Active Streptokinase from the Cloned Gene in *Streptococcus sanguis* Is without the Carboxyl-Terminal 32 Residues†

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ABSTRACT: The streptokinase expressed by the cloned gene in *Streptococcus sanguis* has a molecular weight of about 44 000 [Malke, H., Gerlach, D., Kohler, W., & Ferretti, J. J. (1984) *MGG, Mol. Gen. Genet. 196*, 360–365] while the molecular weight of the native streptokinase is 47 000. The structural and activity differences of the cloned streptokinase (cSK) as expressed by *S. sanguis* and the native streptokinase (nSK) were investigated. From a partially purified cSK, two active fractions were obtained by reversed-phase HPLC. The minor fraction cSK_L was nearly as active as SK in plasminogen activation. The major fraction cSK_s had only about one-fourth of the specific activity. The structures of cSK_L and cSK_s were studied and compared to the known amino acid sequence of SK [Jackson, K. W., & Tang, J. (1982) *Biochemistry 21*, 6620–6625]. From the NH_2- and COOH-terminal sequences and amino acid composition of the cyanogen bromide (CNBr) fragments, it could be deduced that cSK_L and cSK_s are without 31 and 32 residues, respectively, from the COOH-terminal end of SK. Since the cloned gene contained the full SK structure, the missing structures must have been due to posttranslational proteolysis. An SK fragment similar in size to cSK was observed from a chymotryptic digest of SK.

Fibrinolysis is currently the most promising method used for the treatment of coronary thrombosis. One of the agents commonly used in fibrinolytic therapy is the bacterial protein streptokinase (SK).[1] The fibrinolytic properties of SK are manifested via its ability to activate the single-chain serum zymogen plasminogen by converting it to the two-chain plasmin molecule. Interestingly, SK differs from most other plasminogen activators in that it is not a proteolytic enzyme. It activates plasminogen by the formation of a binary complex of SK–plasminogen, which serves as the activator of free plasminogen to form plasmin [for review, see Castellino (1979)]. How the binding of SK and plasminogen generates activator activity is not known, although several hypotheses have been proposed (Jackson & Tang, 1978). The primary structure of streptokinase (Jackson & Tang, 1982) has revealed that SK does have sequence homology to serine proteases. It is not known, however, whether this structural homology contributes to the plasminogen activation. Thus, it is interesting to define the functional roles of various regions of the SK molecule.

Recently, the streptokinase gene from *Streptococcus equisimilus* was cloned (Malke & Ferretti, 1984) and expressed in *Streptococcus sanguis*, an organism that does not normally produce SK (Malke et al., 1984). The amino acid sequence deduced from the SK gene sequence (Malke et al., 1985) was found to be in good agreement with the protein amino acid sequence (Jackson & Tang, 1982); however, the physical properties of the cloned SK molecule as expressed by *S. sanguis* are somewhat different from those of the native SK molecule. The SDS–polyacrylamide gel electrophoretic analysis of cloned streptokinase yielded a mobility corresponding to a molecular weight of 44 000 (Malke et al., 1984), in contrast to the molecular weight of 47 000 for the naturally synthesized SK (Jackson & Tang, 1982). Since a smaller (44K) but active SK provides an opportunity to study the structure and function relationship of this protein, we have investigated the basis for the observed physical differences between native streptokinase and cloned streptokinase, and the results are reported in this paper.

Experimental Procedures

Materials. Native streptokinase (Kabikinase) was provided by A. B. Kabi (Stockholm, Sweden). Cloned streptokinase was obtained by the expression of the *S. equisimilis* streptokinase gene in *S. sanguis* (Malke et al., 1984). The protein was first purified according to methods of Gerlach & Kohler (1977) and Malke et al. (1984). This material was further purified prior to the experiments by the methods described below. Sephadex G-75 superfine was purchased from Pharmacia Fine Chemicals. Cyanogen bromide, α-chymotrypsin, carboxypeptidase A, and the polyacrylamide gel electrophoresis chemicals were purchased from Sigma Chemical Co. The plasmin substrate D-Val-Leu-Lys-*p*-nitroanilide (S2251) was that marketed by A. B. Kabi, Stockholm, Sweden. The HPLC solvents were obtained from Fisher Scientific. Reagents and solvents for the protein sequencer were purchased from

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[1] Abbreviations: SK, streptokinase; nSK, native streptokinase; cSK, cloned streptokinase; HPLC, high-performance liquid chromatography; CNBr, cyanogen bromide; NEMAC, *N*-ethylmorpholine acetate; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PITC, phenyl isothiocyanate; CPA, carboxypeptidase A; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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Beckman Instruments. All other reagents were of the highest purity commonly available.

Purification of Cloned Streptokinase. The cloned SK, which was partially purified from the *S. sanguis* culture filtrate by silica gel absorption, $(NH_4)_2SO_4$ precipitation, and then Sephacryl S200 chromatography (Malke et al., 1984), was further purified according to either of the following procedures. First, when a functionally active protein was not required, the partially purified cloned SK was incubated in 6 M guanidine hydrochloride for 2–3 h, followed by a 3–4-h dialysis against 1.0 M acetic acid. This material was then chromatographed on a column of Sephadex G-75 superfine, equilibrated and eluted with 1.0 M acetic acid. Second, when the functionally intact protein was desired, the partially purified cloned SK was chromatographed on a Bio-Gel TSK-phenyl-5-PW reversed-phase column (7.5 × 75 mm). The component proteins were eluted by a descending salt gradient (0.1 M K_2HPO_4, pH 7.0, to H_2O). Either of the two methods produced essentially homogeneous cloned streptokinase as judged by SDS–polyacrylamide gel electrophoresis and amino acid composition.

SDS–Polyacrylamide Gel Electrophoresis. Samples were analyzed by SDS–polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Sample solutions were mixed with an approximately equal volume of a solution consisting of 10 mM Tris-HCl, pH 6.8, 1% (v/v) glycerol, 3% (v/v) SDS, and 5% (v/v) 2-mercaptoethanol. They were then incubated in a boiling water bath for 2 min. After cooling, 5 μL of 0.05% (w/v) bromophenol blue was added to each sample solution and the samples were applied to the slab gel sample wells. The slab gel was 0.2 cm thick and consisted of a 1 cm long 3% acrylamide stacking gel and a 10 cm long 10% acrylamide separating gel. The gels were stained for 1–2 h in a solution of 0.25% (w/v) Coomassie blue R-250 dissolved in a 1:5:5 (v/v) mixture of glacial acetic acid–methanol–water. Destaining was performed by diffusion in a bath of glacial acetic acid–ethanol–water (1:3:10 v/v), which contained activated charcoal.

Activity for Plasminogen Activation. The plasminogen activation was assayed in a two-stage assay as described by Jackson et al. (1981). Human plasminogen (Glu-plasminogen variant 1) was purified from human serum by the lysine–Sepharose chromatography method of Castellino & Powell (1981). The quantities of plasminogen and streptokinase in the assay were constant; therefore, the molar ratio of plasminogen to streptokinase was constant in all experiments. The molar ratio was 14000 to 1 plasminogen to streptokinase. The amount of plasminogen was calculated from its extinction coefficient at 280 nm (Violand & Castellino, 1976). The quantity of streptokinase was determined by amino acid analysis. All the assays were performed in the linear response range of activity vs. streptokinase.

Amino Acid Analysis. Protein and peptide samples were analyzed for their amino acid compositions following a 24-h hydrolysis at 110 °C, under vacuum, in 5.7 N HCl containing 0.1% (v/v) 2-mercaptoethanol. Samples were evaporated to dryness and then redissolved in 0.01 M HCl for injection. The analysis was performed on a Durram D500 amino acid analyzer by the method of Spackman et al. (1958).

Automated Edman Degradation. The NH_2-terminal sequence determination was performed in a Beckman sequencer, Model 890C. Approximately 50 nmol of cloned SK was used to perform 20 cycles of degradation. The repetitive yield was 93%. A dilute Quadrol program (Brauer et al., 1975) was used with a modification in the concentration of the PITC–heptane solution. The concentration was reduced from 5% (v/v) to 2.5% (v/v). Five milligrams of polybrene and 100 nmol of L-tyrosine were added to the cup, and two precycles were run before application of the actual sample. The conversion of anilinothiazolinones to PTH-amino acids was accomplished with anhydrous methanolic HCl (Tarr, 1975) in a sequemat P-6 autoconverter. The PTH-amino acids were identified by HPLC analysis on a Millipore–Waters 5-μm C_{18} Nova-pak (5 × 100 mm) radical compression column and module. The solvent system and elution procedure were essentially those described by Henderson et al. (1980). Residues not clearly identified by HPLC were also analyzed by thin-layer chromatography (Summers et al., 1973).

Cyanogen Bromide Reaction. Cyanogen bromide cleavage reactions (Steers et al., 1965) were performed on 2.0 mg of either native or repurified cloned streptokinase (a mixture of cSK_s and cSK_L). The reaction was performed with 4 mg of CNBr (200-fold molar excess over methionine content) dissolved in 5 mL of 70% formic acid. The reaction mixture was incubated at room temperature, in the dark, and under nitrogen for 24 h. The mixture was then diluted with 10 volumes of H_2O and lyophilized.

Peptide Isolation. The cyanogen bromide peptides were purified on a column (0.9 × 100 cm) of Sephadex G-75 superfine, which was equilibrated and eluted with 1 M acetic acid. The peptides were detected by adsorption at 280 nm. An additional purification step, reversed-phase HPLC, was performed on the peptide pool representing the lowest molecular weights from the Sephadex chromatography. For this purpose, a Spherisorb 5 ODS-I column (0.46 × 25 cm, HPLC Technology) equilibrated with 0.1% (v/v) trifluoroacetic acid–H_2O was eluted with a linear gradient of acetonitrile, which also contained 0.1% (v/v) trifluoroacetic acid. The peptides were detected by ultraviolet absorption at 215 nm.

Chymotryptic Digestion. Carboxymethylcellulose-immobilized α-chymotrypsin (Sigma) was used to perform a limited digest of native SK. Sixteen milligrams (~8 units of activity) of immobilized enzyme was washed twice with 2 mL of 0.1 M Tris-HCl, pH 8.0. Then 3 mg of native SK was dissolved in 2 mL of the same buffer at room temperature. The digestion was started by addition of the native SK solution to the immobilized enzyme pellet, followed by vortex mixing and incubation at room temperature. At specified times, the reaction mixture was centrifuged briefly (~20 s) to pellet the immobilized enzyme; an aliquot was removed and filtered through a 1-μm (pore diameter) filter membrane to remove any residual enzyme beads. The reaction mixture was vortexed immediately after each aliquot removal and maintained at room temperature.

Carboxypeptidase A Digestion. The cloned streptokinase variants were digested by carboxypeptidase A according to the procedure described by Ambler (1972). The enzyme was solubilized by the addition of 10% (w/v) lithium chloride. The molar ratio of substrate to enzyme was either 60:1 or 100:1. β-Thienylalanine was added as an internal standard for the quantitation of recovery. The reaction was performed in a solution containing 0.2 M *N*-ethylmorpholine acetate and 0.056 M SDS, pH 8.5, at 25 °C. Approximately 3 nmol of cSK_s was digested at a substrate-to-enzyme molar ratio of 60:1. An enzyme blank and three digestion time points of 2, 6, and 10 min were analyzed for their free amino acid content. cSK_L (1.5 nmol) was digested with a 1:100 molar ratio of carboxypeptidase A. An enzyme blank and time points of 2 and 6 min were taken for amino acid analysis. Aliquots were removed at various times and added to an equal volume of 6%

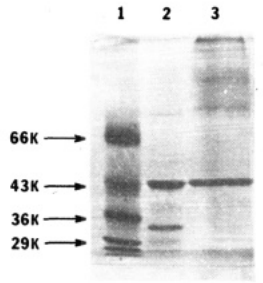

FIGURE 1: SDS–polyacrylamide gel electrophoresis of cloned SK. Lane 1 is a protein standard mixture, lane 2 is crude cloned SK, and lane 3 is phenyl column HPLC purified cloned SK (cSK_s).

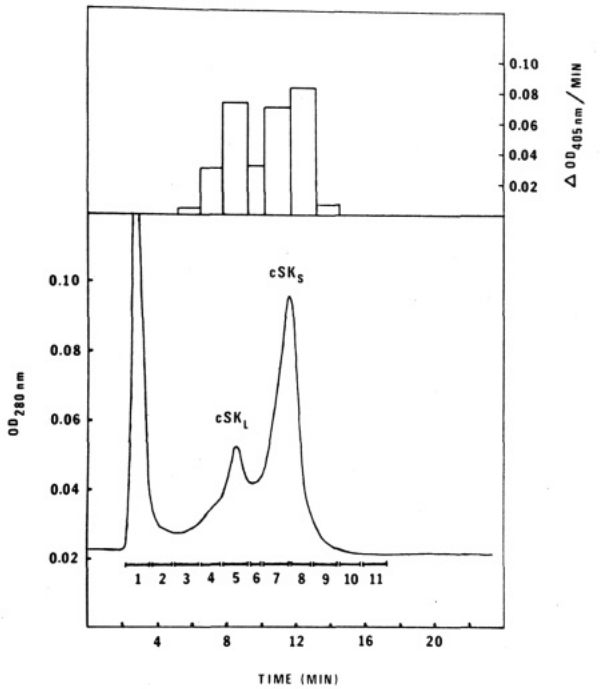

FIGURE 2: The lower panel is the elution profile of crude cloned SK when eluted from a HPLC phenyl column by a 2-min descending salt gradient of 0.1 M potassium phosphate, pH 7.0, to distilled H_2O. The flow rate was 1.0 mL/min. The indicated fractions were collected according to their absorption at 280 nm. Each fraction was assayed for its plasminogen-activator activity as illustrated in the upper panel.

(w/v) trichloroacetic acid–H_2O and placed in a boiling water bath for 1 min. Each sample was then centrifuged briefly to pellet the precipitated protein. The supernatant was filtered through a 0.45-μm filter and then extracted 3 times with 3 volumes of ethyl ether to remove the acid. The aqueous phase was then evaporated to dryness with N_2, redissolved in pH 2.2 citrate sample buffer, and applied to the amino acid analyzer.

RESULTS

Chromatography of Cloned Streptokinase. Partially purified cloned SK (Malke et al., 1984) contained predominantly the 44K SK and lesser quantities of several other contaminants as determined by SDS–polyacrylamide gel electrophoresis (Figure 1). Further purification of active cloned streptokinase was accomplished by reversed-phase chromatography on a phenyl column. At least three major protein peaks were found (Figure 2). The majority of plasminogen-activator activity was found to be associated with two of these peaks: a minor peak contained in fraction 5 and a major peak contained in fractions 7 and 8 in Figure 2. Each of these two peaks was found to produce a single 44K band and was indistinguishable by SDS–polyacrylamide gel electrophoresis and amino acid composition analysis (data not shown). Polyacrylamide gel

Table I: Amino Acid Compositions of Native, Cloned, and Repurified Cloned Streptokinases

amino acid	partially purified cloned SK	repurified cloned SK	native SK[a]	nSK–cSK difference
Asp	56.8	58.8 (59)	65	6
Thr	31.7	25.9 (26)	30	4
Ser	25.8	23.1 (24)	25	1
Glu	59.8	40.3 (40)	44	4
Pro	26.0	17.9 (18)	21	3
Gly	24.0	20.1 (20)	20	
Ala	43.9	22.5 (23)	21	−2
Val	30.6	22.8 (23)	23	
Met	3.5	3.4 (4)	4	
Ile	24.1	21.2 (21)	23	2
Leu	36.1	39.9 (40)	40	
Tyr	16.4	16.8 (17)	22	5
Phe	13.0	15.1 (15)	15	
His	7.6	9.0 (9)	9	
Lys	28.8	30.1 (30)	32	2
Arg	15.4	18.0 (18)	20	2
Trp	ND[b]	ND (1)	1	
total		388	415	27

[a] Values were obtained from the amino acid sequence (Jackson & Tang, 1982). [b] Value was not determined.

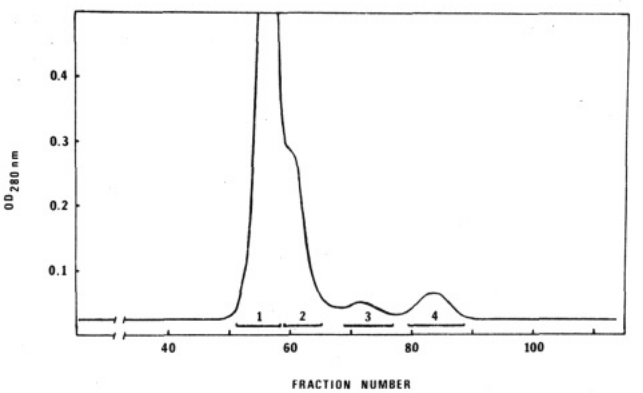

FIGURE 3: Sephadex G-75 superfine chromatography of guanidine hydrochloride treated crude cloned SK. Elution was with 1.0 M acetic acid. The pooled fractions are indicated below the peaks.

electrophoresis of the major peak revealed that only trace impurities remained with an overloaded sample (Figure 1). As shown in Table I, the amino acid composiiton of repurified cloned SK (fractions 5 or 7 and 8 in Figure 2) was very close to that of the native streptokinase obtained from the amino acid sequence (Jackson & Tang, 1982). However, some real differences in amino acid content were observed (Table I). Additionally, the total number of residues was near 388 as compared to the 415 derived from the native molecule sequence. This is in agreement with the smaller molecular weight of the cloned SK (44K, as compared to 47K of native SK) observed by Malke et al. (1984). The inactive peak that eluted early in the chromatogram (fraction 1, Figure 2) had an amino acid composition quite different from that of SK. It was relatively rich in glutamic acid, alanine, and aspartic acid, with these three residues acccunting for more than 40% of its amino acid composition (results not shown).

The results described above indicated that the streptokinase purified from *S. sanguis* was smaller in size than the native SK. In order to establish the structural difference, larger quantities of highly purified inactive cloned SK were obtained by 6 M guanidine hydrochloride denaturation followed by Sephadex G-75 chromatography (Figure 3). The chromatography resulted in four different peaks of absorbance at 280 nm, which were separately pooled. The two incompletely resolved peaks (pools 1 and 2) were found to be equivalent to

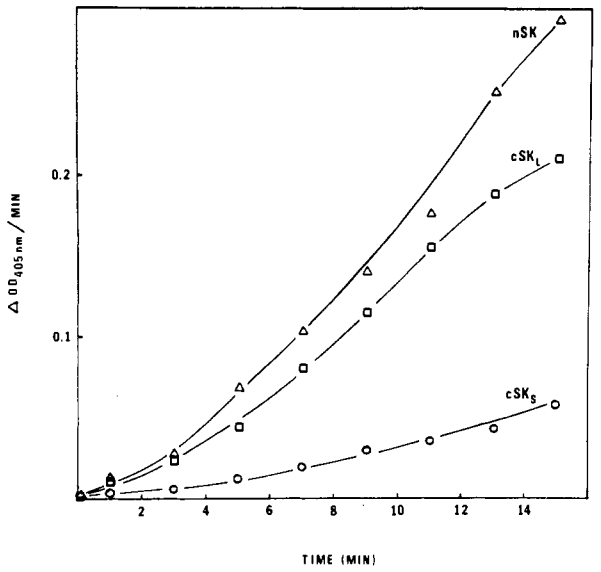

FIGURE 4: Time course of human plasminogen activation by native SK (nSK) and by the two major peaks of activity of the phenyl column purified cloned SK (cSK$_L$ and cSK$_s$). The activation rate was determined by measurement of the change in absorption at 405 nm/min for the hydrolysis of the plasmin synthetic substrate S2251. The plasminogen was present in a large molar excess relative to the streptokinase. Each of the three assays utilized an equivalent molar amount of streptokinase.

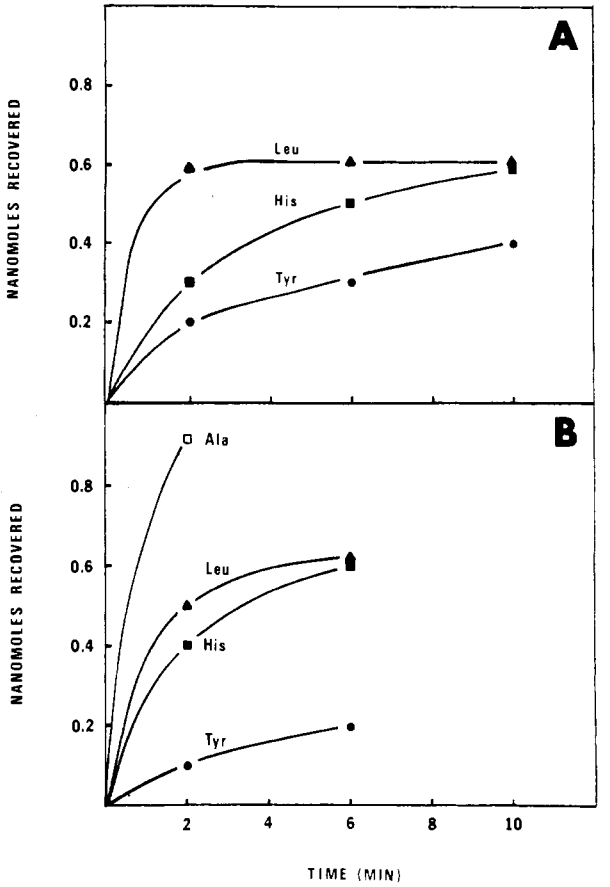

FIGURE 5: (A) Carboxypeptidase A digestion of phenyl column HPLC repurified cSK$_s$. Approximately 3 nm of cSK$_s$ was digested at a substrate-to-enzyme ratio of 60:1 in 0.2 M NEMAC–0.056 M SDS, pH 8.5 at 25 °C. (B) Carboxypeptidase A digestion of phenyl column HPLC repurified cSK$_L$. cSK$_L$ (1.5 nm) was digested at a substrate-to-enzyme ratio of 100:1 under the conditions described in (A).

each other and to the purified cloned SK obtained by phenyl column chromatography (Figure 2) when examined by amino acid analysis and SDS–polyacrylamide gel electrophoresis (data not shown). The two lower molecular weight peaks (pools 3 and 4, Figure 3) had equivalent amino acid compositions that were unlike those of streptokinase; however, they were the same as the non-SK peak eluted from the phenyl column chromatography of partially purified cloned SK (Figure 2, pool 1). These results suggest that Sephadex G-75 chromatography of guanidine hydrochloride treated SK achieved the degree of purity similar to that produced by HPLC (Figure 2).

Plasminogen-Activator Activity of Cloned SK. The plasminogen-activator activity of the two chromatographic forms of cloned SK (cSK$_L$ and cSK$_s$) and of native SK was determined (Figure 4). Both cSK$_L$ and cSK$_s$ were found to be active as plasminogen activators. The specific activity of cSK$_L$ was consistently less than that of native SK in all the assays. The specific activity of cSK$_s$ was dramatically less than that of either native SK or cSK$_L$. The difference in specific activities as well as in apparent sizes of the cSK's suggests differences in the structures of native and cloned SK's. The experiments described below were designed to determine the structural differences.

Amino-Terminal Sequence Analysis. In order to determine the NH_2-terminal sequences, the Sephadex G-75 purified (Figure 3) cloned SK (viz., cSK$_s$ and cSK$_L$) was subjected to automated Edman degradation. The first 20 amino acid residues were identical with those of the NH_2-terminal sequence of native streptokinase (Jackson & Tang, 1982).

Carboxypeptidase A Digestion. To determine the COOH-terminal sequences, the two variants of cloned SK obtained from the phenyl column purification (Figure 2) were independently subjected to carboxypeptidase A digestion. Figure 5 shows the time courses of the amino acid release. Three residues of the COOH-terminal sequence were obtained. From these data the COOH-terminal sequence of cSK$_s$ is -Tyr-His-Leu-COOH. A similar digestion was performed on the cSK$_L$ protein (Figure 5B). The COOH-terminal sequence of cSK$_L$ was found to be -Tyr-His-Leu-Ala-COOH. The carboxypeptidase digestions established that the COOH-terminal sequence of cloned SK's is different from that of native SK, which is -Asn-Asp-Lys.

Cyanogen Bromide Fragmentation and Isolation. Both native SK and cloned SK (a mixture of cSK$_s$ and cSK$_L$, purified by Sephadex G-75 chromatography) were fragmented by reaction with CNBr and chromatographed on a column of Sephadex G-75, as illustrated in Figure 6. As expected, the two chromatographic profiles were similar. The native SK produced five fragments that eluted at the predicted positions (Jackson & Tang, 1982); however, the CB5 peak (residues 371–415) for cloned SK (Figure 6B) was absent at the expected elution position. The material that eluted between fractions 41 and 50 in Figure 6B was partially cleaved SK, which was frequently observed in the chromatography of CNBr-treated nSK (K. W. Jackson and J. Tang, unpublished results). Since no major new peak was seen near the native CB5 position, it was hypothesized that the corresponding CB5 from cSK may have shifted to a lower molecular weight position and eluted with SK fragment CB4 (residues 348–370). The materials corresponding to CB4 (fractions 90–96 in the two chromatographies) were further purified by reversed-phase chromatography as shown in Figure 7. CB4 obtained from the native SK (Figure 6A) gave rise to two major peaks with identical amino acid compositions as expected for this 23-residue fragment (Jackson & Tang, 1982). The two different elution positions are likely due to partial deamidation of one

Table II: Amino Acid Compositions of Two Different Forms of cCB5 from Cloned Streptokinase and a Comparison with CB5 from Native SK

amino acid	$cCB5_a$	residues 371–384	$cCB5_b$	residues 371–383	nCB5[a]	$nCB5–cCB5_b$ difference
Asp	1.3	1	1.2	1	7	6
Thr			0.1		3	3
Ser	1.0	1	1.0	1	2	1
Glu	2.0	2	2.1	2	6	4
Pro	1.0	1	1.0	1	4	3
Gly	2.0	2	2.2	2	3	1
Ala	1.9	2	1.2	1	2	1
Val					1	1
Met/Hse						
Ile					1	1
Leu	0.8	1	0.9	1	2	1
Tyr	0.9	1	1.0	1	6	5
Phe						
His	1.1	1	1.0	1	1	
Lys	1.5	1	1.2	1	3	2
Arg	1.0	1	1.0	1	4	3
total		14		13	45	32

[a] The amino acid composition of native SK CB5 (nCB5) was obtained from its sequence (Jackson & Tang, 1982).

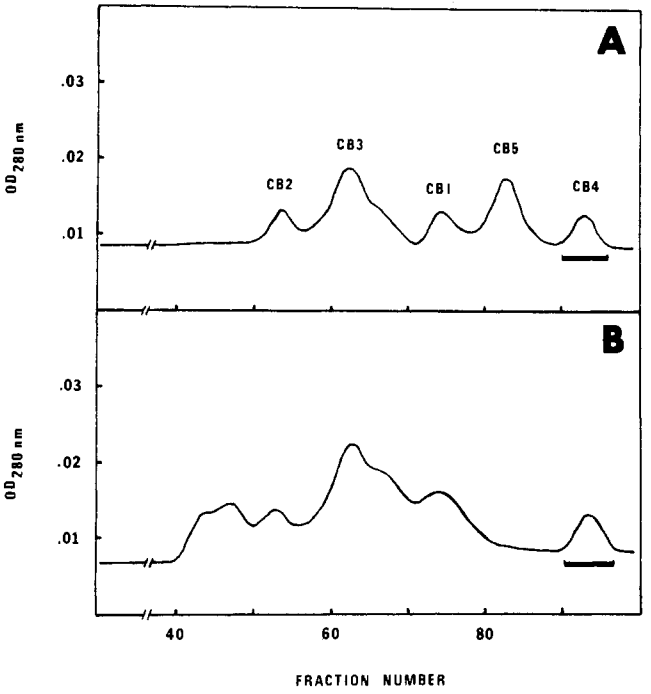

FIGURE 6: (A) Sephadex G-75 chromatography of cyanogen bromide fragmented native streptokinase. Elution was with 1.0 M acetic acid. The CNBr fragments are indicated. The CB4 peak was pooled as illustrated by the bar. (B) Sephadex G-75 chromatography of cyanogen bromide fragmented cloned streptokinase (cSK_s and cSK_L) on the same column and under the same conditions as in (A). All CNBr fragments except CB5 are present in analogous positions as in (A). The peak corresponding to CB4 was pooled as indicated.

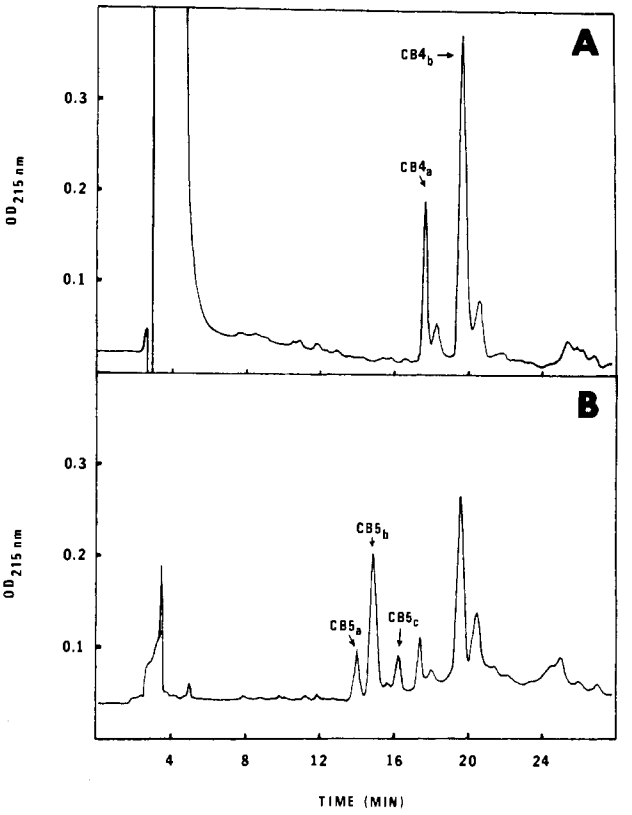

FIGURE 7: (A) HPLC purification of the pooled fractions (CB4) from Figure 6A. The sample was dissolved in 1.5 mL of 0.1% (v/v) trifluoroacetate–H_2O and injected onto a 4.6 × 250 mm C_{18} column equilibrated with 0.1% TFA in 20% acetonitrile–80% H_2O. Upon injection, a linear gradient to 0.1% TFA in 55% acetonitrile/45% H_2O was performed in 15 min at 1.5 mL/min. (B) HPLC purification of the pooled fractions (cCB4 and cCB5) from Figure 6B. The sample volume was 0.95 mL; otherwise, all other conditions are identical with those in Figure 6A. Of the three peaks labeled CB5, $cCB5_a$ had an amino acid composition that matched the first 14 residues of native CB5, while $cCB5_b$ and $cCB5_c$ had compositions that were equivalent to the first 13 residues of native CB5.

of the two Asn residues during the CNBr reaction. When subjected to the same HPLC conditions, the pooled cSK CNBr fractions from Figure 6B that corresponded to CB4 produced the same CB4 peaks (between fractions 17 and 21) as observed in Figure 7A. Their identity as CB4 was confirmed by amino acid analysis (data not shown). In addition, three HPLC peaks that were not observed for native CB4 in Figure 7A were present (Figure 7B). The major peptide of the three, $CB5_b$, had an amino acid composition that was consistent with the composition derived from the CB5 sequence between positions 371 and 383 (Table II). Thus, this peptide represents the COOH-terminal cyanogen bromide fragment of cSK produced by the cleavage at Met-370. Compared to the 45-residue native CB5 (residues 371–415), this peptide consisted of only 13 NH_2-terminal residues of the native CB5. Of the two labeled minor HPLC peaks of Figure 7B, $CB5_c$ had an amino acid composition equivalent to that of the major peak, $CB5_b$, and probably resulted from deamination of an Asn residue. The earliest eluting peak, labeled $CB5_a$, had an amino acid composition equivalent to that of $CB5_b$ plus one additional residue of alanine (Table II). This early eluting peak is most

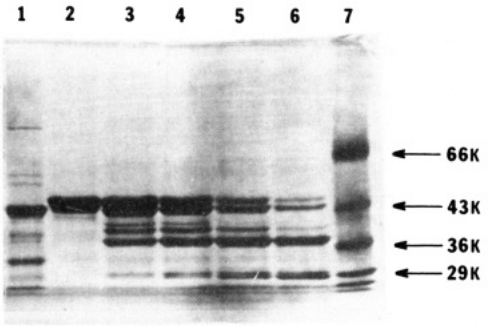

FIGURE 8: SDS–polyacrylamide gel electrophoresis of cloned SK and a time course of the chymotryptic digest of native SK. Lane 1 is crude cloned SK. Lane 2 is native SK. Lanes 3–6 represent the products obtained from digestion times of 1, 2, 4, and 8 min, respectively. Lane 7 is a protein standard mixture.

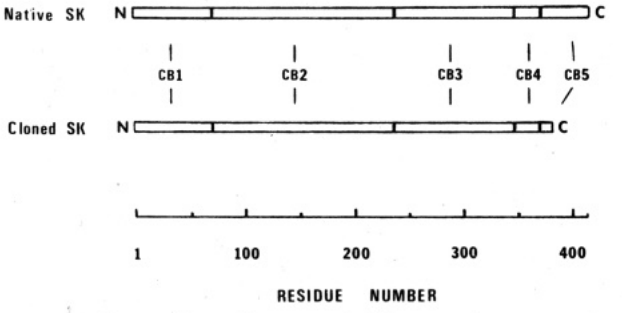

FIGURE 9: Comparison of structural differences between native streptokinase and cloned streptokinase. The positions of the cyanogen bromide fragments in each molecule are shown. The major form of cloned SK (cSK_s) differs from native SK by the absence of 32 residues from its COOH terminus. The minor form of cloned SK (cSK_L) is devoid of 31 residues from the COOH terminus, relative to native SK.

probably due to an alanine residue extension at the COOH-terminus of this cCB5 peptide. This conclusion is supported by the fact that in native SK the residue following Leu-383 is an alanyl residue.

Chymotryptic Digestion of Native Streptokinase. The evidence presented above indicates that as compared to native SK the major form of cloned SK was missing the COOH-terminal 32 residues and terminated primarily at a leucine residue. Since Leu–X bonds are readily cleaved by a protease like α-chymotrypsin, native SK was incubated with this protease in order to determine whether a fragment corresponding to the cloned SK could be produced. Figure 8 illustrates the SDS–polyacrylamide gel electrophoresis of a time course of such a digest. Present in samples from all digestion times (lanes 3–6) is a 44K band with a mobility equivalent to cloned SK (lane 1). However, the 44K band in the chymotryptic digest is eventually degraded to lower molecular weight forms. It is interesting to note that some of the polypeptide bands smaller than 44K in this chymotryptic digest of native SK are also present as minor contaminants in the partially purified cloned SK.

Discussion

The structural difference between cloned streptokinase and native streptokinase is summarized in Figure 9. Native SK consists of 415 amino acid residues, while the majority of cloned SK is missing a 32-residue COOH-terminal peptide and is composed of 383 amino acid residues. A minor form of cloned SK is without the COOH-terminal 31 residues and contains 384 residues. This was supported by several lines of evidence. (1) The molecular weight of the streptokinase cloned in *S. sanguis* is about 44K as compared to 47K for the native protein. (2) The NH_2-terminal sequence of cloned SK is identical with that of the native SK, indicating that the NH_2-terminal region of the cloned SK is not altered. (3) Gel filtration of the five CNBr fragments of cloned SK revealed that only fragment CB5 was different from the corresponding fragment from native SK. By comparison of the elution positions in the gel filtration and the amino acid analysis of the fragment it was evident that the predominant form of CB5 from cloned SK consists of only the first 13 residues of the 45-residue CB5 from native SK. The differences in the amino acid compositions of native SK and cloned SK are summarized in Table I, and the differences between the CB5 molecules from native and cloned SK are shown in Table II. These comparisons illustrate that both intact cSK and cCB5 are devoid of the same 32 amino acid peptide. The data from CB5 were more accurate because they were calculated from the differences of smaller fragments. In spite of the compositions from larger molecular proteins, only the alanine content of the difference composition from the intact molecules is inconsistent. This is probably caused by the presence of a residual amount of the alanine-rich contaminants that were separated from cloned SK by Sephadex G-75 chromatography (see Figure 3). (4) The carboxypeptidase A digestion of the two cSK's revealed that both contained the COOH-terminal sequences of -Tyr-His-Leu. The only location in the streptokinase molecule where this sequence is found (Jackson & Tang, 1982) is at residues 381–383. These results agree completely with that of CNBr fragment CB5 as discussed in (3) above. Again, the conclusion is that the major form of the cSK terminated at residue 383. A minor form, cSK_L, which comprises less than 20% of the total (as judged by the areas in the chromatographies of Figures 2 and 7), contained an additional Ala-384 residue. All evidence considered, it appears reasonable to conclude that cSK_L terminates at Ala-384 and is one residue longer than cSK_s.

Evidence presented in Figure 2 clearly established that the two forms of cSK are both active. Since the smaller of the two forms, cSK_s, is missing the COOH-terminal 32 amino acid residues (residues 384–415), it can be concluded that these 32 residues are not essential for the activity of SK as a plasminogen activator. Interestingly, Ala-384 does appear to have a dramatic effect on the efficiency of plasminogen activation. While the minor form, cSK_L (with Ala-384), is nearly as active as the native SK (Figure 4), the major form, cSK_s (without Ala-384), had only about one-fourth of the specific activity. It is not clear at present why the loss of Ala-384 resulted in the reduction of plasminogen-activation efficiency. This could be due to factors such as (a) less binding affinity between cSK_s and plasminogen, (b) reduction in the ability to induce an effective active site in plasminogen, and (c) ineffectiveness in the cSK_s–plasminogen complex to activate other plasminogen molecules. Active SK fragments produced by the autodigestion of SK–plasminogen complexes have been previously observed (Brockway & Castellino, 1974; Siefring & Castellino, 1976). Although the autodigestion would produce trypsin-like cleavages, in contrast to the chymotrypsin-like cleavages found in this work, some of the active fragments appeared to have lost the COOH-terminal regions of the native SK. Therefore, our current results are in general agreement with those described by Siefring & Castellino (1976).

Although the structural differences between native SK and cloned SK appear clear, the origin of these differences is not. The nucleotide sequence of the native SK gene is in good agreement with the amino acid sequence of the native SK molecule (Malke et al., 1985). However, it seems possible,

although improbable, that the gene cloned into *S. sanguis* did not contain the coding region for the 32-residue COOH-terminal part of the streptokinase molecule. It is more likely that the gene cloned in *S. sanguis* is complete, and the protein molecule initially contains the COOH-terminal 32 residue, but this region is lost due to posttranslational processing by a protease, or proteases, present in *S. sanguis*. Support for this possibility comes from the fact that the COOH-terminal residue of cloned SK is leucine, and it is known that Leu-X bonds are rapidly cleaved by chymotrypsin. Further support for a chymotryptic-like posttranslational processing of cloned SK was demonstrated by the fact that when native SK is incubated briefly with α-chymotrypsin, a degradation product is formed that has a mobility equivalent to cloned SK on SDS-polyacrylamide gel electrophoresis. The protease hypothesis is also supported by the presence of a second minor component, cSK_L.

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Mechanism of a Lipoxygenase Model for Ethylene Biosynthesis†

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ABSTRACT: The model system for ethylene biosynthesis developed by Bousquet and Thimann [Bousquet, J.-F., & Thimann, K. V. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1724-1727], consisting of lipoxygenase, linoleic acid, aminocyclopropanecarboxylic acid, Mn^{2+}, and pyridoxal phosphate, has been studied to identify its products and mechanism. As occurs in ethylene biosynthesis in plant tissue, C1-N1 of aminocyclopropanecarboxylic acid are converted to cyanide. Also as in plant tissue, stereospecifically labeled aminocyclopropanecarboxylic acid is converted to ethylene with loss of stereochemistry. The role of pyridoxal phosphate has been shown not to involve Schiff's base formation with aminocyclopropanecarboxylic acid but chelation of the *o*-hydroxybenzaldehyde unit with manganese. A number of other such compounds may replace pyridoxal phosphate as a chelator in the model system. Chelation has the effect of increasing the reduction potential of the metal ion in order to cleave linoleic hydroperoxide more efficiently. The system shows sufficient fundamental differences with the natural system to confidently discount it.

The study of the process by which aminocyclopropanecarboxylic acid is converted to the plant growth hormone ethylene has been frustrated by the lack of an authentic isolated ethylene-forming enzyme. Consequently, a number of cell-free preparations (Konze & Kende, 1979; Mayak et al., 1981; Mattoo et al., 1982; Guy & Kende, 1984; Vinkler & Apelbaum, 1983; Vioque et al., 1981; Konze & Kwiatkowski, 1981; Boller et al., 1979; Bousquet & Thimann, 1984) have been investigated as model systems. These include peroxidases, microsomal membranes, vacuoles, mitochondria, and others. Often, cofactors (pryidoxal, ascorbate), metal ions (Cu^{2+}, Mn^{2+}), and other ingredients are added to these systems to allow them to operate.

In some cases, these studies have muddied the waters concerning ethylene biosynthesis in vivo. For example, it is now well established that pyridoxal phosphate is required for ethylene biosynthesis and that pyridoxal inhibitors which form

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