Function of streptokinase fragments in plasminogen activation

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Several peptide fragments of streptokinase (SK) were prepared by incubating SK with immobilized human plasmin (hPlm) and purified by h.p.l.c. with a reverse-phase phenyl column. The N-terminal sequences, amino acid compositions and molecular masses of these peptide fragments were determined. The SK peptide fragment of 36 kDa consisting of Ser^{60}–Lys^{387} (SK-p), was the only peptide fragment that could be tightly bound to immobilized hPlm. Another three large SK peptide fragments, SK-m, SK-n and SK-o, with molecular masses of 7 kDa, 18 kDa and 30 kDa, and consisting of Ile^{1}–Lys^{59}, Glu^{148}–Lys^{333}, Ser^{60}–Lys^{333} respectively, were also obtained from the supernatant of the reaction mixture. The purified SK-p had high affinity with hPlm and could activate human plasminogen (hPlg) with a k_{Plg} one-sixth that of the native SK. SK-o had low affinity with hPlm and could also activate hPlg, although the catalytic constant was less than 1 % of the native SK. SK-n, as well as SK-m, which is the N-terminal 59 amino acid peptide of the native SK, had no activator activity. However, SK-m could enhance the activator activity of both SK-o and SK-p and increase their second-order rate constants by two- and six-fold respectively. It was concluded from these studies that (1) SK-o, the Ser^{60}–Lys^{333} peptide of SK, was essential for minimal SK activator activity, (2) the C-terminal peptide of SK-p, Ala^{334}–Lys^{387}, was essential for high affinity with hPlm, and (3) the N-terminal 59-amino-acid peptide was important in maintaining the proper conformation of SK to have its full activator activity.

INTRODUCTION

Streptokinase (SK), a simple polypeptide of 415 amino acid residues containing no disulphide bonds, is a secretory protein of Lancefield group C *Streptococcus* [1,2]. It is now widely used as a thrombolytic agent in treatment of acute myocardial infarction. SK forms complexes with plasminogens (Plgs) of some mammalian species, including human and monkey [3–9]. It was suggested that SK, after binding to the catalytic domain of human plasminogen (hPlg), would induce conformational changes and cause the rearrangement of the catalytic domain [4–6]. The complex of SK and human plasmin (HPlm) is the functional activator that converts Plgs of various animal sources into plasmin (Plm) by the hydrolysis of a specific activating peptide bond of Plg, such as Arg^{560}–Val^{561} of hPlg [3,7,10–16]. Plm or Plg does not autoactivate itself. However, in the absence of SK, non-specific autolysis of hPlm leading to enzyme inactivation was commonly observed [13,14]. Therefore, SK not only induces rearrangement of the catalytic groups in the Plg zymogen molecule and renders Plg catalytically active, but also alters its enzyme specificity so that the activating peptide bond of Plg could be hydrolysed by the activator complex more specifically [10–13].

Although the 36-kDa fragment of SK and other lower-molecular-mass SK fragments were observed in the reaction of SK with Plm, and only the large SK fragment formed in short-time reaction with hPlg can activate hPlg [15,17], there has been no report on the purification and kinetic studies of these peptide fragments. It was demonstrated that the protease active-site domain of hPlg or hPlm was directly involved in the formation of activator complex [10–12,18–20]. However, the exact location of SK involved in this activator complex formation is not known. In the study reported here several purified SK fragments were prepared, and their composition and function studied. The possible reaction sites of SK-peptide fragments with hPlm and hPlg were suggested.

EXPERIMENTAL

Materials

S-2251, urokinase and guanidine hydrochloride were obtained from Sigma. Guanidine hydrochloride was purified by recrystallization from hot methanol. Acetonitrile was obtained from Merck. Trifluoroacetic acid was from Pierce. Blue-Sepharose CL 6B, Sepharose 4B, and CNBr-activated Sepharose 4B were purchased from Pharmacia LKB Biotechnology Inc. All other chemicals were of the highest grade commercially available.

Proteins and enzymes

HPlg was prepared from pooled human plasma by a modification of the Deutsch and Mertz method [21]. Forms 1 and 2 of native human Glu–Plg (i.e. native Plg consisting of Glu^{1}–Asn^{790}) were separated by chromatography on a Lys–Sepharose column [22]. Form 2 of hPlg was used throughout the experiment. HPlm was prepared by activating hPlg with Sepharose-bound urokinase as previously described [11]. Bovine plasminogen (bPlg) was purified from fresh citrated bovine plasma by the same affinity-chromatography method. SK (Behringwerke AG, Marburg, Germany) was further purified by passing through a Blue-Sepharose CL 6B column (0.9 cm × 40 cm) to remove serum albumin [15].

Protein concentration

The protein concentrations were determined spectrophotometrically using the following $A^{1\%}_{280\,nm}$ values and molecular masses respectively: hPlg and bPlg, 17.0 and 84 kDa [23,24]; hPlm, 17.0 and 76.5 kDa [24]; SK, 9.5 and 45 kDa [25]. The amount of

Abbreviations used: Plg, plasminogen; Plm, plasmin; hPlg and bPlg, human and bovine plasminogens respectively; hPlm and bPlm, human and bovine plasmins respectively; SK, streptokinase.

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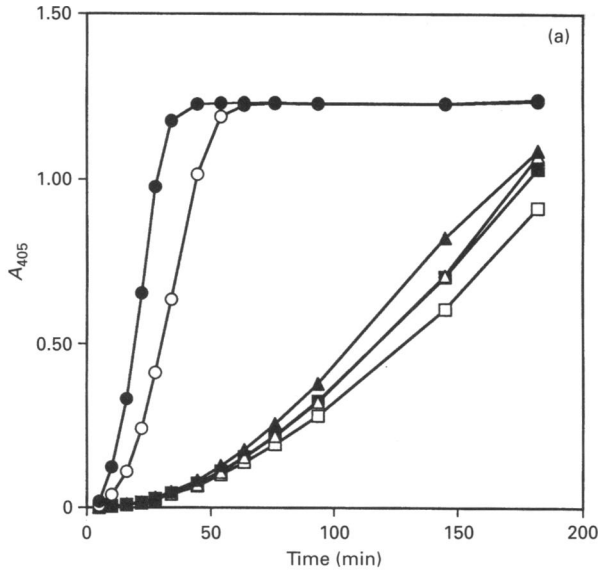

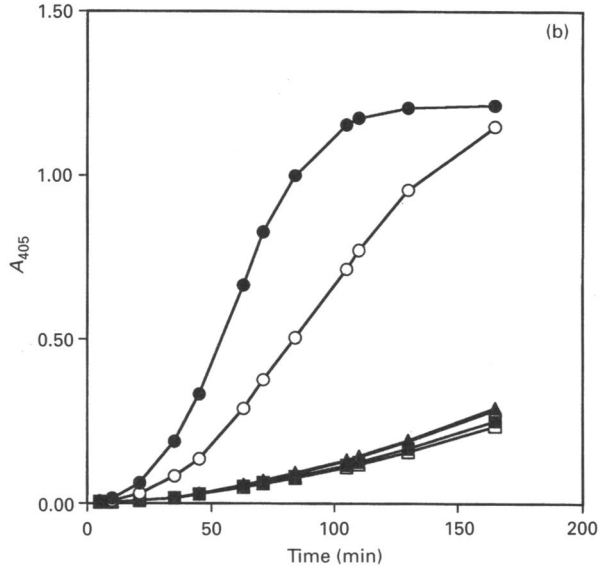

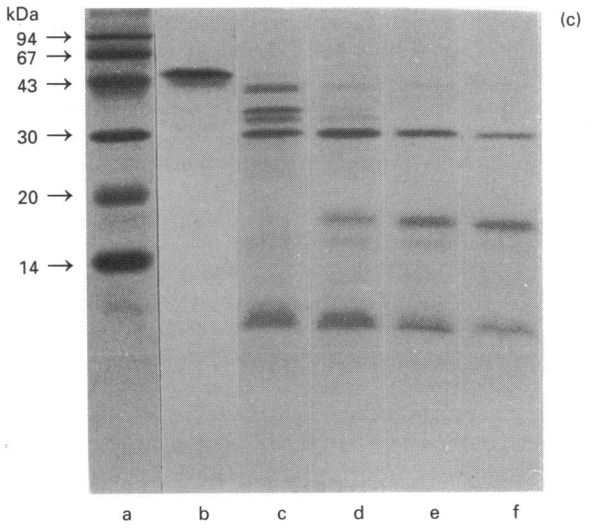

protein on the immobilized gel was determined by amino acid composition analysis. Immobilized hPlg or hPlm (0.1 g) was washed extensively with 30 ml of distilled water, vacuum-dried and hydrolysed in 6 M HCl (Sequanal-grade, Pierce Chemical Co.) at 110 °C for 24 h. Amino acid analysis was performed on a Beckman Model 6300 amino acid analyser. The active concentration of immobilized hPlm was determined by measuring its amidolytic activity according to a standard hPlm calibration curve. Active-site concentration of hPlm was determined by the *p*-nitrophenyl-*p'*-guanidinobenzoate burst titration [11,26]. In all cases, at least 90 %-active immobilized hPlm was obtained.

Preparation of protein-substituted Sepharose

HPlm (6 mg) was coupled to CNBr-activated Sepharose 4B (1 g, from Pharmacia LKB Biotechnology Inc.) in coupling buffer containing 0.1 M $NaHCO_3$/0.5 M NaCl (pH 8.3) at 4 °C overnight. The gel was washed with 1 M ethanolamine in 0.1 M $NaHCO_3$/0.5 M NaCl (pH 8.0) to remove the residual active groups. To remove excess adsorbed protein, the gel was further washed with coupling buffer (pH 8.3) and 0.1 M acetate buffer/0.5 M NaCl (pH 4.0) alternately.

Interaction of immobilized hPlm and SK

Immobilized hPlm (5 μM) and SK (5 μM) were incubated in 1 ml of 0.05 M phosphate/0.1 M NaCl/0.02 M lysine/25 % (v/v) glycerol buffer (pH 6.5) at 25 °C for various durations. Aliquots of the reaction mixture supernatant were collected at intervals, diluted and assayed for hPlg and bPlg activator activity. Diluted samples at a final concentration of 2 nM and hPlg (0.19 μM) were added to wells of a flat-bottomed 96-well polystyrene microtitre plate containing 0.2 mM S-2251 (NH_2-D-Val-Leu-Lys-*p*-nitroanilide) in 50 mM Tris/HCl (pH 7.4) containing 0.1 M NaCl and 0.01 % Tween 80 at a total volume of 250 μl. The plate was incubated at 37 °C and the absorbance change at 405 nm was measured with a Molecular Devices Thermomax Microplate Reader. For the bPlg activator activity measurement, bPlg (0.19 μM) was incubated with SK-peptide fragment premixed with hPlg (at a final concentration of 2 nM) for 1 min, and the activator activity was measured in the same way as for hPlg. Parallel samples were also taken for SDS/PAGE.

Preparation of SK-peptide fragments

Immobilized hPlm (10 μM) and SK (5 μM) were incubated in 1 ml of 0.05 M phosphate/0.1 M NaCl/0.02 M lysine/25 % (v/v) glycerol buffer (pH 6.5) at 25 °C for 90 min. The reaction mixture supernatant was collected and applied to an h.p.l.c. column. The immobilized gel was washed with 20 ml of phosphate buffer (pH 6.5) to remove the unbound peptide fragments and incubated in 1 ml of 6 M guanidine hydrochloride/0.25 M Tris/0.003 M EDTA (pH 8.6) for 30 min. After incubation the dissoci-

Figure 1 The hPlg activator activity (a), bPlg activator activity (b) and SDS/PAGE pattern (c) of the reaction mixture supernatant of immobilized hPlm and SK

Immobilized hPlm (5 μM) and SK (5 μM) were incubated in 1 ml of 0.05 M phosphate/0.1 M NaCl/0.02 M lysine/25% glycerol buffer (pH 6.5) at 25 °C for various durations. Samples of the reaction mixture supernatant were collected at 0 (●), 10 (○), 30 (▲), 60 (△), 103 (■), and 130 (□) min. Diluted samples at a final concentration of 2 nM were assayed for hPlg and bPlg activator activity as described in the Experimental section. For the bPlg activator activity measurement, samples were premixed with 2 nM hPlg for 1 min before incubating with bPlg. Parallel samples were also taken for SDS/PAGE as shown in (**c**). Lane a, molecular-mass marker; lane b, 0 min; lane c, 10 min; lane d, 30 min; lane e, 60 min; lane f, 103 min.

ated peptide fragments were applied to the same h.p.l.c. column. The amount of SK-peptide fragment eluted from the h.p.l.c. column was determined by amino acid composition analysis.

Peptide analysis by h.p.l.c.

H.p.l.c. was carried out on a μ-Bondapak phenylalkyl column (0.4 cm × 30 cm) in a Beckman system Gold. Solvent A was 0.05% trifluoroacetic acid in water while solvent B was 0.05% trifluoroacetic acid in acetonitrile. Sample volume was 100 μl. The detector was set at 230 nm. The flow rate was kept constant at 0.8 ml/min. A linear acetonitrile gradient to 60% solvent B was run over 60 min for the separation of the 36 kDa SK-peptide fragment (SK-p). For the separation of SK-peptide fragments recovered from the reaction mixture supernatant, a linear gradient to 37% solvent B was established over 37 min, followed by isocratic elution at 37% solvent B for 20 min.

SDS/PAGE analysis

SK-peptide fragments eluted from the h.p.l.c. column were processed with a EYELA Centrifugal Vaporizer CVE-2000D to evaporate the solvent and were subjected to SDS/PAGE as described by Laemmli [27].

Sequence analysis

Sequence determination was carried out by Edman degradation in an Applied Biosystems Sequencer (Model 477A).

Steady-state kinetic parameters of activation of hPlg by SK fragments

SK-peptide fragments eluted from the h.p.l.c. column were collected in Eppendorf tubes, the solvent was removed by evaporation, and they were then dissolved in 50 μl of 50 mM Tris/HCl (pH 7.4) containing 0.1 M NaCl and 0.01% Tween 80. The method used to measure the kinetics of human Glu–Plg activation by SK fragments was as previously described [19,28]. This method was classified as a one-stage assay. Briefly, hPlg at final concentrations ranging from 0.04 to 4 μM was incubated with 0.5 mM S-2251 in an assay cuvette containing 1.0 ml of 0.05 M Tris buffer (pH 7.4) and 0.1 M NaCl. Activation was initiated by adding SK fragment of fixed concentration, and the change in absorbance at 405 nm was monitored at 37 °C in a Hitachi 330 spectrophotometer. The increments of absorbance between 5 and 360 s after addition of Plg activator were used to measure the initial rate of hPlg activation. Initial reaction rates were determined from the slopes of plots of absorbance versus t^2 and double-reciprocal plots were then constructed. HPlg activation parameters, K_{Plg} (the apparent Michaelis constant for the hPlg substrate) and k_{Plg} (the catalytic rate constant of activation), were calculated as described [28].

RESULTS

Fragments of SK which had Plg activator activity were observed in reaction of SK with hPlm in solution. In this study immobilized hPlm was used to prepare SK fragments to avoid contamination by hPlm and its autolytic fragments in solution, so that the purification process could be simplified. The Plg activator activity of SK in reaction with immobilized hPlm declined as SK was gradually degraded (Figures 1a and 1b). The SK molecule was cleaved into peptide fragments, and the peptides of 36, 33, 32, 30 and 7 kDa were found in the supernatant after 10 min of incubation (Figure 1c). The 36 kDa peptide fragment decreased, while peptides of 30, 18 and 7 kDa increased, when the reaction proceeded for more than 30 min (Figure 1c). Slight decline of the large SK fragments was observed up to 103-min incubation (Figure 1c). The SK fragments in the supernatant, as well as on the gel of immobilized hPlm, were purified by h.p.l.c. (Figure 2). A typical h.p.l.c. pattern is shown in Figure 2. Eighteen peptides, a to p, were identified on the chromatogram and analysed for N-terminal sequences and amino acid compositions (Figure 3, Table 1). The molecular masses of the SK-peptide fragments were determined by SDS/gel electrophoresis as well as by amino acid compositions (Figure 3, Table 1). The major peptide retained by the immobilized hPlm and eluted out in 6 M guanidine hydrochloride solution was SK-p. The other peptides could all be recovered in the supernatant. Analysis of the N-terminal amino acid sequences at various time points revealed the order of the

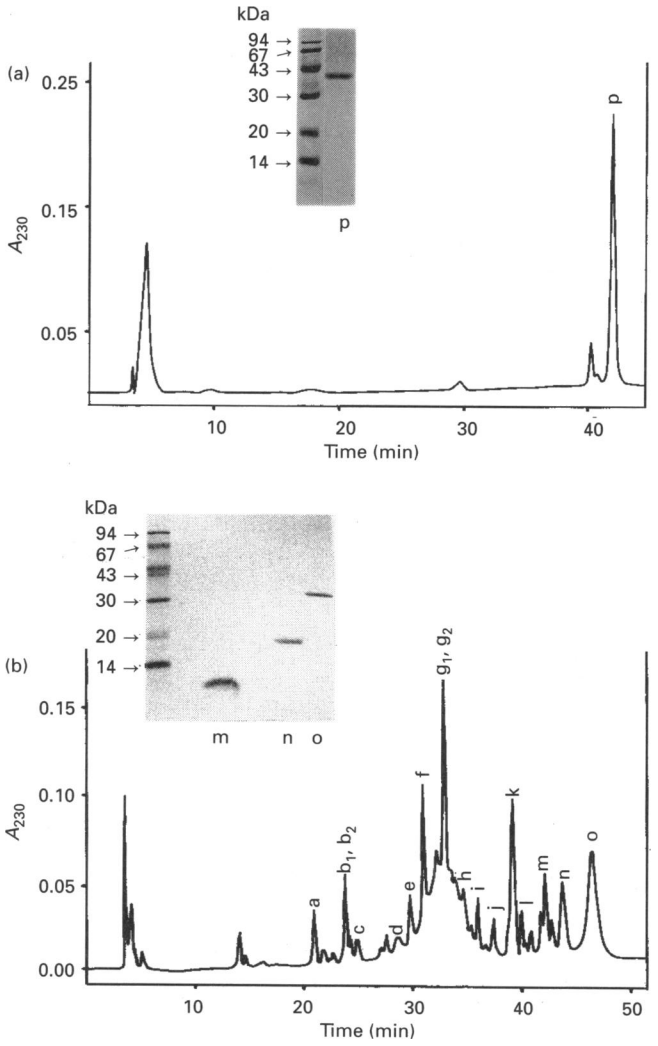

Figure 2 H.p.l.c of SK peptide fragments after reacting with immobilized hPlm

(**a**) Peptide fragments associated with the immobilized gel. (**b**) Peptide fragments in the supernatant of the reaction mixture. SK (5 μM) was incubated with immobilized hPlm (10 μM) at 25 °C for 90 min. The SK-peptide fragments were separated by h.p.l.c. The acetonitrile gradient systems used were as described in the Experimental section. The molecular masses of SK-m, SK-n, SK-o and SK-p were 7, 18, 30 and 36 kDa respectively, as analysed by SDS/PAGE (inset).

Table 1 Amino acid composition of SK-m, SK-n, SK-o and SK-p

The results represent mol of amino acid/mol of peptide after hydrolysis with 6 M HCl/0.1% phenol at 110 °C. The composition was normalized to one residue of histidine for SK-m, 18 residues of lysine for SK-n, 24 residues of lysine for SK-o, and eight residues of histidine for SK-p. The number of residues/mol of peptide (in parentheses) was calculated based on the amino acid sequences for SK [2] from residues Ile^{1}–Lys^{59} for SK-m, from residues Glu^{148}–Lys^{333} for SK-n, from residues Ser^{60}–Lys^{333} for SK-o, and from residues Ser^{60}–Lys^{387} for SK-p. Abbreviation: ND, not determined.

	Content (no. of residues/mol of fragment)			
Amino acid	SK-m	SK-n	SK-o	SK-p
Asp	6.74 (6)	30.33 (32)	41.70 (42)	52.58 (53)
Thr	2.64 (4)	12.54 (14)	18.16 (19)	20.23 (23)
Ser	4.93 (6)	7.67 (10)	18.26 (17)	14.20 (18)
Glu	7.17 (7)	24.22 (22)	34.39 (30)	35.70 (32)
Pro	3.42 (4)	10.62 (9)	13.95 (13)	14.93 (14)
Gly	5.13 (6)	8.59 (5)	11.02 (9)	14.40 (13)
Ala	3.02 (3)	8.07 (7)	16.37 (14)	18.69 (18)
Val	4.46 (5)	8.64 (8)	15.49 (15)	17.16 (17)
Met	0.00 (0)	0.38 (1)	1.02 (2)	1.97 (4)
Ile	2.74 (3)	13.03 (12)	15.28 (16)	17.12 (19)
Leu	6.21 (6)	16.85 (20)	24.55 (29)	31.61 (34)
Tyr	0.00 (0)	10.22 (10)	12.56 (13)	17.00 (18)
Phe	2.40 (2)	6.77 (7)	9.83 (12)	12.70 (13)
Lys	3.43 (3)	17.00 (18)	24.00 (24)	27.63 (28)
His	1.00 (1)	3.06 (3)	6.69 (6)	8.00 (8)
Arg	2.40 (2)	9.04 (10)	11.27 (13)	14.88 (15)
Trp	ND (1)	ND (0)	ND (0)	ND (0)

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        326
a:      Asp-Leu-Tyr-Asp-Pro-Arg-Asp-Lys-

        403
b1:     Tyr-Thr-Gly-Thr-Pro-Ile-Pro-Asp-Asn-Pro-Asp-Asp-Lys-

        311
b2:     Ser-Glu-Gln-Leu-Leu-Thr-Ala-Ser-Glu-Arg-Asn-

        210
c,g2:   Asn-His-Pro-Gly-Tyr-Thr-Ile-Tyr-Glu-Arg-Asp-Ser-Ser-Ile-Val

        258
d:      Ser-Gly-Leu-Asn-Glu-Glu-Ile-Asn-Asn-Thr-Asp-Leu-Ile-Ser-

        300
e,i:    Tyr-Val-Asp-Val-Asp-Thr-Asn-Glu-Leu-Leu-Lys-Ser-Glu-Gln-

        365
f:      Ile-Ile-Thr-Val-Tyr-Met-Gly-Lys-Arg-Pro-Glu-Gly-Glu-Asn-Ala-

        388
g1:     Asp-Arg-Tyr-Thr-Glu-Glu-Glu-Arg-Glu-Val-Tyr-Ser-Tyr-Leu-

        233
h:      Thr-Ile-Leu-Pro-Met-Asp-Gln-Glu-Phe-Thr-Tyr-

        113
j:      Asn-Gly-Lys-Val-Tyr-Phe-Ala-Asp-Lys-Asp-Gly-Ser-Val-Thr-

        334
k,l:    Ala-Lys-Leu-Leu-Tyr-Asn-Asn-Leu-Asp-Ala-Phe-Gly-Ile-Met-

        1
m:      Ile-Ala-Gly-Pro-Glu-Trp-Leu-Leu-Asp-Arg-Pro-Ser-Val-Asn-

        148
n:      Glu-Lys-Pro-Ile-Gln-Asn-Gln-Ala-Lys-Ser-Val-Asp-Val-Glu-

        60
o,p:    Ser-Lys-Pro-Phe-Ala-Thr-Asp-Ser-Gly-Ala-Met-Ser-His-Lys-
        Leu-Glu-Lys-Ala-Asp-Leu-Leu-Lys-Ala-Ile-Gln-Trp-Gln-Ile-Leu-
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Figure 3 N-terminal amino acid sequences of SK-peptide fragments eluted from the h.p.l.c. column of Figure 2

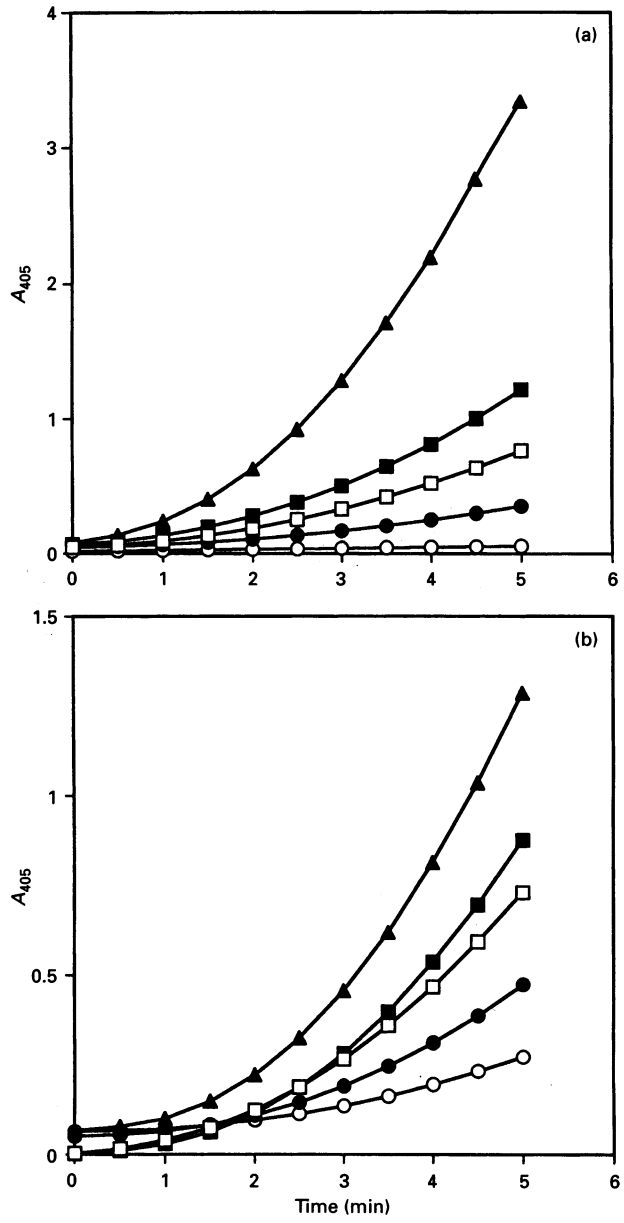

Figure 4 Effect of SK-m on hPlg activation by SK-p and SK-o

(**a**) HPlg (2 μM) was activated by SK-p (0.002 μM) in the absence of SK-m (●), and in the presence of 0.1 μM SK-m (▲), 0.01 μM SK-m (■), or 0.005 μM SK-m (□). SK-p and SK-m were preincubated at 25 °C for 1 min before adding to the assay mixture. An incubation with SK-m (0.1 μM) alone was used as control (○). (**b**) HPlg (2 μM) was activated by SK-o (0.092 μM) in the absence of SK-m (●), and in the presence of 1.2 μM SK-m (▲), 0.6 μM SK-m (■), or 0.3 μM SK-m (□). SK-o and SK-m were preincubated for 1 min at 25 °C before adding to the assay mixture. An incubation with SK-m (1.2 μM) alone was used as control (○).

peptide-bond cleavage. The early cleavage sites of SK by immobilized hPlm were at Lys^{59}–Ser^{60}, Lys^{333}–Ala^{334}, Lys^{387}–Asp^{388}, and Arg^{402}–Tyr^{403}. Peptide bonds at Arg^{112}–Asn^{113}, Lys^{147}–Glu^{148}, Lys^{209}–Asn^{210}, Arg^{232}–Thr^{233}, Lys^{257}–Ser^{258}, Lys^{299}–Tyr^{300}, Lys^{310}–Ser^{311}, Arg^{325}–Asp^{326} and Arg^{364}–Ile^{365} of SK were also cleaved by immobilized hPlm after more than 90 min of incubation (Figures 2 and 3).

The SK peptide of molecular mass 36 kDa (SK-p) was found in association with the gel of immobilized hPlm (Figure 2a). This peptide could not be washed off the gel with phosphate buffer

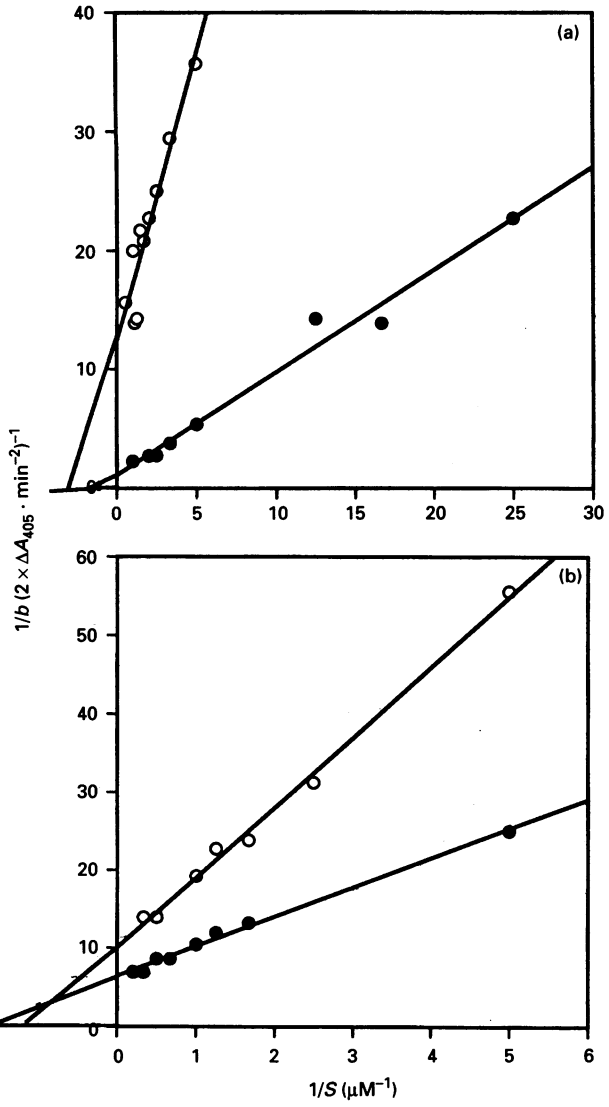

Figure 5 Lineweaver–Burk plots of hPlg activation kinetics by SK-p (a) and SK-o (b) in the presence (●) and absence (○) of SK-m

HPlg at concentrations ranging from 0.04 to 2.0 μM was activated by SK-p (0.004 μM) with and without SK-m (0.085 μM) and by SK-o (0.18 μM) with and without SK-m (0.6 μM) as described in the Experimental section.

Table 2 Steady-state kinetic parameters of the activation of human Glu-Plg by SK peptide fragments with and without SK-m

SK peptide fragments were purified from an h.p.l.c. column as shown in Figures 2(a) and 2(b). Values are the mean ± S.E.M. of four experiments.

	Activator parameters		
Activator	K_{Plg} (μM)	k_{Plg} (min^{-1})	k_{Plg}/K_{Plg} ($\mu M^{-1} \cdot min^{-1}$)
SK	0.21 ± 0.02	17.33 ± 0.88	82.5
SK-p	0.17 ± 0.03	2.23 ± 0.10	13.1
SK-p + SK-m	0.30 ± 0.05	24.28 ± 0.90	80.9
SK-o	0.37 ± 0.06	0.058 ± 0.003	0.16
SK-o + SK-m	0.25 ± 0.04	0.099 ± 0.005	0.40

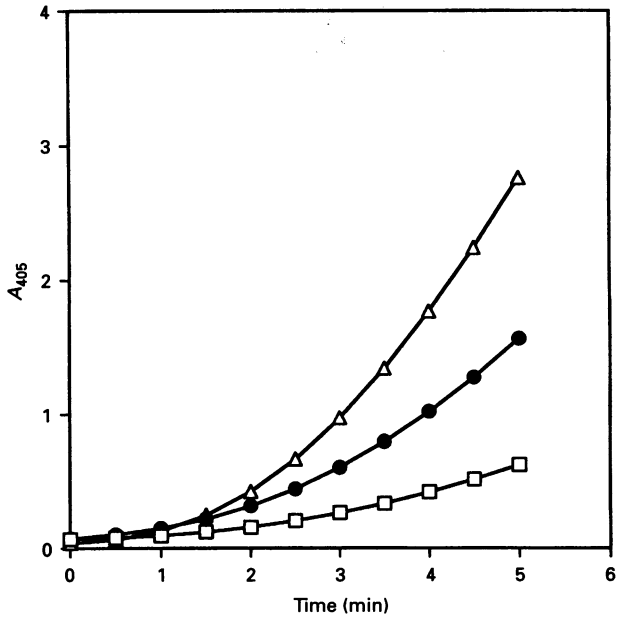

Figure 6 Dependence on the preincubation of SK-m and SK-p in hPlg activation

HPlg (2 μM) was activated by: △, a SK-p (0.002 μM) and SK-m (0.085 μM) mixture preincubated at 25 °C for 1 min; ●, adding SK-p (0.002 μM) and SK-m (0.085 μM) sequentially; □, adding SK-p (0.002 μM) only.

(pH 6.5) and could be eluted from the gel by 6 M guanidine hydrochloride. Three of the peptide fragments in the supernatant (SK-m, SK-n and SK-o), of molecular mass 7, 18, and 30 kDa, were weakly associated with immobilized hPlm and could be eluted from the gel by phosphate buffer. The relative amount of SK-o and SK-n present in the reaction mixture was dependent on the length of incubation; with increasing duration, the amount of SK-o decreased while that of SK-n increased. The N-terminal sequence analysis of these peptides indicated that both SK-p and SK-o started from amino acid residue Ser^{60} of SK (Figure 3). The N-terminals of SK-m and SK-n were Ile^{1} and Glu^{148} of SK respectively (Figure 3). The result of amino acid composition analysis implied that SK-m, SK-n, SK-o and SK-p were peptides of Ile^{1}–Lys^{59}, Glu^{148}–Lys^{333}, Ser^{60}–Lys^{333} and Ser^{60}–Lys^{387} respectively (Table 1).

Purified SK-m, which is the N-terminal 59 amino acid peptide of SK, could not activate hPlg (Figure 4). However, SK-m could dose-dependently enhance the hPlg activator activities of SK-p and SK-o (Figures 4a and 4b). Steady-state kinetic analysis of the activation of hPlg by SK, SK-p and SK-o in the absence and presence of SK-m is summarized in Table 2 and Figure 5. The result indicated that the second-order rate constant, k_{Plg}/K_{Plg}, of SK-p and SK-o was increased 6- and 2-fold respectively, in the presence of SK-m, while their K_{Plg} did not change significantly (Table 2, Figure 5). The k_{Plg}/K_{Plg} of SK-p in the activation of hPlg was about one-sixth that of the native SK. However, the k_{Plg}/K_{Plg} of SK-o was less than 1 % of native SK (Table 2). SK-n could not activate hPlg either with or without SK-m. SK-m also did not alter the activator activity of native SK–hPlg complex (results not shown). It is possible that SK-m stimulated hPlg activation by SK-p either through interaction with SK-m or hPlg. To differentiate these two possible mechanisms, SK-m and SK-p were added to hPlg either sequentially or in preincubated

mixture and assayed for hPlg activation. The result indicated that the maximal enhancement effect of SK-m on the hPlg activation by SK-p was obtained when SK-m and SK-p were premixed before adding to hPlg (Figure 6).

DISCUSSION

The SK and hPlg can form an activator complex that activates Plgs from different animal sources [7,15,24,29]. The SK molecule exerts this function presumably by altering the conformation of the catalytic domain of HPlg. The hPlg moiety in the complex expressed similar catalytic activity as hPlm and was named virgin enzyme [16,30]. SK can be considered as a co-enzyme of the activator molecule and is involved in the binding of the substrate hPlg molecule to the activator complex. The interaction of SK with hPlg or hPlm provides an intriguing example of modulation of enzymic activity by means of a tightly bound protein cofactor. It has been suggested that SK consists of at least three domains with different stabilities and is able to exist in a structured state independent of other regions of the protein [31]. However, the exact interaction sites of SK with hPlg and their function have not been determined.

SK can be degraded in reaction with hPlm. A 36-kDa fragment isolated from an hPlg activator complex, and named altered SK, behaved in a manner analogous to that of the native SK in direct Plg activation assay [15,17]. It was also demonstrated that the altered SK and native SK had different circular dichroism spectra and thus had different conformations [17]. SK-p in our preparation corresponds to the 36 kDa peptide in previous reports. However, samples of the altered SK in previous reports might be contaminated with some unidentified smaller peptides (Figure 2 in ref. [17]). An N-terminal peptide(s) with a cumulative molecular mass of 8 kDa might have been incorporated into the altered SK in the previous report [17]. Functional studies of the purified N-terminal peptide and other SK fragments have never been reported.

In this study the peptide fragments of SK from the reaction of SK with immobilized hPlm were purified by h.p.l.c. N-terminal amino acid sequencing identified 13 cleavage sites. All the cleavage sites identified were located in the hydrophilic regions of SK; nine out of 13 were at the β-turn regions [32].

Several major degraded peptide fragments were formed in the solution of SK incubated with immobilized hPlm as analysed by SDS/gel electrophoresis (Figure 1). The activator activity of the incubation mixture supernatant assayed after 10 min of reaction was similar to that at time zero, suggesting that degradation of SK in the first 10 min to SK-p (altered SK) and other unidentified fragments did not cause significant loss of activator activity. There was an obvious decrease of the activator activity of the incubation mixture supernatant between 10 and 30 min in reaction with immobilized hPlm. No dramatic changes of the activator activity were observed during 30 and 130 min of the reaction. However, a major peptide fragment of 30 kDa was observed during this period of incubation. The SK-o (30 kDa) was relatively stable in reaction with hPlm and a slow degradation of SK-o to smaller fragments was observed. SK-o might represent the core portion of the SK molecule and was the smallest purified fragment of SK which still had detectable activator activity.

The gel of immobilized hPlm, after reaction with SK, was eluted with 6 M guanidine hydrochloride and purified by h.p.l.c. The only peptide retained was SK-p. The hPlg activator activity of SK-p was about one-sixth of that of native SK. The affinity of SK-p with hPlm was so high that it could not be washed off the gel of immobilized hPlm by extensive washing with buffers. SK-o, which was purified from the supernatant of the incubation mixture, had only 1% of activator activity in comparison with SK-p. SK-o did not have strong binding with hPlm, so that SK-o was mostly recovered from the solution, not from the immobilized gel. SK-p consisted of peptide Ser^{60}–Lys^{387} and SK-o consisted of peptide Ser^{60}–Lys^{333} of SK. These results suggested that the 54 amino acid residues of the C-terminal peptide of SK-p, Ala^{334}–Lys^{387}, were essential for binding with hPlm and for the activator activity of SK.

The peptide bond at Lys^{59}–Ser^{60} of SK was immediately cleaved in reaction with hPlm. The function of the N-terminal peptide was not clear, although alteration of the Gly at position 24 with either His or Glu caused complete loss of SK activity to activate hPlg [33]. In this study, the purified SK-m, the N-terminal 59 amino acid peptide, was prepared and was shown to dose-dependently enhance the activity of SK-p to an extent close to that of native SK. SK-m could also enhance the activator activity of SK-o. The k_{Plg} values of SK-p and SK-o increased significantly in the presence of SK-m, while the K_{Plg} did not change. If the affinity with the substrate hPlg were reflected on change of K_{Plg}, SK-m might not affect the binding of substrate hPlg to the activator moiety. If the SK molecule is involved in the binding of hPlg substrate, the binding site should be located in the SK-o, that is, SK peptide Ser^{60}–Lys^{333}, since this peptide is the common peptide fragment of SK-p and native SK and they all have the same K_{Plg}.

The maximum enhancement effect of SK-m on the hPlg activation by SK-p was dependent on the preincubation of SK-m and SK-p. Therefore, SK-m might interact only with the activator moiety and not with the hPlg substrate, and most probably works on SK-p by altering its conformation. Consequently, the activator catalytic constant k_{Plg} of SK-p was enhanced. One possible explanation which can be speculated from this study is that the activator conformation is in equilibrium between two forms, and SK-m causes the activator to shift to the more active one.

It was noticed that native SK was very rapidly converted into SK-p in just a few minutes in the hPlg–SK complex. A shift to a more random or less helical conformation for the altered SK in c.d. analysis than the native SK was also observed [17]. The altered SK which could activate hPlg had the same molecular mass and N-terminus as SK-p. Our results showed that the purified SK-p had a lower k_{Plg} than native SK in activating hPlg. The SK-p in the presence of SK-m became as effective an activator as native SK in reaction with hPlg. It is highly possible that the peptide bond at Lys^{59}–Ser^{60} of native SK was cleaved in the reaction with hPlg to form SK-m and SK-p. The SK-m might remain on the hPlg–SK complex and maintain the high activator activity. This could explain why SK-m could not further stimulate the activator activity of native SK. A satisfactory method is not at present available to determine whether the cleavage of the peptide bond at Lys^{59}–Ser^{60} is essential for the activator activity, since a mutant at that specific site is as yet unavailable. However, our results provide strong evidence that SK-m might interact with SK-p in the hPlg activator moiety and function as a modulator of the SK-p.

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