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Plasminogen Activator Activities of Equimolar Complexes of Streptokinase with Variant Recombinant Plasminogens†

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ABSTRACT: The steady-state kinetic characteristics of the amidolytic and plasminogen activator activities of equimolar streptokinase (SK)–human plasminogen (HPg) and SK–human plasmin (HPm) complexes have been determined, exploiting the generation and use of cleavage site resistant mutants of HPg to stabilize plasminogen within the complex. Whereas amidolytic kinetic constants for equimolar complexes of SK with the following proteins, viz., plasma HPm, insect (i) cell-expressed wild-type (wt) recombinant (r) HPm, R^{561}E-irHPg, and Chinese hamster ovary cell (c)-expressed R^{561}S-crHPg, are similar, it has been found that the various SK–HPg complexes are far better enzymes than SK–HPm complexes for activation of bovine plasminogen, a species of plasminogen that is resistant to activation by SK, alone. In addition, it is emphasized that as a result of mutating the cleavage site in plasminogen, it is possible to express this protein in mammalian cells, and thus provide it for use in complex with SK as a more efficient plasminogen activator than plasma plasminogen, which is rapidly converted to HPm within the SK complex. This finding has important implications in the assessment of thrombolytic therapeutic reagent employing SK–plasminogen and SK–plasmin complexes.

Human plasminogen (HPg)[1] is a plasma-derived zymogen of the fibrinolytic and fibrinogenolytic enzyme plasmin (HPm) and exists in the circulation as a single-chain glycoprotein containing 791 amino acids (Wiman, 1973, 1977; Sottrup-Jensen et al., 1978; Malinowski et al., 1984; Forsgren et al., 1987; McLean et al., 1987), with Glu at its amino terminus ([Glu^1]Pg).[1] Activation of HPg results from cleavage of the Arg^{561}-Val^{562} peptide bond in the zymogen, producing the two-chain, disulfide-linked serine protease [Lys^{78}]Pm, which also lacks the amino-terminal 77 amino acids as a result of autolysis by HPm formed during the activation (Violand & Castellino, 1976). This activation is catalyzed by a variety of proteins, which include streptokinase (SK), urokinase (UK), and tissue plasminogen activator (tPA) [for a review, see Castellino (1983)]. Whereas the latter two proteins are enzymes that directly catalyze cleavage of the appropriate peptide bond in HPg, providing HPm, SK has no such inherent activity, and its plasminogen activator relies on its ability to form complexes with HPg and HPm utilizing the actual or latent

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[1] Abbreviations: HPg, any form of variant of human plasminogen; HPm, any form or variant of human plasmin; [Glu^1]Pg, native human plasminogen with Glu, residue 1, at the amino terminus; [Lys^{78}]Pg, proteolytically derived form of human plasminogen with Lys, residue 78, at the amino terminus; [Lys^{78}]Pm, human plasmin, which arises from [Glu^1]Pg by cleavage at the activation site Arg^{561}-Val^{562}, and also at Lys^{77}-Lys^{78}; BPg, bovine plasminogen; SK, streptokinase; tPA, tissue plasminogen activator; UK, urokinase; EACA, ϵ-aminocaproic acid; r, recombinant; wt, wild type; i, insect cell expressed; c, CHO cell expressed; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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plasmin active sites of these latter two molecules in order to function as an activator (Castellino, 1983).

[Glu^1]Pg exists in plasma in the form of two major variants, which differ in their extent of glycosylation at Asn^{289} (Hayes & Castellino, 1979a,b,c; Castellino, 1983). In [Glu^1]Pg, the latent plasmin heavy chain, residues 1–561, contains 5 highly homologous regions (Sottrup-Jensen et al., 1978), each of approximately 80 amino acids, designated as *kringles*, which most likely exist as independent domains (Castellino et al., 1981), and which are of importance to the functional properties of HPg and HPm. As examples, the *kringle 1* domain (amino acid residues 84–162) is believed to be important in the interaction of plasmin(ogen) with fibrin(ogen) (Lucas et al., 1983), with the negative activation effector Cl^- (Urano et al., 1987) and with the positive activation effector EACA (Markus et al., 1978). Additionally, this same domain is responsible for the initial rapid binding of HPm to its major plasma inhibitor, α_2-antiplasmin (Moroi & Aoki, 1976). Other *kringles* contain the weak EACA binding site(s) present on [Glu^1]Pg, which is involved in the large ligand-induced conformational alteration of [Glu^1]Pg (Violand et al., 1975) and the concomitant increase in the activation rate of the zymogen in the presence of this positive effector (Claeys & Vermyelin, 1974).

We have been utilizing site-directed mutagenesis of the cDNA of HPg to design molecules that will reveal important structure–function relationships of this protein and have employed an insect cell expression system to obtain fully functional [Glu^1]Pg (Whitefleet-Smith et al., 1989). We believed that this system would be appropriate for expression of activation cleavage site resistant variants of HPg that could be fruitfully employed to investigate enzymatic properties of stable SK–HPg complexes, a very difficult task to accomplish with plasma or recombinant wt-HPg due to the rapid conversion of HPg to HPm within the SK complex. This report summarizes our findings regarding the enzymic nature of the SK–HPg complex, stabilized as such as a result of employing variant recombinant plasminogens that were resistant to conversion to HPm.

Materials and Methods

Proteins. Native human plasma [Glu^1]Pg, affinity chromatography form I, was purified according to the method of Deutsch and Mertz (1970) as modified by Brockway and Castellino (1972). Bovine plasminogen (BPg) was purified from fresh bovine plasma in the same manner. Recombinant (r) wild-type (wt) [Glu^1]Pg and R^{561}E-r[Glu^1]Pg insect cell (i)-expressed plasminogens were purified as above from the culture media of *Spodoptera frugiperda* cells, infected with the recombinant baculovirus PAV6 (Whitefleet-Smith et al., 1989) containing the [Glu^1]Pg (wt-irHPg) cDNA or R^{561}E-[Glu^1]Pg (R^{561}E-irHPg) cDNA (Whitefleet-Smith et al., 1989). R^{561}S-[Glu^1]Pg (R^{561}S-crHPg) was similarly purified from the culture supernate of CHO cells (c) transfected in a manner similar to that previously described (Goeddel et al., 1983). SK was prepared according the method published previously (Castellino et al., 1976). Restriction endonucleases were purchased from Promega (Madison, WI).

Site-Directed Mutagenesis of the cDNA for HPg. In vitro mutagenesis was accomplished with the cDNA for HPg inserted in pUC119 (p119PN127.6) (McLean et al., 1987). The nucleotide sequence of p119PN127.6 showed that the translated protein would have a Val^{475} in place of the Ala^{475} deduced from direct amino acid sequence analysis. While this is probably a normal isoform of HPg, we nonetheless wished to alter it to Ala, since this latter residue seems to be the more common amino acid at position 475, based on protein sequencing. The mutagenic prime (the bold and underlined bases represent the mutations imposed) used to make the V^{475}A mutation was

5′-GTAACAGTGGT**TGC**CCTCTTGCCTC-3′

Positive colonies were screened by using an *Eco*RI/*Bst*EII restriction endonuclease digest. Clones with the proper size fragments were sequenced (vide infra) over the region corresponding to amino acid positions 455–502.

The mutagenic primer employed for generation of R^{561} change in the cDNA for HPg was

5′-CCCCCCTACAAC**GGA**TCCAGGACATTTCTT-CGG-3′

The colonies were screened by the presence of the new *Bam*HI restriction endonuclease site also inserted in the cDNA as a result of these mutations.

The mutagenic primer used for construction of R^{561}E-HPg was

5′-CCCCCCTACAACCCT**C**CC**G**GGGACATTTCTT-CGG-3′

Positive colonies for these mutations were screened for the presence of the newly generated *Sma*I restriction endonuclease cleavage site which accompanies the alterations made.

Amidolytic Assays of Stoichiometric Complexes of SK–HPg and SK–HPm. A quantity of 0.2 mL of a buffer consisting of 100 mM Hepes–NaOH/10 mM EACA, pH 7.4, was placed in a spectrophotometer cuvette, maintained at 25 °C. Following this, the desired concentration of the chromogenic substrate H-D-Val-L-Leu-L-Lys-pNA (S2251; Helena Laboratories, Beaumont, TX) was added, followed by the required amount of H_2O. The hydrolysis of the substrate was accelerated by addition of the desired preformed stoichiometric SK–HPg or SK–HPm complex (final concentration) 6–10 nM). The rate of hydrolysis of S2251 was recorded continually for 2–5 min at 405 nm. The absorbancies were converted to initial activation rates as described previously (Urano et al., 1987), and the rate data were analyzed according to usual Lineweaver–Burk plots. The enzyme complexes were generated by incubation at 25 °C of stoichiometric amounts of SK and the desired plasminogen.

Plasminogen Activator Assays of Stoichiometric Complexes of SK–HPg and SK–HPm. A quantity of 0.2 mL of a buffer consisting of 100 mM Hepes–NaOH/10 mM EACA, pH 7.4, was placed in a spectrophotometer cuvette, maintained at 25 °C. After this, 0.08 mL of S2251 (final concentration 0.5 mM) was added, followed by the required amount of H_2O, various concentrations of BPg, and, lastly, the SK–HPg or SK–HPm activator complex (final concentration 0.2 nM). The rate of activation of BPg was monitored in continuous assay (Urano et al., 1987) by recording the release of *p*-nitroanilide resulting from the hydrolysis of S2251 by the bovine plasmin generated. The data were analyzed by Lineweaver–Burk plots as we have published for previous studies of this type (Urano et al., 1987). Under these conditions, BPg was not activated by SK alone.

It should be noted that all kinetic assays contained EACA as a buffer component. This agent was present to provide maximal activation rates and to eliminate any consideration of possible differences in activation rates being due to variable amounts of [Glu^1]Pg and [Lys^{78}]Pg in the assay mixtures with any of the plasminogens examined in this report.

Deglycosylation of HPg. The desired HPg preparation (in 10 mM sodium phosphate, pH 7.4) was treated with glycopeptidase F (Boehringer Mannheim, Indianapolis, IN) at a concentration of 0.4 unit of enzyme/μg of HPG. The reaction

was allowed to proceed for 24 h at 37 °C. These conditions were found suitable for removal of the Asn^{289}-linked carbohydrate from all of the samples investigated in this report.[2] The mixture was then subjected to centrifugation in molecular weight 10000 cutoff (Centricon 10) microconcentrator tubes (Amicon, Danvers, MA) to separate the liberated oligosaccharide from the protein sample.

Amino Acid Sequencing. Proteins were subjected to amino-terminal amino acid sequence analysis on a Porton Instruments gas-phase sequencer, after adsorption of the protein onto the peptide support disks. PTH-amino acids were separated on a Beckman reverse-phase ODS column (5 μm, 4.6 mm × 250 mm), employing a Spectra Physics HPLC system. The latter consisted of a Model 8800 ternary HPLC pump, a Model 8480 UV/Vis detector, a Model 4270 recording integrator, and a PI2030 interface for on-line injections of the samples onto the HPLC column. Resolution of the 20 PTH-amino acids was accomplished at 55 °C, under the following linear gradient conditions: 88% solution A (1 mL of glacial acetic acid/20 mL of tetrahydrofuran/0.05 mL of triethylamine/H_2O to 500 mL, pH adjusted to 4.10 with 3 N NaOH)/12% solution B (1% tetrahydrofuran in CH_3CN) as the start solution, to 60% solution A/40% solution B (limit solution) over a period of 24.5 min at a flow rate of 1 mL/min. Solution B was then continued for an additional 5.5 min at the same flow rate during which time the last four PTH-amino acids were eluted.

DNA and Protein Analytical Methods. Oligonucleotides were synthesized by using phosphoramidite chemistry on a Biosearch (San Rafael, CA) Cyclone two-column DNA synthesizer. All reagents were purchased from this same source. The oligonucleotides were purified by using the Applied Biosystems (Foster City, CA) oligonucleotide purification cartridges. cDNAs were sequenced by the dideoxy technique (Sanger et al., 1977) with aid of the Sequenase reagent kit (United States Biochemicals, Cleveland, OH). Cell transfections were performed by the calcium phosphate method (Kingston, 1987).

Plasmid DNAs were purified by CsCl/ethidium bromide (EtdBr) gradient centrifugation (Moore, 1987), with a Beckman (Palo Alto, CA) L5 65 preparative ultracentrifuge. We used vertical rotor (VTi.65.1) centrifugation for 7 h at 55000 rpm, 15 °C, to separate the DNA bands. After the desired material was obtained from the centrifuge tube, EtdBr was removed from the plasmid DNA by extraction into a solution of 2-propanol saturated with CsCl. The DNA was then dialyzed against a buffer of 1 mM Tris-HCl/0.1 mM EDTA, pH 7.1, prior to cell transfections.

The cDNAs and cDNA fragments were purified by excising the appropriate bands after their electrophoretic separation on 1% agarose. Recombinant molecules were created by the method of Struhl (1985).

Single-strand plasmid DNAs were generated as described (Vieira & Messing, 1987), and site-specific mutagenesis was conducted according to published techniques (Kunkel et al., 1987).

Western Analysis. Protein samples were separated by $NaDodSO_4$/PAGE (Laemmli, 1970) on 10% (w/v) polyacrylamide gels under nonreducing conditions. The separated protein bands were transferred to Immobilon-P (Millipore, Bedford, MA) membranes according to established procedures (Burnette, 1981) and then incubated at 37 °C for 1 h in 1% (w/v) gelatin (Bio-Rad EIA grade) in TBS (blocking buffer).

[2] D. J. Davidson and F. J. Castellino, unpublished results.

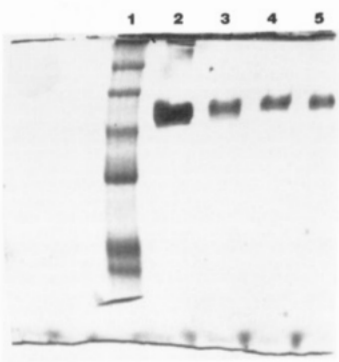

FIGURE 1: $NaDodSO_4$/PAGE electrophoretograms of the plasminogens employed in this investigation. (Left to right) Lane 1, molecular weight standards containing (from top) the following molecular weights: 180000 (human α_2-macroglobulin subunit), 116000 (*Escherichia coli* β-galactosidase subunit), 84000 (rabbit muscle fructose-6-phosphate kinase subunit), 58000 (chicken muscle pyruvate kinase subunit), 36500 (rabbit muscle lactate dehydrogenase subunit), 26600 (rabbit muscle triosephosphate isomerase subunit). Lane 2, human plasma [Glu^1]Pg. Lane 3, R^{561}S-cr[Glu^1]Pg. Lane 4, wt-ir[Glu^1]Pg. Lane 5, R^{561}E-ir[Glu^1]Pg.

Our exact conditions for transfer were 4 °C in 25 mM Tris-HCl/200 mM glycine/15% (v/v) methanol, pH 8.3, at 20 V for 12 h. This solution was replaced with another containing 4 μg/mL monoclonal murine anti-HPg (Whitefleet-Smith et al., 1989), in blocking buffer, and incubated at room temperature for 2 h with mixing. The filter was washed with three changes of 0.05% (v/v) Tween-20 in TBS at room temperature, over a 15-min period. It was next incubated with rabbit anti-mouse IgG–alkaline phosphatase conjugate (Sigma) in blocking buffer for 2 h at room temperature, with mixing, and then washed as above. Positive bands were visualized after incubations, at room temperature, with the substrate solution [16.5 mg of nitro blue tetrazolium/0.5 mL of 70% (v/v) aqueous DMF/8.5 mg of bromochloroindolyl phosphate in 1 mL of H_2O, which is added to 50 mL of 0.1 M Tris-HCl/0.1 M NaCl/0.005 M $MgCl_2$, pH 9.5].

Results

Two recombinant human plasminogens were obtained from infection of insect cells with a recombinant baculovirus containing the wild-type [Glu^1]Pg cDNA (providing wt-irHPg) and the cDNA for HPg containing an R^{561}E mutation in the protein (R^{561}E-irHPg). Another recombinant [Glu^1]Pg was produced in CHO cells with an R^{561}S mutation in the protein (R^{561}S-crHPg). The proteins were purified from the culture media by affinity chromatography on Sepharose–lysine, and $NaDodSO_4$/PAGE of the purified proteins, compared with human plasma HPg, is shown in Figure 1. Their electrophoretic behavior suggests that all have been highly purified and possess the molecular weight characteristics associated with human [Glu^1]Pg. Amino-terminal amino acid sequence analysis of all plasminogens reported herein provided the sequence NH_2-Glu-Pro-Leu-Asp-Asp, suggesting that all have been correctly processed with regard to cleavage of the signal polypeptide and also suggesting that the small mobility differences seen in Figure 1 may be reflections of slight molecular weight variations resulting from differences in glycosylation between the plasma-, insect cell-, and CHO cell-derived plasminogens.[2]

The steady-state amidolytic activities of stoichiometric complexes of SK with the wild-type and variant plasmin(ogen)s are listed in Table I and compared with that of the stoichiometric complex of SK and human plasma plasmin(ogen). We found that full amidolytic activity of each of the complexes

Table I: Steady-State Kinetic Constants at 25 °C for the Amidolytic Activity toward S2251 of Equimolar Complexes of SK with Plasminogens and Plasmins

activator species	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM\ min)^{-1}$
SK–HPm[a]	0.35 ± 0.05	312 ± 16	891
SK–wt-irHPm	0.33 ± 0.04	312 ± 12	945
SK–R^{561}E-irHPg	0.17 ± 0.05	272 ± 18	1600
SK–R^{561}S-crHPg	0.28 ± 0.06	368 ± 20	1314
SK–R^{561}S-crHPg-ΔCHO[b]	0.50 ± 0.06	377 ± 18	754

[a] Human plasma plasmin. [b] HPg variant deglycosylated at Asn^{289}.

developed in 1–5 min, and we employed 5-min incubation times for generation of the enzymes used in this study. In the case of wt-irHPg and human plasma HPg, $NaDodSO_4$/PAGE analysis of the components of the complexes at 5-min incubation times clearly showed that they were composed of SK and [Lys^{78}]HPm, as expected (Bajaj & Castellino, 1977), whereas for the activation cleavage site variant plasminogens, the complexes consisted of SK and the relevant HPg, as also would be expected since the nature of the mutation precludes the conversion of HPg to HPm within the complex. The data of Table I show that the SK–HPm complexes, containing either human plasma HPm or wt-irHPm, possess virtually identical steady-state kinetic constants toward S2251. Similary, amidolytic activity is present in the stoichiometric complexes of SK with the two variant HPg preparations, viz., R^{561}E-irHPg and R^{561}S-crHPg. Comparison of the values of the kinetic constants for these latter two SK–HPg complexes with those of the former SK–HPm complexes suggests that only small differences exist in the amidolytic steady-state properties of the various SK–HPg and SK–HPm complexes. To determine whether the Asn^{289}-linked carbohydrate of HPg played a role in the small differences observed in the steady-state amidolytic kinetic constants, we have deglycosylated R^{561}S-crHPg with glycopeptidase F. The stoichiometric complex of SK with this form of HPg possessed only a slightly higher K_m than its glycosylated counterpart, suggesting that the presence of carbohydrate of Asn^{289}, at least with respect to the type present on the CHO cell-expressed material, does not play a large role in this kinetic property of the SK–R^{561}S-crHPg complex.

Table II provides steady-state kinetic parameters reflecting the respective abilities of the various preformed stoichiometric SK–HPg and SK–HPm complexes, at catalytic levels, to serve as activators of plasminogen. BPg has been selected as the source of plasminogen due to its insensitivity to activation by SK, alone. Comparison of the kinetic properties of the SK–[Lys^{78}]Pm (generated with plasma HPg) complex (3-min preincubation time, Table II) with the same complex composed of ir[Lys^{78}]Pm (3-min preincubation time, Table II) shows that the complex containing the insect-expressed HPm is considerably more active than that containing human plasma HPm, due primarily to an increase in the k_{cat} of activation. From comparison of the data obtained with the SK–irHPm complex (3-min preincubation time) and the SK–R^{561}E-irHPg complex, it would appear that the complex containing HPg is considerably more effective as a plasminogen activaor than that same complex containing HPm. Additional evidence for this view is obtained from analysis of the effect of time of preincubation of stoichiometric levels of SK both with plasma [Glu^1]Pg and with wt-irHPg on the ability of the resulting complexes to activate BPg. The results are illustrated in Figure 2. At very early times of preincubation (≤30 s), $NaDodSO_4$/PAGE shows that the complex still contains approximately 80% HPg, in the case of each plasminogen, and approximately 20% HPm (data not shown). At times of 1 min, and thereafter, all HPg is converted to HPm within the complex. Figure 2 shows that a much higher level of BPg activator activity is present in the 30-s sample than in samples preincubated for >1 min, strongly suggesting that the SK–HPg complex is more effective than the SK–HPm complex in activation of BPg. Kinetic constants for the activation of BPg with the SK complexes prepared from human plasma HPg and

Table II: Steady-State Kinetic Constants at 25 °C for the Plasminogen Activator Activity toward Bovine Plasminogen of Equimolar Complexes of SK with Plasminogens and Plasmins

activator species	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M\ min)^{-1}$
SK–HPg[a]	1.72 ± 0.06	1.79 ± 0.07	1.04
SK–HPm[b]	7.00 ± 1.14	0.61 ± 0.09	0.09
SK–wt-irHpg[c]	0.98 ± 0.12	4.14 ± 0.52	4.22
SK–wt-irHPm[d]	9.80 ± 1.06	2.28 ± 0.23	0.32
SK–R^{561}E-irHPg	0.72 ± 0.07	7.00 ± 0.84	9.72
SK–R^{561}S-crHPg	0.98 ± 0.10	3.54 ± 0.36	3.61
SK–R^{561}S-crHPg-ΔCHO[e]	0.49 ± 0.11	8.52 ± 1.00	17.4

[a] Human plasma plasminogen. This complex was formed as a result of preincubation of SK and HPg for 30 s. Estimates from reduced $NaDodSO_4$/PAGE show that the relative percentage of the original HPg remaining at this time in the complex was 80%. Approximately 20% existed as HPm in this complex. [b] Human plasma plasmin. This complex was formed as a result of preincubation of SK and HPg for 3 min. [c] Insect cell expressed wild-type human plasminogen. This complex was formed as a result of preincubation of SK and wt-irHPg for 30 s. Estimates from reduced $NaDodSO_4$/PAGE show that the relative percentage of the original HPg remaining at this time in the complex was 80%. Approximately 20% existed as HPm in this complex. [d] Insect cell expressed wild-type human plasmin. This complex was formed as a result of preincubation of SK and wt-irHPg for 3 min. Estimates from reduced $NaDodSO_4$/PAGE show that the relative percentage of HPm in complex with SK was approximately 100%. [e] Stoichiometric complex of SK with R^{561}S-crHPg, deglycosylated at Asn^{289}.

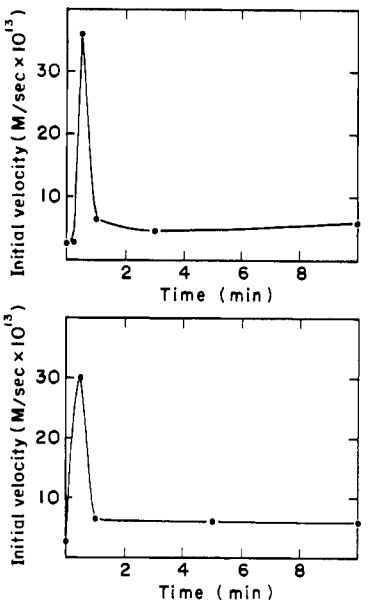

FIGURE 2: Activation time course of BPg with various activators. Stoichiometric levels of SK and the relevant HPg were preincubated for the times listed on the abscissae of the graphs. At these times, an aliquot (final concentration 0.2 nM) was added to a cuvette containing BPg (final concentration 0.8 μM) and S2251 (final concentration 0.5 mM). The activation rate of the BPg was assayed as described under Materials and Methods. (Top) The activator complex was generated with human plasma plasminogen. (Bottom) The activator complex was generated with insect recombinant wild-type plasminogen.

wt-irHPg at short preincubation times (30 s) are listed in Table II. As seen from these data, the K_m values are dramatically decreased from those obtained with the corresponding samples wherein all HPg was converted to HPm within the complexes (compare the data for the 30-s and 3-min preincubation times in Table II). When similar experiments were performed with R^{561}E-irHPG and R^{561}S-crHPg, no such early time activity peak was noted; only temporally constant activator activities of the complexes were observed.

Finally, the mammalian cell expressed variant HPg (R^{561}S-crHPg), in stoichiometric complex with SK, possessed similar, but not identical, steady-state kinetic values toward BPg activation as the insect-expressed protein (R^{561}E-irHPg). The k_{cat} for the enzyme complex containing this latter protein is approximately 2-fold higher than that of the complex formed with the former HPg. That differences in glycosylation of the variant recombinant HPg preparations may play a role in the plasminogen activator activities of the SK complexes containing these same plasminogens can be observed from the data of Table II wherein the k_{cat} for activation of BPg for the SK–R^{561}S-crHPg complex is approximately 2.4-fold lower and the K_m approximately 2-fold higher than the corresponding values for the same complex prepared with the glycopeptidase F-deglycosylated R^{561}S-crHPg. These differences were not revealed in analysis of the amidolytic activities of the same complexes (Table I).

Discussion

We have produced recombinant human plasminogen in insect cells (irHPg) (Whitefleet-Smith et al., 1989) which, by amino-terminal amino acid sequence analysis, molecular weight estimation on $NaDodSO_4$/PAGE, Sepharose–lysine affinity chromatography behavior, activation characteristics, antibody reactivity, and activity of the resulting plasmin, appears to be comparable in properties to human plasma [Glu^1]Pg. This is a significant finding since to date that has not been successful expression of wt-rHPg in mammalian cells, most likely due to the ubiquitous presence of plasminogen activators in such cells, and their resulting ability to convert HPg to HPm, which will autodegrade. However, the nature of the glycosylation at Asn^{289} in different for each of the plasminogens in that the human plasma protein contains complex-type carbohydrate at this position (Hayes & Castellino, 1979b) and the insect-expressed protein contains complex and high-mannose moieties[2] at Asn^{289}. In this paper, we compared in detail kinetic properties of the equimolar complexes formed from SK with plasma HPg and recombinant insect cell-expressed HPg. In each case, $NaDodSO_4$/PAGE gels of the temporal events within the respective complexes were identical with results already published (Bajaj & Castellino, 1977) in that a rapid conversion of HPg to HPm occurred, suggesting that HPg is not a stable component of the complex. Thus, it has been very difficult to investigate the enzymic properties of the SK–HPg complex, and its properties have only been determined at low temperature (Chibber et al., 1986). In order to circumvent this problem, we have prepared a variant HPg by in vitro mutagenesis of one of the amino acid residues (Arg^{561}) positioned at the critical cleavage site in the conversion of HPg to HPm, and expressed the resulting cDNA in insect cells, as described previously for wt-irHPg (Whitefleet-Smith et al., 1989). The resulting plasminogen, R^{561}E-irHPg, was stable as such in complex with SK and rapidly (≤30 s) developed both amidolytic and plasminogen activator activity.

Steady-state amidolytic kinetic constants for hydrolysis of the plasmin chromogenic substrate (S2251) by complexes containing equimolar levels of SK with plasma HPm, irHPm, and R^{561}E-ir[Glu^1]Pg (Table I) do not display many differences. Importantly, the equimolar SK complexes with either human plasma HPm or the insect-expressed HPm are nearly identical, demonstrating by another means that the insect cells are producing a highly functional protein, comparable in properties to human plasma HPg. The K_m for the SK complex with the variant cleavage site resistant irHPg is slightly lower than this value for the same complexes with either of the plasmins, suggesting possible kinetic differences in the SK–HPg complex, as compared to the SK–HPm complex.

Such possible kinetic differences are greatly magnified in comparing the plasminogen activator activities of these same SK–HPg and SK–HPm complexes (Table II). From examination of this table, it is first observed that the k_{cat} for the plasminogen activator activity of the equimolar SK–irHPm complex is nearly 4-fold greater than that for the corresponding SK–plasma HPm complex, while the K_m values are nearly the same. These differences result in a higher specificity constant (k_{cat}/K_m) for the enzyme containing the insect-expressed protein. The reasons for this are not entirely clear without extensive characterizations of these proteins, but may be related to a known difference between then, i.e., the presence of the high-mannose oligosaccharide in the latter protein, and of complex-type oligosaccharide in the plasma protein. Also, of great importance, it is clearly seen in Table II that the second-order specificity constant for plasminogen activation for the SK–R^{561}E-irHPg complex is approximately 30-fold greater than the same value for the SK-wt-irHPm complex, due to both a decrease in the K_m and an increase in the k_{cat} values. These data strongly suggest that the SK–HPg complex is more efficient than the SK–HPm complex for activation of plasminogen. Further evidence for this conclusion is clear from the results of Figure 2, wherein the activity of the complex formed from incubation of SK and either plasma HPg or wt-irHPg is greatest at very short incubation times, where plasminogen remains as a major component of the complex. At longer times, this HPg is converted to HPm, and the activity decreases and remains stable. Estimations of the kinetic constants for early (30 s; 80% HPg/20% HPm in the equimolar complex with SK) and late (3 min; 100% HPm in the equimolar complex with SK) incubation times also show decreased K_m values and increased k_{cat} values at the early times, providing very strong evidence for the greater efficacy of the SK–HPg complex in plasminogen activation, as compared to the SK–HPm complex. In addition, the insect cell expressed HPg behaves in this regard in an identical fashion as does the plasma HPg (Figure 2), further demonstrating the strong equivalence of these two proteins.

Finally, given the above results showing the potency of SK–HPg complexes in activation of plasminogen, it became of great interest to us to determine whether cleavage site resistant plasminogen mutants could be expressed in mammalian cells. Success in this regard would allow provision of a mammalian source for expression of the human plasminogen cDNA, which, importantly, does not require human plasma fractionation. The resulting HPg, in combination with SK, would possibly provide a thrombolytic agent more effective than SK–HPm complexes. Current reagents of this type most probably contain HPm, since the conversion of HPg to HPm within the complex is so rapid. In order to examine this possibility, we attempted expression in CHO cells of a variant HPg, viz., R^{561}S-crHPg. This expression was successful. Using the equimolar SK complex with this variant HPg in amidolytic kinetic assays (Table I), we find no substantial

differences in its catalytic properties from the other plasminogens and plasmins employed in this study. The ability of this same enzyme complex to activate BPg (Table II) also has been examined, and its steady-state kinetic constants are similar to those of the other SK–HPg complexes. Thus, the greater efficiencies of SK–HPg complexes toward activation of plasminogen, as compared to SK–HPm complexes, appears to be of general nature. In order to determine whether differences in glycosylation of HPg, resulting from expression of its cDNA in different cell systems, could play a role in its kinetic properties, we have deglycosylated the CHO-expressed protein at its Asn-linked oligosasccharide sites with glycopeptidase F and reexamined the steady-state kinetic properties of this form of the protein (SK–R^{561}C-crHPg-ΔCHO) in equimolar complex with SK. While only small differences were observed in the amidolytic kinetic constants (Table I) of the resulting enzyme complex, more substantial differences were seen in its steady-state kinetic constants for plasminogen activator (Table II). The deglycosylated form of the CHO-expressed variant HPg, in equimolar complex with SK, allowed the complex to become an even more efficient activator of BPg. This indicates that the nature of the carbohydrate may play some role in this particular property of HPg.

In conclusion, we have shown that stabilization of HPg within the SK complex results in its ability to activate plasminogen to be greatly increased. In addition, we have also described a manner to express stable forms of plasminogen in mammalian cells for possible use in this regard. These results are of great utility in investigating structure–function relationships with plasminogen, as well as of possible use in designing reagents for application in thrombolytic therapy.

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