Activation of Human Plasminogen by Staphylokinase. Direct Evidence That Preformed Plasmin Is Necessary for Activation to Occur

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To directly determine whether the mechanism of activation of human plasminogen (HPg) by staphylokinase (Sak) required formation of an active complex of Sak and HPg, recombinant (r) variants of HPg were examined that allowed dissection of the steps involved in this activation. The rate of activation of wild-type (wt) r-HPg by equimolar levels of Sak was enhanced when small amounts of human plasmin (HPm) were included, suggesting that a Sak-HPm complex was a more effective plasminogen activator than a putative Sak-HPg complex. Incubation of equimolar Sak with a cleavage site resistant mutant of HPg (r-[R561 A]HPg) did not result in generation of amidolytic activity of the complex, in contrast to a similar experiment with streptokinase (SK) in place of Sak, where substantial amidolytic activity was generated. This result supplies evidence that an active complex of Sak and HPg does not form, as is the case with SK. Another mutant, r-[D646E]HPg, which, upon activation, would lead to a form of HPm defective in enzymatic activity, is also not converted to its two-chain form by Sak, but is converted to the inactive two-chain form by urokinase, a direct plasminogen activator, and by equimolar complexes of SK or Sak with wt-HPm. This shows that the active site of HPm is the functional plasminogen activator entity in the Sak-HPm complex. These results show that the mechanism of activation of HPg by Sak proceeds in a distinctly different manner than the similar activation by SK. Although SK does not require the presence of HPm for this activation, a necessary condition for the activation by Sak is formation of a small amount of HPm generated via another activation pathway. These different mechanisms have significant implications in production of the fibrinolytic state by these two indirect bacterial plasminogen activators.

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Human plasminogen (HPg) is the plasma protein precursor of the serine protease plasminogen (HPm), an enzyme that functions in blood clot dissolution. The full-length human-derived zymogen (E1-HPg) contains 791 amino acids in a single polypeptide chain. HPm is formed from HPg as a result of activator-catalyzed cleavage of the R561-V562 peptide bond in the zymogen. The resulting full-length plasmin (E1-HPm) contains a heavy chain of 561 amino acid residues, originating from the amino-terminus of E1-HPg, disulfide-linked to a light chain of 230 amino acid residues. This latter chain, containing the carboxyl-terminus of HPg, is homologous to serine proteases such as trypsin and elastase. The catalytic triad of amino acids that define serine proteases is entirely present within the light chain of HPm, and consists of H520, D646, and S444. The heavy and light polypeptide chains of HPm are covalently linked by two disulfide bonds. One such bond bridges C558 of the heavy chain and C656 of the protease chain, and another spans the sequence of amino acids residing between C558 of the heavy chain and C466 of the light chain. A second functionally significant hydrolytic reaction, catalyzed by HPm, occurs between residues K77 and K78 of E1-HPg, with additional assorted peptide bond cleavages within this 77-amino acid polypeptide, particularly at K82 and R102. Hydrolysis of this peptide bond from the amino-terminus of E1-HPg, and/or the amino-terminus of the heavy chain of E1-HPm, provides K77-HPg and K77-HPm, respectively.

From these considerations, it is clear that plasminogen activators must possess proteolytic activity, a property of two major mammalian enzymes that function in HPg activation, namely urokinase (uPA) and tissue-type plasminogen activator (tPA). Two other important HPg activators, obtained from bacterial sources, namely streptokinase (SK) and staphylokinase (Sak), are not proteases. Therefore, their participation in this activation process must be indirect. The mechanism of action of SK in HPg activation has been clarified through intense study. A critical first step in this process is generation of an equimolar complex of SK with HPg, in which an active site subsequently develops in the HPg moiety of the complex without cleavage of peptide bonds. In catalytic amounts, this complex serves as an efficient HPg activator. Other proteolytic events within the SK-HPg complex occur, after which another HPg activator complex, namely SK-HPm, forms as the most stable entity. Although it is logical to assume that Sak would function in the same manner, kinetic evidence has been forthcoming that suggests that a Sak-HPg functional complex does not form, and the HPg activator consists of an equimolar complex of Sak and preformed HPm.

Due to the potential importance of Sak as a thrombolytic agent, and development of new drugs based on this structure, it is of considerable importance to understand its mechanism of action. We believed that generation of a cleavage site-resistant form of HPg and a study of its ability to function as a plasminogen activator with Sak would directly address the issue of whether a functional Sak-HPg complex was formed. This report provides a summary of the results of this investigation.

MATERIALS AND METHODS

Proteins. A recombinant Sak, Sak-STAR, was provided by Dr H.R. Lijnen (Leuven, Belgium). SK was obtained from AM Kabi.
Restriction endonucleases were purchased encoding wtr-\(E_1\)-HPg, r-[\(R\ 561\ A\)]\(E_1\)-HPg, r-[\(D\ 646\ E\)]\(E_1\)-HPg, after insertion of the respective heterologous gene into the baculovirus genome from Promega (Madison, WI).

A low-molecular-weight derivative of uPA (lmw-uPA) was a gift from Abbott Laboratories (North Chicago, IL). This protein lacked the amino-terminal 135 amino acid residues from the wild-type (wt) form, and was cleaved at K\(158\)-I\(159\), resulting in a disulfide-linked two-chain enzyme.

Protein concentrations were determined from the absorbancies at 280 nm, using an \(e\) of 1.70 for HPg, 0.98 for \(S\alpha\kappa\), 0.95 for SK, and 1.32 for lmw-uPA. These values were determined from the amino acid compositions of the proteins.

Construction of mutant plasminogens. The cDNA for HPg in pUC119 was used as the starting material for mutagenesis strategies.

The variants, r-[\(R\ 561\ A\)]\(E_1\)-HPg and r-[\(D\ 646\ E\)]\(E_1\)-HPg, were generated by primer-directed mutagenesis of single-stranded pl119/HPg, as previously described. The oligonucleotide primers used for this purpose follow (the mutagenic changes are in lowercase letters): (1) \([R\ 561\ A]\); 5'-AA TGT CCc GGg gcG GTT GTA G (\(\text{Sma}\ I\)); (2) \([D\ 646\ E]\); 5'-GAG CCC ACg CGt AAA GAg ATT GCC TTG (\(\text{Mlu}\ I\)).

Primer 1 encodes the last two bases of K\(557\), followed by codons for \(E\ 558-V\ 563\) and the first base of the codon for G\(564\). Primer 2 codes for E\(561-L\ 569\).

Positive Escherichia coli transformants were identified with use of the indicated restriction endonucleases (the recognition sites are underlined).

The mutants were subcloned into the \(\text{BamHI/HindIII}\) polylinker sites of plasmid pBlueBacIV and pBlueBacIII (Invitrogen, San Diego, CA) for the \([R\ 561\ A]\) and \([D\ 646\ E]\) mutants, respectively. All mutant cDNAs were subjected to oligonucleotide sequence analysis to approximately 100 nucleotides on each side of the mutations. The cDNAs in the transfer vectors were then inserted by homologous recombination into the baculovirus genome in such a manner as to disrupt the polyhedrin gene. Recombinant viruses were detected as blue plaques in the presence of the chromogenic substrate, X-Gal (5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactoside), purchased from Promega.

Expression and purification of mutant plasminogens. Expression in Trichoplusia ni cells (High Five cells; Invitrogen) of cDNAs encoding wtr-\(E_1\)-HPg, r-[\(R\ 561\ A\)]\(E_1\)-HPg, r-[\(D\ 646\ E\)]\(E_1\)-HPg, after insertion of the respective heterologous gene into the baculovirus genome, and purification of the mutants by affinity chromatography on Sepharose-lysine, was accomplished as previously described. The insect cells were infected for 96 hours with the above r-baculoviruses at multiplicities of 4 plaque-forming units/cell using the infection and cell handling protocols described earlier.

Activation assays. All HPg activation assays were performed with the E\(1\)-HPg form of the zymogen in buffers lacking Cl\(-\), or any other anion that inhibits E\(1\)-HPg activation. The exact experimental conditions have been detailed in the relevant figure legends. Samples from the activation mixtures were also analyzed by 9% \(\text{DodSO}_4/PAGE\) gel electrophoresis (PAGE) under reducing conditions.

RESULTS AND DISCUSSION

Two HPg mutants were expressed in baculovirus-infected lepidopteran insect cells, and were purified by lysine-Sepharose affinity chromatography. One, r-[\(R\ 561\ A\)]\(E_1\)-HPg, possessed a mutation at the activation cleavage site and it would not be expected that this form of plasminogen would be activatable via mechanisms that require cleavage at R\(561\)-V\(562\). Confirmation of this point is obtained from the data of Fig 1, where it is seen that neither two-chain HPm nor HPm activity was generated from this mutant by activation with the serine protease, lmw-uPA, under conditions where wtr-\(E_1\)-HPg was readily activated. Another mutant, r-[\(D\ 646\ E\)]\(E_1\)-HPg, was able to be transformed to two-chain HPm (vide infra), but did not possess HPm activity due to mutation of the aspartic acid component of the serine protease catalytic triad.

Activation of HPg by SK, a protein without inherent enzymic activity, proceeds first through generation of equimolar complexes of SK with HPg and HPm (for a recent review, see ref 23), which, at catalytic levels, function as activators of plasminogen. The initial complex of SK-HPg, in which HPg has developed the active site in the absence of peptide

![Fig 1. Activation of HPg by lmw-uPA. wtr-\(E_1\)-HPg (100 nmol/L) was activated by lmw-uPA (0.5 nmol/L) in the presence of the chromogenic substrate, S2251 (0.6 mmol/L). The HPm generated as a function of time was detected by the liberation of p-nitroanilide from the substrate and is depicted here as the absorbance at 405 nm. The curves illustrated are for wtr-\(E_1\)-HPg and for the mutant, r-[\(R\ 561\ A\)]\(E_1\)-HPg. The reduced \(\text{DodSO}_4/PAGE\) gel insert shows the protein components present after 10 minutes of activation. The buffer was 10 mmol/L HEPES-NaOH/150 mmol/L NaOAc, pH 7.4, at 37°C.](from www.bloodjournal.org by guest on September 8, 2017. For personal use only.)
bond cleavage, is a relatively unstable complex and rapidly generates the more stable species, SK-HPm, by autocatalytic cleavage of the R\textsuperscript{561}V\textsuperscript{562} peptide bond in the HPg moiety of the complex.\textsuperscript{9} As would therefore be predicted, the data presented in Fig 2 show that r-[R\textsuperscript{561}A]E\textsuperscript{1}-HPg forms a functional complex with SK, with some proteolytic degradation of SK by the active site of the resulting enzyme complex, and this species possesses enzymatic activity despite the inability of the complex to undergo transformation to SK-HPm. Thus, this mutant was judged to be very suitable for investigation of the possibility of formation of an active Sak-HPg complex.

Figure 3 shows that whereas wtr-E\textsuperscript{1}-HPg is activated by Sak, neither r-[R\textsuperscript{561}A]E\textsuperscript{1}-HPg nor r-[D\textsuperscript{646}E]E\textsuperscript{1}-HPg underwent activation by this protein under the same conditions. In addition, these two mutants were not converted to two-chain HPm forms by Sak (see Fig 3, gel insert), while, as would be expected, r-[D\textsuperscript{646}E]E\textsuperscript{1}-HPg is converted to the two HPm chains when it is incubated with catalytic levels of either SK-HPm or Sak-HPm (Fig 4). Sak has no inherent proteolytic activity and, in addition, it is obvious from the lack of development of amidolytic activity in the Sak/r-[R\textsuperscript{561}A]E\textsuperscript{1}-HPg incubation mixture that an active Sak-HPg complex does not initially form, in contrast to the case of similar activation by SK. Therefore, we propose that minor contaminating levels of HPm that are usually present in HPg preparations complex with Sak, forming a Sak-HPm plasminogen activator complex, which then activates the re-

![Figure 2. Activation of HPg by SK.](image1)

![Figure 3. Activation of HPg by Sak.](image2)
Fig. 4. Activation of r-[D646A]E1-HPg by SK-HPm and Sak-HPm. Lane 1, wtr-E1-HPg. Lane 2, wtr-E1-HPg (200 ng) + SK-HPm (10 ng). Lane 3, wtr-E1-HPg (200 ng) + Sak-HPm (10 ng). Lane 4, r-[D646A]E1-HPg. Lane 5, r-[D646A]E1-HPg (200 ng) + SK-HPm (10 ng). Lane 6, r-[D646A]E1-HPg (200 ng) + Sak-HPm (10 ng). Lane 7, molecular-weight markers (phosphorylase b, 94,000; bovine serum albumin, 66,200; rabbit muscle aldolase, 39,200; and triose phosphate isomerase, 26,600). The slightly lower molecular weight of the HPm heavy chain in lane 5 compared with lane 6 is due to a small amount of free HPm in the SK-HPm mixture that was released consequent to degradation of the SK within the complex, and which would then catalyze conversion of the E1-HPm heavy chain to the E2-HPm heavy chain. The buffer for the activations was 10 mmol/L HEPES-NaOH/150 mmol/L NaOAc, pH 7.4, at 37°C.

To test this hypothesis, we added a 5% level of contamination of HPm to the wtr-E1-HPg preparation during the lag period of the Sak-wtr-HPg incubation and continued to monitor the activation. As seen from Fig 3, an immediate increase in activity was observed, demonstrating an increase in the activation rate of wtr-HPg. This suggests that placement of HPm in the sample resulted in a higher concentration of HPg activator at the same concentration of Sak. The fact that r-[D646E]E1-HPg did not activate with Sak, nor was it converted to a two-chain HPm (gel insert, Fig 3), shows that it is the active site of HPm in the Sak-HPm complex that functions as a HPg activator in this complex. A control experiment with lmw-uPA (gel insert, Fig 3) shows that the mutation used to generate r-[D646E]E1-HPg did not adversely affect the ability of this variant to be converted into the polypeptide chains of HPm.

Clearly then, as shown by the use of these strategic mutant plasminogens, a critical step of the SK-catalyzed activation of HPg is absent in the case of activation by Sak, namely an initial Sak-HPg active complex is not formed. This conclusion is in agreement with kinetic studies of HPg activation with Sak.11 Thus, while SK is able to activate HPgzymogen, Sak would not have that ability because it relies on small amounts of HPm generated in other manners. This has importance in considerations of production of a fibrinolytic state with Sak because such a condition is dependent on the presence of other HPg activators to allow development of some HPm to complex with Sak.

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