THE MECHANISM OF ACTIVATION OF HUMAN PLASMINOGEN BY STREPTOKINASE

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SUMMARY

The mechanism of activation of human plasminogen to human plasmin by streptokinase was investigated by the use of the active site titrant p-nitrophenyl-p'-guanidinobenzoate. These studies show that (a) human plasmin is not required to initiate this activation, (b) streptokinase and human plasminogen combine in a one to one molar ratio to yield a complex which undergoes a time and temperature dependent change generating an active site, (c) the formation of this active site does not require cleavage of plasminogen into two peptide chains.

The mechanism of activation of human plasminogen (HPg)[1] to plasmin (HP) by streptokinase (SK) has been the subject of many investigations. It has been deduced from sequential rate studies that SK first combines with a "proactivator" in human plasma to form an "activator" complex (SKX). This complex then functions catalytically to convert HPg to HP [see Amery and Claeys for a recent review of the subject (1)]. It has been shown by physical methods (2,3) that at least one of the proactivators is in fact HPg. In the course of our characterization of purified HPg's the active site titrant p-nitrophenyl-p'-guanidinobenzoate (NPGB), was used (4). The rapid and irreversible nature of the active site acylation by this agent made it possible to follow the course and the extent of the formation of the activator complex while at the same time preventing extraneous auto-digestion, since the acylated material lacks proteolytic activity. The

[1] Abbreviations used are: HPg, human plasminogen; HP, human plasmin; SK, streptokinase; NPGB, p-nitrophenyl-p'-guanidinobenzoate; SKX, streptokinase activator complex; NP, p-nitrophenol; ME, 2-mercapto-ethanol; DFP, diisopropyl phosphoro-fluoridate; CTA, NHI Committee on Thrombolytic Agents.

results of this direct kinetic study are the subject of this communication.

MATERIALS AND METHODS

The HPg was purified from Fraction III [plasma alcohol fractionation method 9 (5)] using either the method of Robbins et al. (6) or a modification of the affinity chromatography procedure of Deutsch and Mertz (7) as the first step. These preparations were further fractionated on carboxymethylcellulose as described by Wallén (8). The last HPg protein peak eluted from this column was used for these studies. These purified HPg's were stored frozen at -70°, at a concentration of 5-6 mg/ml, in 0.05 M ammonium acetate and 0.02 M lysine buffer at pH 5.0. Samples were either diluted into, or dialyzed against the buffers indicated without significant inactivation.

After catalytic activation with SK the proteolytic activities of these HPg's were compared with the CTA primary HP standard (9). These data along with active site titration results are shown in Table I. Unless otherwise specified, the HPg prepared by the methods of references 7 and 8 were used for the studies reported.

Streptokinase was prepared as described by De Renzo et al. (10) and stored as the lyophilized powder.

Table I. Plasminogen activity.

Purification Sequence (Ref. Cited)	CTA Units/mg[a]	NPGB Titration	
		Moles of Site/mg[a]	% of Theory[b]
6 + 8	35	10×10^{-9}	85
7 + 8		12×10^{-9}	100

[a] Calculated from absorbence 280 nm using $E_{cm}^{1\%} = 17$.

[b] Assuming a M. W. of 85,000.

NPGB was purchased from Cyclo Chemical Co., Los Angeles (Lot No. K 5965) and used without further purification. All other chemicals were of reagent grade quality.

The active site titrations ("burst assays") as described by Chase and Shaw (4) were carried out at pH 8.3 in veronal buffer or at pH 7.4 in phosphate buffer. All rate studies were at pH 7.4 in 0.1 M phosphate and 0.1 M lysine. The time dependence of active site formation in the presence of excess NPGB was obtained with a Gilford Model 2000 at 410 nm using a thermostated cuvette and cell compartment (11). Initial velocities were obtained directly from the recorder tracing after correction for any spontaneous hydrolysis of NPGB. Specific conditions are given in the legends of each figure.

Starch gel electrophoresis studies were essentially as described by Robbins et al. (12). Samples (ca. 3-6 mg/ml protein) were reduced with 4 M ME for one hour at 37° in 8 M urea at pH 8.0, after which the pH was adjusted to 3.2 with 1 M HCl. A volume of 0.05 ml was applied to the gel. Electrophoresis was in 8 M urea + 0.01 M ME, 0.067 M formate buffer, pH 3.2, 19.5 hours, 25°, and 5 volts/cm.

RESULTS AND DISCUSSION

Preliminary studies with the active site titrant NPGB indicated that this acylating agent did not react with either HPg or SK. NPGB did, however, react very rapidly with either catalytically generated HP or pre-formed activator[2] with the concomitant liberation of nitrophenol (NP). In fact, the reaction with pre-formed active sites was essentially complete within the mixing time of our apparatus and therefore appeared as a "burst" of NP. On the other hand, when SK was added to a sample of HPg containing an excess of NPGB, a readily measurable rate of NP production was observed.

[2] Pre-formed activator was obtained by mixing stoichiometric amounts of HPg and SK and allowing this mixture to stand for several minutes prior to addition of NPGB.

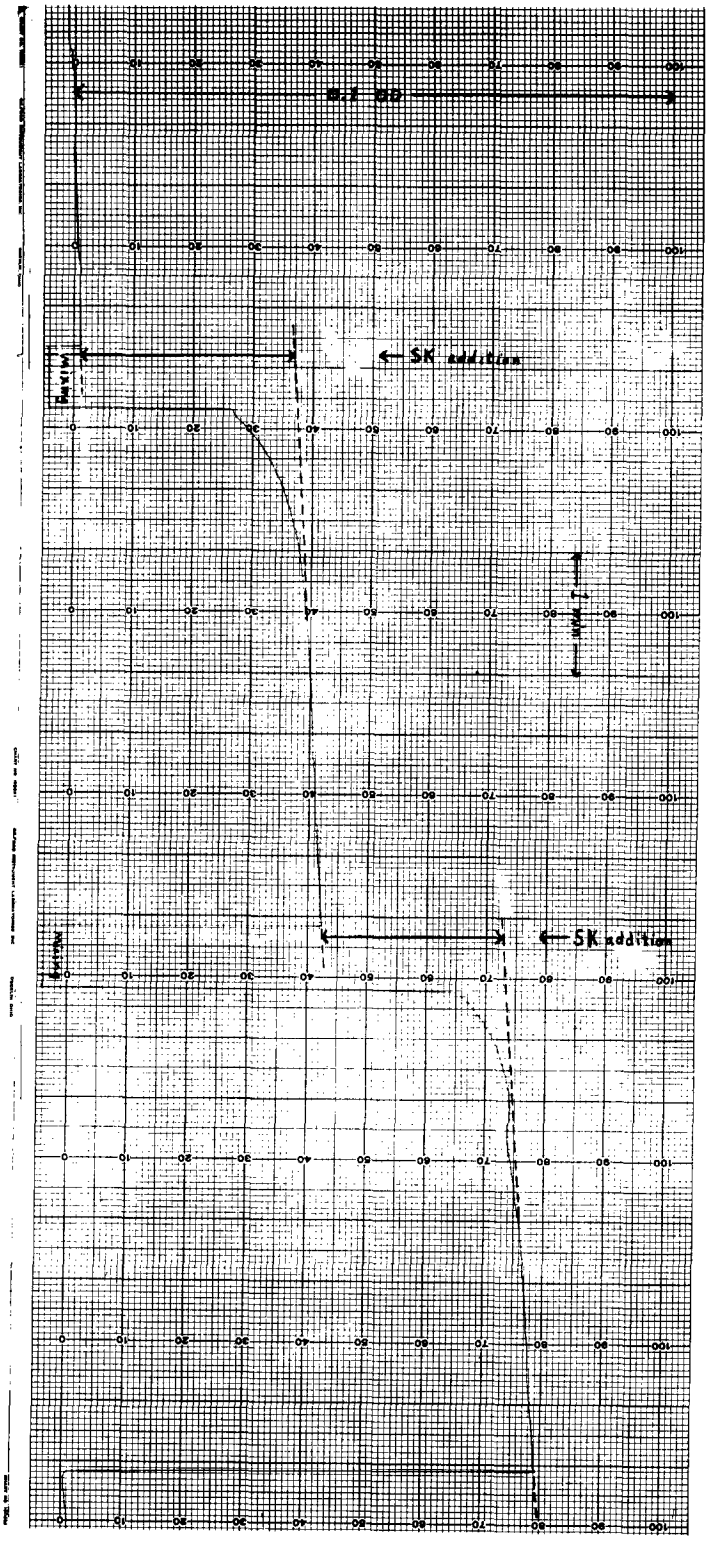

Figure 1. Incremental addition of SK to HPg pretreated for 5 minutes with excess NPGB. HPg present: 14×10^{-9} moles in 1.0 ml of 0.1 M phosphate and 0.01 M lysine at pH 7.4 plus 15 μl 0.01 M NPGB at 25°. 10 μl samples of 2.8×10^{-4} M SK were added at 5 minutes intervals. A total of nine SK additions were made, only the first two being shown.

This rate was not dependent on the NPGB concentration. These results showed conclusively that pre-formed HP was not a requirement for the generation of an active site since HP (which never represented more than 5% of the HPg present) was inactivated by NPGB prior to addition of SK. Earlier studies using DFP had led to the same conclusion (13,14). These data further suggested that the appearance of NP could be used to monitor the rate limiting step involved in the formation of the active site.

The data of Figure 1 show that the incremental addition of SK to an HPg-NPGB mixture leads to the generation of NP, the extent of which is limited by the quantity of SK added. It is clear that under these conditions, SK is functioning stoichiometrically and is not being regenerated.

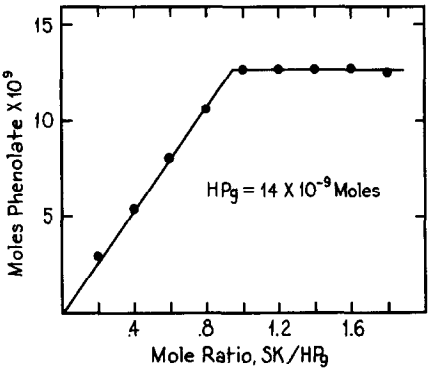

Figure 2. Total moles of phenolate formed (total moles of active site) versus SK/HPg mole ratio. Data taken from the experiment of Figure 1.

The sum of the incremental responses from the experiment of Figure 1 is shown plotted as moles of NP formed against the SK/HPg ratios in Figure 2. A maximum number of sites, which is nearly equivalent to the moles of HPg added initially, is obtained when the mole ratio of SK to HPg is equal to one.

An important question concerns the identity of the rate limiting step leading to the formation of active site. Information on this point was obtained from a kinetic study of the formation of the NPGB susceptible

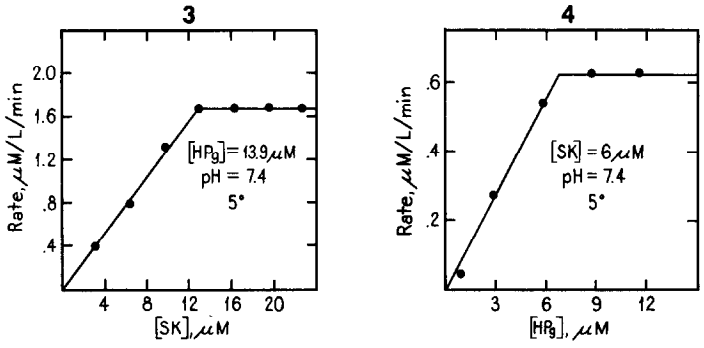

Figure 3. Initial rates of active site formation as a function of streptokinase concentration. [HPg] = 13.9 μM in 0.1 M phosphate and 0.1 M lysine, pH 7.4 at 5°. [NPGB] = 2.2×10^{-4} M, [SK] as indicated.

Figure 4. Initial rates of active site formation as a function of plasminogen concentration. [SK] = 6 μM in 0.1 M phosphate and 0.1 M lysine, pH 7.4 at 5°. [NPGB] = 2.2×10^{-4} M, [HPg] as indicated.

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site. The initial rates of active site formation as a function of [SK] or [HPg] are shown in Figures 3 and 4, respectively. If the rate limiting step were the bimolecular reaction SK + HPg $\longrightarrow$ SK·HPg, the sharp breaks in these curves would not be expected. In addition, these results suggest that the initial complex between SK and HPg does not contain the active site. These data, however, are consistent with a two-step mechanism whereby SK and HPg first combine rapidly in a one to one molecular ratio to form a simple complex with no active site. This complex is then converted in a second unimolecular rate limiting step to the species containing the NPGB susceptible site.

In further support of this mechanism, Figure 5 shows a first-order plot of a representative reaction. The assumption is made that all available HPg has been rapidly and completely converted to the SK·HPg complex. The plot is linear for at least 60% of the reaction as would be expected for the proposed mechanism.

The HPg prepared by the procedures given in references 6 and 8 gave rate data which was qualitatively the same as that shown above. Rate

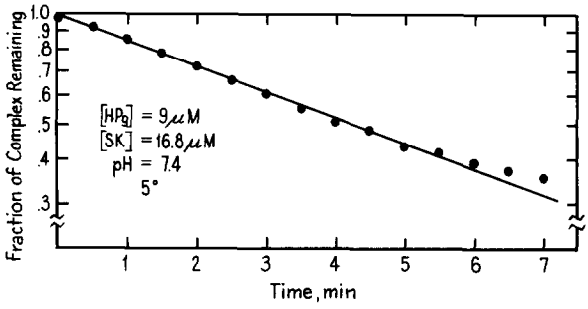

Figure 5. First order plot of the reaction leading to formation of active site. [HPg] = 9 μM, [SK] = 16.8 μM, [NPGB] = 2.2 x 10^{-4} M in 0.1 M phosphate and 0.1 M lysine, pH 7.4 at 5°.

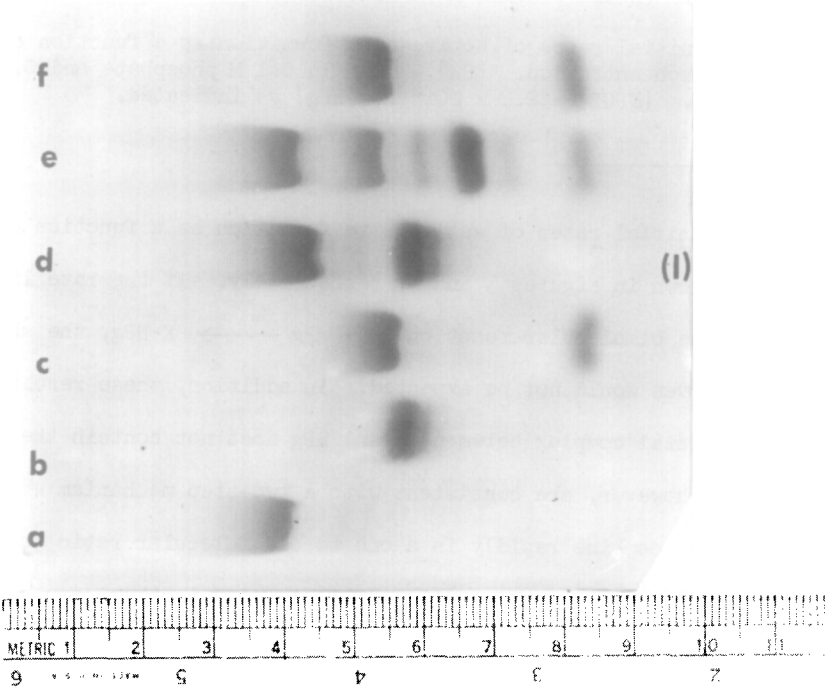

Figure 6. Examination by starch gel electrophoresis of the products formed from HPg and SK after reduction and dissociation in urea-ME.
(a) HPg, control, (b) SK, control, (c) HP, catalytically formed with SK and treated with NPGB; HPg:SK (40:1), 45 minutes, 25°, pH 7.4; then 10-fold excess NPGB, (d) Complex between HPg and SK, formed in the presence of a 10-fold molar excess NPGB; 20 minutes, 5°, pH 7.4, (e) Complex between equimolar amounts of HPg and SK, formed in the absence of NPGB; 6 minutes, 25°, pH 7.4; then treated with a 10-fold molar excess of NPGB, (f) As in c; activation for 80 minutes before addition of excess NPGB.

studies with this HPg, over a range of temperature (5°-25°) showed that an energy of about one kilocalorie was required for the formation of one mole of active site.

Starch gel electrophoresis in urea-ME, after reduction, has been shown by Robbins et al. (12) to be a very useful method for detecting peptide bond cleavages in the HPg molecule. Application of this technique is shown in Figure 6. These results demonstrate that while active site formation in the absence of NPGB leads rapidly to a number of split products (c, e and f), no such behavior is observed if the site is generated in the presence of NPGB (d). Therefore, it is clear that it is possible to generate this active site without the peptide bond cleavage which leads to the two-chain type structure of HP.

If it is assumed that there is a single latent active site in the plasminogen molecule, NPGB must be reacting with the same site as is found in activator and plasmin. However, it may be, as proposed by De Renzo and coworkers (15, 16) that SK first induces a reversible conformational exposure of this active site which in the absence of NPGB may subsequently be permanently exposed by a peptide bond cleavage.

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ERRATA

Volume 42, No. 3 (1971) in the Communication, "Nitrogenase from Vanadium-grown Azotobacter: Isolation, Characteristics, and Mechanistic Implications", by R. C. Burns, W. H. Fuchsman and R. W. F. Hardy, pages 353-358, page 353 - METHODS - the first sentence should read:

"Azotobacter vinelandii OP (ATCC 13705) was grown on a modified Burke's medium supplemented with 1 mg Mo/liter as $Na_2MoO_4 \cdot 2H_2O$ (3) or 1 mg V/liter as $Na_3VO_3 \cdot 16H_2O$."

Volume 42, No. 5 (1971), in the Communication, "Control Properties of Yeast Glycogen Phosphorylase", by F. Sagardia, I. Gotay, and M. Rodríguez, pages 829-835, page 830 in section "Materials and Methods', first sentence should read:

"Bakers yeast phosphorylase (Fleischman's) was assayed as described previously for blue crab muscle phosphorylase (5), except that no AMP or cysteine was required, and.....".

Volume 42, Number 6 of March 19, 1971, due to a technical error pages 1039 and 1139 have been interchanged.