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Site directed mutagenesis is helping of the central domain of streptokinase in substrate plasminogen

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Abstract

Role of the central β-domain residues 151-287 of streptokinase was probed by site specifically altering two charged residues at a time to alanines in a region residues 230-290 previously identify by peptide walking to play role in PG plasminogen activation .these mutants were then screened for altered ability to activate equimolar human PG with substrate PG resulting in an overall compromised capability for substrate PG processing.SK kk256.257AA showed roughly 20-fold reduction in PG activator activity in comparison to wild type SK expressed in Escherichia coli .these feature were drastically accentuated when the charges on the two Lys residues at position 256 and 257 of SK were reversed to obtain SK kk256.257EE.this mutant showed PG activator activity approximately 10-fold less than that of SK.small amounts of human plasmin in the PG activation reaction of SK resulted in dramatic. Rejuvenation of its PG activation capability .indicating that it required pre-existing to from functional activator since it could not effect active site exposure in partner PG on its own. Further conclusion confirmed by its inability to show of p-nitrophenol released in the presence of equimolar human PG and p-nitrophenyl guanidine benzoate. The steady-state kinetic parameters for HPG activation of its 1 ratio 1 complex with human PN revealed that although it could form a highly functional activator .SK mutants carrying simultaneous two and three site charge-cluster alterations SK RE248.249AA; EK281.282 (SK(CH))SK EK272.273AA;EK281.282AA SK (FH)and SK RE248.249AA; EK272.273AA;EK281.282AA SK(CFH).these result provide direct assistance by the SKβ-domain in the doocingand processing of substrate PG by activator complex.

Key words: Plasminogen; Plasminogen activation mechanism; Site-specific mutagenesis ;Substrate plasminogen.

Introduction

Activators of plasminogen such as streptokinase and urokinase are huge use as thrombolytic agent for the treatment of diverse circulatory disorders including myocardial infarction .however unlike UK and TPA which are proteases .SK is enzymatic ally inert .it act by forming a non covalent high affinity complex with HPG.this complex is then believed to undergo an intra molecular conformational change that expose the active site in the zymogens.SK –HPG now become capable of acylation and can be titrated by hydrolysis of p-nitrophenyl p-guanidino benzoate to p-nitrophenol burst or catalyze the processing of small –MW amidolytic substrate,an understanding of the structural processes involved in first the exposure of the active site in the virgin SK-HPG complex followed by its transformation to highly specific protease that unlike free plasmin displays a very high substrate preference for HPG.(Markus & Werkheiser.1964)is crucial to the design of improved SK-based thrombolytic agents.

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SK has been shown to be composed of three distinct domains designated α,β,and separated by two coiled coils and small region at the N and C terminal of the protein with disordered flexible structures. Although it has been demonstrated that the three domains retain significant amount of their original *native like structure. In contrast to the absence of any HPG activator activity in* isolated domains .the characteristic high affinity bind of SK with HPG more recently using physico-chemical approaches it has been elegantly demonstrated that the ß domain contributes in a major way toward the generation of the high affinity interaction between SK and HPG that lead to the formation of the equimolar activator complex between the two protein.(conejero-lara et al .1998)therefore the extract role of this domain particularly in terms of the epitopes that may may be involved in interacting with HPG to translate the primary event in SK-HPG interaction namely the avid binding between these two protein to the catalytic high efficiency conversion of substrate

molecules of HPG to HPN by the activator complex needs to be explored further.

In the present paper using site-directed mutagenesis as a tool.we present experimental evidence for an important role of the ß-domain in substrate docking and processing by the SK plasmin(ogen)activator complex and probably in zymogen activation as well.

Material and Methods

Glu-HPG was procured from boehringer-mannheim. Where albumin –free HPG was specifically needed Glu-HPG was purified from human plasma by affinity chromatography on lysine –agarose in the presence of protease inhibitors as describe.both preparation contained less than 2% of lys-HPG and less than 0.01%free HPN.where needed HPG was converted to HPN by the action of agarose –immobilized .in 50mM tris-cl buffer,pH8.0,containing 10mg/mlHPG,25mML-lysine & 25%glycerol for 12 h at 22.C(3mg STL/ml agarose)used to prepare HPN-deficient HPG .protein concentration were determined by the method Bradford s.equisimilis SK.used for the determination of specific activities of SK .chromogenic plasmin substrate tosyl-Gly-pro-Lys-anilide was obtained from Boehringer-mannheim.the T 7 RNA polymerase promoter based vector pET23(d)used for the intracellular expression of SK in e coli.was product of novageninc. STI was procured from sigma chemical.thermostable DNA polymerase with proof-reading activity plasmid bluescript ll KS.and E.coli XL-blue were procured from stratagene lnc.restriction endonucleases and other enzyme used for rDNA experiment were procured from new England biolabs.oligonucleotide primer were either synthesized in-house on an applied biosystems.DNA synthesized model 492 by Ransom Hill biosciences lnc.N-terminal protein sequencer was done on perkin elmer /applied biosystem sequencer.model 476 A.PCR generated DNAs from agarose gels.&plasmid DNAs were routinely purified using kits from Qiagen lnc.Automated fluorescence-dye DNA sequencing was carried out an applied biosystems /perkin Elmer DNA sequencer system.model 377 at the university of delhi.all other reagents used were of the highest analytical grade available..

Design and construction of SK mutants

The cloning of the SK of S.equisimilis strain H46 in E coli was carried out essentially by the approach of Malke and Ferretti (1984)the different SK mutant were constructed by PCR-based strategy using the megaprimer method of oligonucleotide directed site-directed mutagenesis using one mutagenic and two flanking primer followed by cloning into plasmid vector by standard methodologies.the primer were designed to be complementary to the known DNA sequence of the SK gene from S.equisimilis.the first PCRs were carried out in volume of 100 µ L.and contained 200 µ MdNTPs 100ng of template DNA pET23(d)-SK the T 7 polymerase based expression vector in which the full-length SK gene had been cloned.the PCR cycling condition were in accordance with the denaturation temp.of different primer.DNA cassettes were then purified by agarose gel electrophoresis by excising the required DNA band and further purifying by qiagen gel extraction kit.60ng template pET23(d)-SK 200 µM dNTPs 10µL of 10 X pfu buffer and varying concentration of megaprimer mix were held for 5 min.at 95CĊ and the reaction addition of 5.0Upfu DNA polymerase per reaction .this was followed by seven cycle of denaturation and extension to ensure build –up of mutated stand DNA.after the last cycle 20pmol of downstream primer was add and cycling continued as before for 15 cycles followed by a final extension at 72 Ċ for 10 min.

Expression and purification of SKmutant

The native like recombinant SK mutant were expressed intracellularly in E.coli.the bacterial pellet obtained by centrifugation 1 L shake flask culture were suspended in 80mL of 20mM Na Po4 buffer pH7.2 and subjected to ultrasonication.for 10 min.after centrifugation at 4Ċ.at 12000x g for 15 min.the supernatant were made 0.5M in NaCl and loaded onto colum of phenyl-agarose in equilibrating buffer.the column was then washed successively with five bed volumes each of equilibrating buffer 20 mM NaPo4 buffer.pH 7.2 distilled water and finally 8M urea.the SK was more then 95% pure at this stage.the major SK-containing fraction were pooled and stored at-70 Ċ…until analyzed.

Results & Discussion

The sequence of residues 230-290 of SK from streptokinase equisimilis that has been previously implicated in the interaction of SK with HPG by peptide walking studies is depicted in figure 1.

IIe Phe Arg Thr IIe Leu Pro Met Asp Gln
230 239
• + + + -
Glu Phe Thr Tyr Arg Val Lys Asn Arg Glu
240 249
+ +
Gln Ala Tyr Arg IIe Asn Lys Lys Ser Gly

250 259
- -
Leu Asn Glu Glu IIe Asn Asn Thr Asp Leu
260 269
- + + +
IIe Ser Glu Lys Tyr Tyr Val Leu Lys Lys
270 279
- +
Gly Glu Lys Pro Tyr Asp Pro Phe Asp Arg Ser
280
289 290

Fig.1. Primary structure of target sequence for mutagenesis in the β-domain of SK.Amino acid sequence of the residues 230-290 of the β – domain of SK from S.equisimilis H46A is shown.the charged cluster that were reversed by mutation are bold underlined..

Charge –cluster mutation Oligonucleotide sequence R.E.[b] site

DE 238.240 AA 5'-ATTTTACCcATGGcgCAAGcGTTTACTTACC-3' Nco I

RK 244.246AA 5'-AAGAGTTTACgTACgcTGTTgcAAATCGGGAAC-3' Bsi WI

RE 248.249AA 5'-CGTGTTAAAAATgctGcgCAAGCTTATAGGAT-3' FspI

KK 256.257AA 5'-GCTTATAGGATtAATgcAgcATCTGGTCTGAATGAA-3' Vsp I

KK 256.257EE 5'-AGGATCAATgAAgAATCTGGTCTcAATGAAGAAATA-3 Bsm A I

EE 262.263KK 5'-AAAAAATCTGGTCTcAAaAAaAAATAAACAACACT-3' Alw26 1

EE 262.263AA 5'-AATAAAAAATCTGGgCTcAATgcAgcAATAAACAACACT-3' Bsp12861

EK 272.273AA 5'-ACCTGATCTCTGcagcgTATTACGTCCTT-3' Pst I

KK 278.279AA 5'-AGAAATATTACGTaCTTgcAgcAGGGGAAAAGC-3' Bsa AI

EK 281.282AA 5'-AAAAAAGGGGcggcGCCGTATGATCC-3' Nar I

Upstream primer 5'-ATTTATGAACGTGACTCCTCAATCGTC-3' Bse RI

Downstream primer 5'-ATAGGCTAAATGATAGCTAGCATTCTCTCC-3' Bsm I

Table 1. sequence of PCR primers used for the construction of streptokinase mutant…

The mutant primer shown this table are for constriction of single charge-cluster mutant.

These primer had sequences ,carrying unique R.E.site present in the nSK gene were used to mutant PCR,into the SK expression vector.

This region of primary structure of SK show several positively and negatively charged clustered residues.the possible role of these side chain site specific mutation of eight such clustered charged to alanine residues were carried out using a PCR –based megaprimer.the mutated cassettes were then docked back into the native SK gene in the expression plasmid and the gene expressed intracellulary in E.coli and purified to homogeneity .you can see Table 1.for sequence of primer used for mutagenesis.

Perhaps the most question with the mode of action of PG activators in general and SK Plasmin activator complex in particular .in the case of the latter the identification of the mechanism underlying the conversion of the relatively nonspecific trypsin like substrate specificity of plasmin to that of high PG specific protease upon complexing with SK still constitutes a challenge despite the recent availability of the three dimensional structure of the SK- human plasmin light chain complex.SK could produce a change in the specificity of the plasmin active site by change its conformation by providing altered secondary substrate binding subsites into the active centre.it is also possible that more than one of the above possibilities is operative together in the function of SK. However the structure of the SK-plasmin light chain complex strongly support the third possibility. Which we had also advanced earlier on the basis of biochemical studies .that SK switches the substrate specificity of plasmin because the SK plasmin complex provides two substrate specific sites for the optimal binding of substrate PG onto the complex.

Our mutagenesis studies show that the replacement of the two Lys residues at position 256 and 257 in the core region with either Ala residues affects the HPG activation properties of the molecule. In that study the Lys residues were altered to Ala residues and a decrease in HPG activation kinetics by the mutant was noted. However in the present investigation by mutating the lysyl to glutamly side chains a structurally much more nonconservative alteration.

Greater insight into the structure function inter relationship of residues 256 and 257 could be obtained. The presence of two consecutive negative charges at this locus turned out to be highly detrimental to the function of the molecules. Which now showed a very low overall HPG activation capability even though its apparent affinity for HPG to form 1¦1 complex was largely unaffected.. in the present study a direct experimental evidence of the involvement of the β-domain in such a protein cofactor mediated catalytic assistance and substrate docking mechanism for SK action is being reported.

Conclusion

At the last we can conclude this study that possible role of the β-domain of streptokinase. These features were drastically accentuated when the charge on the two Lys residues at position 256 and 257 of streptokinase. Substitution of negatively charged side chain at position 256 and 257 of SK had adversely affected the protein ability to rapidly form a stoichiometric complex with HPG. However the apparent affinity of SK for HPG when determined by the solid phase radioassay. Was found to be virtually unaltered 1.5 fold decrease compared to Nsk indicating that its observed inability to activate equimolar zymogen did not emanate from a correspondingly lowered affinity between the complex partners.

These results thus suggest the absence of grossly misfolded structure in the mutated SK derivatives. It should be mentioned however that the native like CD spectra do not necessarily rule out the presence of conformational alteration in the vicinity of the mutation since the spectral contribution arising from these changes could easily have been averaged out by the spectral contribution emanating from the remainder of the molecule.

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