin. *Methods Enzymol 80*:379–387.

Schaller J, Rickli EE. 1988. Structural aspects of the plasminogen of various species. *Enzyme 40*:63–69.

Sottrup-Jensen L, Zajdel M, Claeys H, Petersen HE, Magnusson S. 1975. Amino-acid sequence of activation cleavage site in plasminogen: Homology with "pro" part of prothrombin. *Proc Natl Acad Sci USA 72*(7):2577–2581.

Summaria L, Hsieh B, Robbins KC. 1967. The specific mechanism of activation of human plasminogen to plasmin. *J Biol Chem 242*(19):4279–4283.

Summaria L, Robbins KC. 1976. Isolation of a human plasmin-derived, functionally active, light (B) chain capable of forming with streptokinase an equimolar light (B) chain-streptokinase complex with plasminogen activator activity. *J Biol Chem 251*(18):5810–5813.

Tillett WS, Garner RL. 1933. The fibrinolytic activity of hemolytic streptococci. *J Exp Med 58*:485–502.

Wang J, Brdar B, Reich E. 1995. Structure and function of microplasminogen: I. Methionine shuffling, chemical proteolysis, and proenzyme activation. *Protein Sci 4*:1758–1767.