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Role of N-Terminal Domain of Streptokinase in Protein Transport

Jitesh Pratap, Jagdeep Kaur, G. RajaMohan, Dawinder Singh, and Kanak L. Dikshit[1]

Institute of Microbial Technology, Sector 39 A, Chandigarh 160014 India

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Streptokinase (SK), an extracellular protein of several haemolytic strains of *Streptococcus,* is utilized as a potent thrombolytic agent for the treatment of various myocardial disorders. Functional properties of SK remain unchanged when the first 13 N-terminal amino acid (aa) residues are removed. At present, role of this segment in protein structure function is unclear. *skc* gene encoding for the mature SK and its deletion variant, lacking its first 13 aa residues, were cloned and expressed in *E.coli.* Full length SK, deprived of any leader sequences, was able to translocate slowly, across the cyto-plasmic and outer membranes of *E.coli.* Whereas, SK derivative, devoid of its first 13 N-terminal aa residues, could not do so. Cell fractionation studies as well as genetic evidences utilizing alkaline phosphatase fusion, point towards the existence of additional information for protein transport, within the N-terminal domain of SK. To further investigate the role of this region in protein secretion, genetic fusions were created in between full length and 13 aa deleted SK with *OmpA* leader peptide. Studies on kinetics of SK export from *E.coli,* revealed that translocation of protein is 3-4 times faster when the first 13 N-terminal residues of SK are intact. On the basis of results obtained, it has been proposed that the N-terminus of mature SK maintains the export competent status of protein and, thus, confer speed and efficiency upon the translocation process of streptokinase.

Streptokinase, one of the potent fibrinolytic agents, utilized routinely for thrombolytic therapy, is produced as a secreted 47 kD protein by some pathogenic strains of *Streptococcus* (1, 2). With the importance of SK as a potential thrombolytic agent, the techniques of genetic engineering are currently being applied by several groups of investigator in attempt to understand molecular mechanisms of its function (3, 4, 5). From the point of view of protein function, SK represents a unique system. Unlike most plasminogen activators such as, tPA and Urokinase, it does not display any inherent catalytic activity, instead, it activates plasminogen through forming a strong stoichiometric complex with plasminogen, which catalyzes the formation of proteolytically active product, plasmin (6, 7). Thus, SK provides an interersting model for the study of protein-protein interaction and modulation of protein folding. In the past, most attempts towards understanding the protein structure function relationship in SK, have mainly focused on understanding the interaction of SK with plasminogen. Biochemical and genetic studies, collectively, have revealed that the loci of interaction with plasminogen resides within two distinct regions of SK (5,8,9). Studies involving interaction of SK fragments with plasminogen suggested that the N-terminus of SK may be an independent domain with functions which are unrelated to binding or activation of plasminogen (5,10). This presumption got support with the findings that the recombinant SK fusion protein in which the 13 or 14 N terminal aa residues of SK were replaced with fragments of unrelated proteins, retained the full plasminogen activator capability (11). Additionally, the chemical cleavage of 15 N-terminal residues of mature SK does not result in the loss of its biological function (12). Thus, functional role of N-terminal domain of SK remains obscure at present.

Gene (*skc*), encoding for SK, has been cloned and expressed in different heterologous hosts

[1] Corresponding author. Fax + 9(0172) 690585. E-mail: kanaklata@imtech.ernet.in.

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by various groups of investigators (13,14). Post-translational processing of 47 kD SK at its C-terminus results in truncation of SK into 44 kD form which retains its capability of plasminogen activation (15). Similarly, gene fusion studies utilizing SK and β-galactosidase, revealed that the first 13 aa residues of mature SK may be substituted with several hundreds of unrelated amino acids without preventing SK from activating human plasminogen (11). Thus, both N and C termini of SK are not involved in the main biological function of protein and may have some other role (s), unrelated to plasminogen activation.

In an attempt to study extracellular production of SK in heterologous host systems, we found that the transport capability of SK and its variants, lacking a part of its N-terminal region, are significantly different in *E.coli.* It prompted us to explore the role of N-terminal domain of SK in protein trans-location. In the present study, we have demonstrated that the N-terminus of mature SK carries additional information for protein secretion and allows slow transport of SK in *E.coli* even in the absence of any leader peptide. During signal peptide directed transport of SK, it further aids in efficiency of protein translocation across the cellular membranes.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Streptococcus equisimilis H46A(ATCC 12449) was utilized for retrieving gene encoding for the SK. *E. coli* strains, AG1 (Stratagene, Germany), JM109 and BL21DE3 (Invitrogen) and plasmids, pBR322 and pUC 19 (New England Biolabs) were obtined from commercial sources and utilized for various cloning purposes. Plasmids, ptac85 (16) and pTON (17) were utilized as expresion and secretion vectors, respectively. All restriction and DNA modifying enzymes were obtained from Promega (WI, USA) or New England Biolabs (Beverly, USA). Standard recombinant techniques were applied for genetic manipulations (18).

Construction of SK expression plasmids. A 2.5 kb Pst I fragment carrying the entire structural gene of streptokinase (*skc*) along with its flanking regulatory region, was cloned from *Streptococcus* H46A into *E.coli* on the plasmid vector pBR322 in an approach similar to that described by Malke and Ferretti (13). This plasmid construct was designated as pJKD-8 (Fig.1A) and used as a source for retrieving *skc* gene for the construction of various SK expression plasmids. Plasmids, pJKD-38 and pJKD-39 (Fig.1A), were created to express mature SK and its truncated derivative (carrying deletion of 13 N-terminal aa residues), lacking any signal peptide, under the control of *tac* promoter. Sequence of oligomers used for the PCR amplification of *skc* gene is given as 1). 5′-GCCATGGCATGATTGCTG-GACGTGAG-3′; 2) 5′-GCCATGCCATGAACAACAGCCAATTA-3′; 3) 5′-GTCGACGGATCCTTATTT-3′; 4) 5′-GCCATGGCTATTGCTGGACGTGAG-3′, 5) 5′-GCCATGGCTAACAACAGCCAA-3′. Oligomer sets, 1-3 and 2-3 were used to amplify full length and 13 aa deleted form of SK, respectively. Through 5′-PCR primer an ATG codon was introduced for the intracellular expression of SK and its variant form. Maps of these plasmids are given in Fig.1. Plasmid pTON (17), which carries *OmpA* signal peptide sequence under the control of T7 promoter, was used to create fusion with full length and truncated form of SK. Utilizing the oligomer sets, 4-3 and 5-3, full length and truncated *skc* gene was amplified and cloned at NcoI-Bam HI site of pTON. Nucleotide sequence of fusion junction was determined for checking the correct linkage between *OmpA* and *skc* gene. Detail maps of these plasmid constructs are given in Fig.1A.

Cell fractionation. Quantitative fractionation of cells into periplasmic and cytoplasmic fractions was done by osmotic shock method (19). β-Lactamase (20) and β-galactosidase (21) were assayed as periplasmic and cytoplasmic control markers, respectively. The cytoplasmic protein fraction was obtained by subjecting the osmotically shocked cells to sonication in 0.5mM magnesium chloride at 0°C with four intermittent 20 s pulses.

Western blot analysis. Polyclonal antisera against purified SK (Sigma) were raised by immunizing rabbits following the standard protocol (22). Protein samples were analysed by SDS-PAGE according to the method of Laemmli (23). The electrophoresed proteins on the gel were transferred onto nitrocellulose paper and cross reacted with antiSK to visualize specific SK band.

Assay of Streptokinase activity. SK activity was assayed by the method of Jackson *et.al.* (24) using chromozyme PL (Boehringer Mannheim, Germany) as an artificial substrate. Human plasminogen (Boehringer, Mannheim) was activated with SK at 37°C for 15 min and amydolytic activity of this complex was monitored at 405 nm after the addition of synthetic plasmin substrate, chromozyme PL. Purified SK (WHO) was used as standard to quantitate the protein.

Construction of SK-phoA fusions. The procedure, developed by Manoil and Beckwith (25) for constructing *phoA* fusions on plasmid encoded genes using λ Tn *phoA,* was utilized to create SK-*phoA* fusions. *E.coli* C118 and Tn*phoA* were kindly provided by Prof. C.Manoil (University of Washington, Seattle, USA). Plasmid used for this purpose was pJKD-38 which carries entire *skc* gene for the production of full length SK. Integration of alkaline phosphatase

with SK was obtained by selecting blue colonies on 5-bromo-4-chloro-3-indolyl phosphate (Sigma) plate. Purity of clones that expressed active *phoA* was checked by restriction mapping of the resulting plasmid and loss of SK activity due to Tn *phoA* integration.

Proteinase K accessibility test. To demonstrate that SK is translocated across the cellular membranes of *E.coli,* and is not the result of leakage due to cell lysis, we performed proteinase K accessibility test as described by Rasmussen *et. al.* (26). with slight modification. Here instead of immunoprecipitating the protein prior to analysis, samples were directly solubilized in SDS sample buffer and assayed through Western blotting.

RESULTS

(a) Streptokinase deprived of its signal sequence is partially translocated into E.coli. Utilizing the earlier established procedure developed by Malke and Ferretti (13), *skc* gene encoding for the SK was cloned from *Streptococcus* into *E.coli* on a 2.5 kb Pst I fragment resulting in the plasmid construct, pJKD-8. This plasmid carries entire *skc* gene along with its flanking regulatory region and is essentially similar to that reported by Malke and Ferretti (13). To construct an expression vector for leader sequence-deleted SK, *skc* gene, coding for the full length and 13 aa residues deleted SK, was isolated by PCR. Through 5′ amplification primer an ATG codon was introduced for translation initiation in *E.coli.* The amplified product was cloned on the expression plasmid, ptac 85 (16), at NcoI - Sal I site. It resulted in the plasmid construct, pJKD-38 and pJKD-39, capable of expressing full length and 13aa residues deleted form of SK, respectively, after IPTG induction. Inspite of leader peptide deletion, significant level of SK secretion was detected in *E.coli* carrying the plasmid construct, pJKD-38, encoding for the full length SK (Fig.1 B). Whereas, when 13 aa residues deleted SK was expressed in *E.coli,* release of SK into the culture broth was not observed. To further verify the secretion of SK through these plasmid constructs, localization of SK activity was monitored in different cellular compartments at various stages of growth (Fig.2 A) through monitoring the level of SK in different cellular fractions. Results indicated that 20-30 % of SK is transported into the periplasmic and extracellular medium, when full length SK, lacking any leader sequences, is expressed in *E.coli.* In contrast, presence of SK could not be detected in the periplasm and culture supernatant of *E.coli* in the case of SK deletion derivative, lacking its 13 N terminal aa residues. Western blotting of total proteins obtained from various cellular fractions, further substaintiated this result. The absence of β-galactosidase activity in the extracellular fraction indicated that the detection of SK in the periplasm and culture supernatant is not due to any cell lysis. These results demonstrated that the mature form of SK has got the capability to traverse slowly through the cellular membranes of *E.coli,* and this capability is reduced or lost when at least 13 aa residues are removed from the N-terminus of SK.

(b) Evidence for the partial translocation of mature SK from proteinase K accessibility test. To further check the possibility of partial transport of mature SK, in the absence of any full fledged transport signal and demonstrate the role of its N-terminus in this process, various cellular fractions of *E.coli,* carrying plasmid constructs, pJKD-38 and pJKD-39, were analysed through proteinase K accessibility experiments. In this experiment, cells were spheroplasted by the lysozyme-EDTA treatement and divided into two equal parts, one of which was lysed by sonication. Each sample was then further divided into two samples, one of which was treated with proteinase K (20 min) and other with an equal volume of distilled water. As can be seen from the Fig 3 that extent of SK degradation is more or less similar in lysed and intact spheroplast of *E.coli* carrying the plasmid construct, pJKD-38, capable of producing full length SK. In contrast, *E.coli,* carrying truncated form of SK, did not show significant degradation of SK in the fraction carrying only spheroplast. A minor 44 kD band appeared in the western blot represented the active, C-terminally processed, product of SK which has also been reported earlier by other workers (15). These results gave an indication that significant amount of mature SK is transported outside the cytoplasmic membrane of *E.coli,* but when

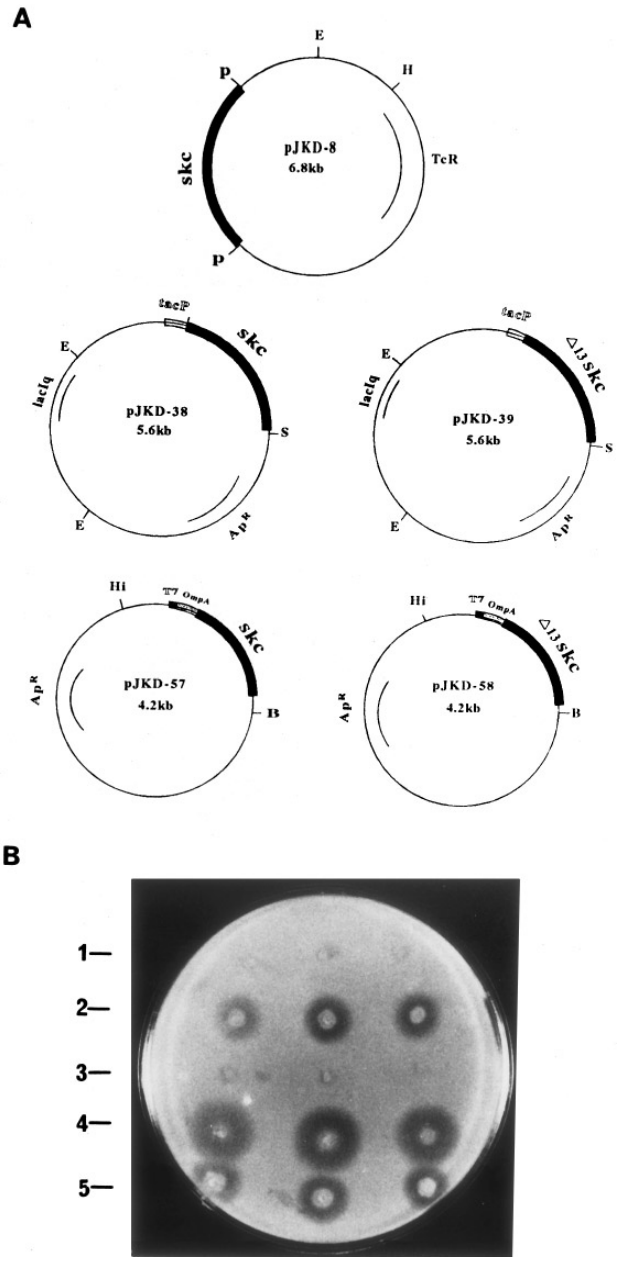

FIG. 1. (A) Restriction maps of recombinant plasmids carrying *skc* gene. Sites for restriction enzymes are denoted by, B. Bam HI, E. EcoRI, H, Hind III, Hi. HincII, P. Pst I. and S. Sal I. Designation for genetic markers are Ap^r, ampicillin resistance ; Tc^r, tetracycline resistance; *skc*, gene encoding for the Streptokinase; Δ13 *skc*, gene encoding for the streptokinase deletion derivative, lacking its 13 N-terminal codons. *OmpA*, sequence coding for *OmpA* leader peptide; ptac, *tac* promoter; T7, T7 promoter. (B) Plasminogen specific caseinolytic activity of *E.coli*, carrying different plasmid constructs.Single colony of *E.coli*, carrying their respective recombinant plasmid, was patched on agar plate (LB+ 0.05mM IPTG) and incubated for overnight at 37°C. Amydolytic activity of recombinant strains was detected by plasminogen-skim milk overlay technique (13).1. *E.coli* AG1 2. *E.coli* AG1 (pJKD-38), 3. *E.coli* AG1 (pJKD-39), 4. *E.coli* BL21DE3 (pJKD-57), 5. *E.coli* BL21DE3 (pJKD-58).

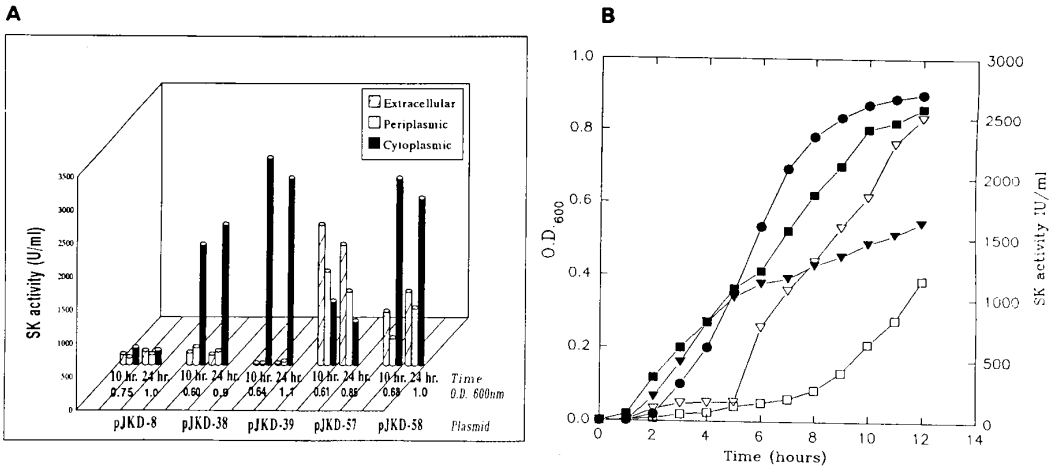

FIG. 2. (A) Distribution of SK activity in different cellular fractions of *E.coli* carrying various recombinant plasmids at different stages of growth. (B) *OmpA* directed export of SK from *E.coli* carrying plasmid constructs, pJKD-57 and pJKD-58. Overnight culture of *E.coli*, raised from a single colony, was inoculated into 100 ml of LB with 100 μg/ml ampicillin, and cultured at 37°C for 2 h. IPTG (0.05mM) was then added in the culture broth to induce the production of SK. Culture supernatant was checked periodically to detect extracellular level of SK. Pattern of growth and SK production is represented as ; *E.coli* (pJKD-57), OD_{600} without IPTG (●) and with IPTG (▼), Secretion of SK into the medium (▽) : *E.coli* (pJKD-58) OD_{600} with IPTG (■), Secretion of SK into the medium (□).

its13-N terminal aa residues are removed, protein loses its capability to translocate in the absence of any leader peptide.

(c) Genetic evidence for the transport of mature SK in the absence of any leader peptide. Since some inner and outer membrane proteins lack a proper leader peptide that is cleaved during translocation process, we checked for the possibility of SK N-terminus mimicking as a transport signal. We undertook a genetic analysis, based on well developed techniques of

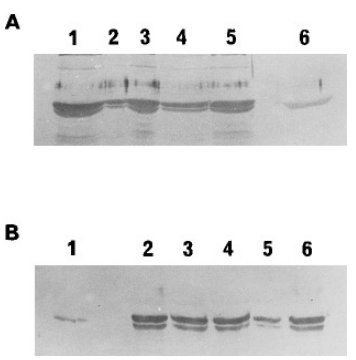

FIG. 3. Proteinase K accessibility test in intact and lysed spheroplasts of *E.coli* carrying plasmids, pJKD-38 and pJKD-39. A 5 ml sample of culture of each strain was harvested and treated according to the protocol recommended by Rassmussen *et. al.* (26). Equal amount of cell sample was loaded on 10% SDS PAGE and analysed through western blotting using polyclonal anti-SK serum. (A) Cells carrying pJKD-37 : Lane1. Whole cells without any treatment 2. intact spheroplast treated with proteinase K 3. intact spheroplast without any treatment 4. lysed spheroplast treated with proteinase K, 5. lysed spheroplast without any treatment, and 6. standard SK. (B) Cells carrying pJKD-38. Lane 1. Standard SK 2. Whole cells, no proteinase K treatment ; 3. intact spheroplasts treated with proteinase K; 4. intact spheroplast, no proteinase K treatment ; 5. Lysed spheroplast treated with proteinase K; 5. Lysed spheroplast, No proteinase K.

TABLE 1
Periplasmic Export of SK-*PhoA* Fusion Proteins

Enzymatic activity (U/ml)	Cellular fraction	Strains		
		CC118/pJKD-37	CC118/pSK1	CC118/pSK2
Alkaline phosphatase	Periplasmic	0.02	2.15	1.53
	cytoplasmic + membrane	0.001	6.88	8.80
β-lactamase	Periplasmic	288	316	298
	cytoplasmic + membrane	28	15	9
β-galactosidase	Periplasmic	21	15	—
	cytoplasmic + membrane	266	284	245

CC 118 contains deletion in *phoA.* Plasmid pJKD-38 carries *skc* gene under the control of *tac* promoter, pSK1 and pSK2 are the derivatives of this plasmid carrying SK-*phoA* in frame fusions generated by the procedure of Manoil and Beckwith (25).

gene fusions to identify the location of the signal(s) within the protein that can direct its transport. Tn *phoA*, which can conveniently be used to generate *in vivo* fusion products with plasmid encoded gene, was utilized to create fusion of *phoA* with *skc* gene on the plasmid construct, pJKD-38, carrying full length gene, coding for the mature SK. Few hundred kanamycin resistant colonies were isolated and out of that two fusion constructs, capable of exporting the hybrid protein, were isolated. These two constructs did not show any SK activity, indicating that fusion has occurred within the *skc* gene. Restriction mapping of these two plasmids indicated that. fusion has occurred near the N-terminus of *skc* gene. Since this region carries an unique HincII site, localization of *phoA* gene was relatively easy. Partial sequencing of two fusion constructs indicated that integration of Tn*phoA* has taken place near the 15 and 20 codons of *skc* gene. These constructs were able to display low level of periplasmic alkaline phosphatase activity (Table 1), indicating that the transport of SK-*phoA* fusion product has occurred in the periplasmic fraction. It suggested that the N-terminal domain of SK contained at least one important determinant for the export process. It should be noted that analysis presented over here relies on results from a limited number of fusions which does not exactly define the limit of this region, but provide strong evidence that N-terminal region of this polypeptide is important in this process.

(d) Signal peptide directed transport of mature and truncated form of SK in E.coli. SK is synthesised as a preprotein in its natural host, *Streptococcus equisimilis.* Since gram-positive signal peptides do not show its optimal efficiency in gram-negative host, we utilized well established transport signal of *E.coli* outer membrane protein, *OmpA,* to create fusion between full length and truncated SK, in order to study the kinetics of SK transport through these fusion constructs. Plasmid constructs, pJKD-57 and pJKD-58 (Fig.1 A), carry fusion of full length and 13 aa residues deleted SK with *OmpA* leader peptide, respectively, under the transcriptional control of T7 promoter. Secretion of SK through these constructs were monitored after IPTG induction by estimating the level of SK in the extra-cellular fraction, at different time intervals. Detection of SK through quantitative estimation and western blotting indicated that SK starts appearing in the extracellular fraction within 2 h of IPTG induction in the case of *ompA*-mature SK fusion. Release of SK in the culture broth increased sharply during next 3-4 h, finally reaching to steady state level, after10 h of IPTG induction (Fig.2 B). In contrast, *OmpA* Δ 13SK fusion product was detectable in the culture supernatant of *E.coli,* only after

3 h of IPTG addition resulting in a slow release of SK in the culture supernatant. At the end of 10 h, extracellular level of mature SK was three times higher as compared to its deletion product. Time course study on the kinetics of SK export through these two constructs, thus, indicated that translocation of SK via *OmpA* leader sequences was 2-3 times more efficient in *E.coli,* when the N terminal residues of SK are intact. Truncation of 13-N terminal aa residues from the mature SK reduces its capability to traverse across the cellular membranes. Since, it has been established earlier that the loss of these residues, does not affect functional properties of SK, it could be concluded that slow transport of SK detected in the case of truncated SK, is due to low amount of protein translocation rather than the loss of its functional activity. This result was substantiated by immunoblot analysis of exported protein in both the cases (Result not presented here).

(e) Proposed role of SK N-terminus. We analysed the N-terminal sequences of SK to look into the possibility of its acting as a surrogate transport signal. There is no homology between prokaryotic signal sequences at the level of primary structure, but all of them possess positively charged N-terminus, followed by a central stretch of hydrophobic residues. Overall charge of first 15 N-terminal aa residues of SK (I A G P E W L L D R P S V N N) is negative, but its hydrophobicity index is relatively high. Analysis of exported and membrane proteins have indicated that the most hydrophobic segments of protein corresponds to trans-membrane region (27). It may be that these short sequences, if present at the N-terminus, pass for signal sequences, either by interfering directly with the folding of proteins or by recruiting factors that do so. Alternatively, these sequences may also act like a pro-region which has been shown to display several functions including maintenance of export competent structure of proteins by modulating its overall folding properties.

DISCUSSION

Streptokinase is synthesised as preprotein with a 26 aa residues signal peptide flag, which directs its transport across the cellular membranes. Results presented here indicate that the amino-terminal region of SK is required for maitaining its transport efficiency in *E.coli.* Whether it performs the similar function in its natural host, *Streptococcus,* remains to be elucidated. Our presumption is based on the following facts : (a) SK, deprived of any leader peptide, exhibits slow translocation across the cellular membranes of *E.coli* and deletion of13 amino-terminal residues from SK, prevents this activity, (b) transport efficiency of fusion product of *OmpA* signal peptide with mature SK is higher as compared to 13 aa residues deleted SK, and (c) amino terminal (1-13 aa residues) of SK is not essential for its biological function (s). No functional role has been assigned to this region of protein. It is possible that full length SK fold slowly in the cytoplasm as compared to its deletion product and is targeted efficiently to the export machinery in the cytoplasmic membranes. Slow folding is likely to be critical feature that distinguishes exported from cytoplasmic proteins (28). Salient feature of exported proteins is the tendency to fold slowly in the cytoplasm when attached to their signal sequences (29). SK, in the absence of any signal peptide, was found to be released slowly into the periplasmic space of *E.coli.* Probability is there that the N′ terminus of SK maintains its loosely folded structure and keeps the protein in export competent state, thus, maintaining the export competant status of SK in *E.coli.* However, at present there is no direct evidence to support this hypothesis. It is relevant to indicate here that SK displays significant similarity with serine proteases and most of them carry a N-terminal region (pro region) which plays important role in protein folding (30).

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