A Characterization of Native Streptokinase and Altered Streptokinase Isolated from a Human Plasminogen Activator Complex†

William J. Brockway‡ and Francis J. Castellino*

ABSTRACT: The properties of native streptokinase and streptokinase purified from the human plasminogen activator complex (altered streptokinase) have been compared. Native streptokinase possesses a molecular weight of 44,000 and a $s^0_{20,w}$ value of 3.03 S whereas the values for altered streptokinase are 36,000 and 2.82 S, respectively. Native streptokinase possesses an amino-terminal isoleucine and a carboxyl-terminal lysine residue whereas altered streptokinase possesses an amino-terminal serine and a carboxyl-terminal lysine residue. The amino-terminal amino acid sequence of native streptokinase is NH_2-Ile-Ala-Gly-Pro-Glu-Trp-Leu-Leu-Asp-Arg-Pro-Ser- and the amino-terminal amino acid sequence for altered streptokinase is NH_2-Ser-Lys-Pro-Phe-Ala-X-Asp-Ser-Gly-Ala-Met-Ser-. These results suggest that native streptokinase loses an amino-terminal peptide(s) with a cumulative molecular weight of approximately 8000 upon complex formation with human plasminogen or plasmin. Circular dichroism and optical rotatory dispersion analysis suggest differences in the conformations of native and altered streptokinase. Finally and importantly, we feel that both native and this particular form of altered streptokinase behave in an analogous manner in direct plasminogen activation assays and the plasminogen activator active site most likely does not reside in the streptokinase moiety of the activator complex. This latter issue has been the subject of some recent controversy.

The mechanism of the activation of the single-chain proenzyme, plasminogen to the two-chain enzyme, plasmin by streptokinase has been a widely studied subject. Any study of this mechanism must include the fact that at least one bond in the plasminogen molecule must be cleaved in the activation process (Robbins *et al.*, 1967; Summaria *et al.*, 1967). Although synthetic ester substrates have been found for all other plasminogen activators, none has been found to be a substrate for streptokinase (De Renzo *et al.*, 1967a). This finding appears to rule out direct activation of plasminogen by streptokinase; however, such a mechanism has been nonetheless proposed (Summaria *et al.*, 1969). On the other hand, most investigators have suggested an indirect activation mechanism *via* an activator complex. It was found that streptokinase could form a 1:1 complex with human plasmin and this complex could in fact directly activate bovine plasminogen (Zybler *et al.*, 1969; Blatt *et al.*, 1964; Ling *et al.*, 1965, 1967). This same activator complex was formed whether streptokinase and human plasmin or streptokinase and human plasminogen were used as the starting materials (Ling *et al.*, 1967). More recently, these considerations have been incorporated into a unified mechanism for the streptokinase induced activation of human plasminogen (Reddy and Markus, 1972) and for the streptokinase-induced activation of rabbit plasminogen (Schick and Castellino, 1973). These studies demonstrate that the principal activator of human plasminogen is a complex of streptokinase and human plasminogen, containing an active site. Activation of plasminogen by complexes of streptokinase and human plasmin can also proceed equally well. For the rabbit plasminogen system, the most important, if not the sole activator was a complex of streptokinase and rabbit plasminogen, containing an active site.

Recently, a controversy has arisen concerning the active site of the activator complex. In the streptokinase–human plasmin activator complex, many investigators have proposed that the active site resided in the plasmin moiety. This is based on the fact that reagents such as diisopropyl fluorophosphate were incorporated into the plasmin moiety of the complex at rates which paralleled the rate of loss of plasminogen activator activity of the complex (De Renzo *et al.*, 1967b; Summaria *et al.*, 1968). Further, in the streptokinase–human plasminogen activator complex, it is thought that binding of streptokinase to human plasminogen induces a conformational alteration in the plasminogen moiety leading to formation of the activator active site (Werkheiser and Markus, 1964; Hummel *et al.*, 1966; McClintock and Bell, 1971; Reddy and Markus, 1972). However, it was recently concluded by Taylor and Beisswenger (1973) that the plasminogen activator complex consisted of altered streptokinase and human plasmin with the activator active site residing in the altered streptokinase moiety. These authors proposed that altered streptokinase, purified from the activator complex, possessed direct proteolytic activity on plasminogen, resulting in conversion to plasmin.

It was our feeling that the conclusions derived by Taylor and Beisswenger required verification, based on the wealth of apparently contradictory evidence presently in the literature. We, therefore, undertook our own investigation on this important topic and this manuscript reports our findings.

Materials and Methods

Proteins. Native streptokinase was prepared from Kabikinase, generously donated by Dr. Hugo Nihlén. Approximately 1.2 g of Kabikinase was first exhaustively dialyzed against cold water and lyophilized. The residue was dissolved in 10 ml

† From the Department of Chemistry, Program in Biochemistry and Biophysics, University of Notre Dame, Notre Dame, Indiana 46556. *Received December 17, 1973.* Supported in part by Grants HL-13423 and HL-15747 from the National Heart and Lung Institute, National Institutes of Health; a grant-in-aid from the Indiana Heart Association and a cooperative grant-in-aid from the Indiana and American Heart Associations.

‡ Present address: Hematology Department, Mayo Clinic, Rochester, Minn.

of cold water. To this was added 2.77 g of solid $(NH_4)_2SO_4$ in small aliquots over a period of 1.5 hr; after which the suspension was allowed to stand overnight. The mixture was centrifuged in a Sorvall RC-2 centrifuge at 10,000 rpm for 15 min at 4°. The precipitate was washed twice with 5 ml of 45% $(NH_4)_2SO_4$ in water. The residue was dissolved in 10 ml of cold water, exhaustively dialyzed againt cold water, and lyophilized. The streptokinase was then subjected to isoelectric focusing, utilizing a pH 3–6 linear gradient, as described below, in order to obtain high-purity streptokinase in a total yield of 70%.

Altered streptokinase was isolated by first incubating solutions of human plasminogen and streptokinase for 2–5 min at 25°. Each protein was dissolved in 0.05 M Tris-HCl–0.05 M L-lysine (pH 8.0). The final concentrations were 2.83×10^{-4} M plasminogen and 2.88×10^{-4} M streptokinase. The reaction mixture was then frozen and lyophilized. In order to isolate high-purity altered streptokinase from the complex, the residue was subjected to isoelectric focusing in 6 M urea in a pH 3–6 gradient as described below. The overall yield of altered streptokinase was 85%.

All plasminogens used in this study were isolated as previously described for rabbit plasminogen (Sodetz *et al.*, 1972).

Isoelectric focusing experiments for purification of native streptokinase were performed on a LKB 110-ml column utilizing a temperature control system maintained at 4°. The anode solution consisted of 0.4 ml of 18.1 M H_3PO_4, 26 ml of H_2O, and 26 g of sucrose. The cathode solution contained 0.5 g of NaOH dissolved in 50 ml of H_2O. After addition of the anode solution, a linear ampholine gradient was layered directly above the anode solution with the aid of a peristaltic pump. The starting solvent contained 21 g of sucrose dissolved in 28.7 ml of H_2O to which was added 2.82 ml of pH 3–6 ampholines in 40% sucrose (LKB). The limit solvent contained 2.75 g of sucrose in 51.1 ml of H_2O to which was added 1.15 ml of the same ampholine solution. When approximately 40 ml of the gradient was added, 12–18 mg of partially purified streptokinase was added directly to the remaining starting solution. The entire gradient was then allowed to form. At this point, a few extra ml of the limit solution was added, followed by the cathode solution. The run was then started and allowed to proceed for 68 hr. Fractions of 1 ml were then collected from the column and the tubes were analyzed for absorbance at 280 mm. The pH of each tube was then determined using the expanded-scale capabilities of a Radiometer PHM 26C pH meter. An extinction coefficient $[E^{1\%}_{1\,cm}]$ of 9.49 at 280 mm was used for streptokinase (Taylor and Botts, 1968).

In order to prepare altered streptokinase by isoelectric focusing, an exactly analogous procedure was used except that previously deionized 6 M urea was substituted for H_2O in all the required solutions.

Ultracentrifuge Experiments. Sedimentation coefficients of native and altered streptokinase were performed on a Spinco Model E analytical ultracentrifuge at 52,000 rpm and 20°. Scanner optics were used at 280 mm with protein concentrations of 0.2–0.3 mg/ml; thus, circumventing the necessity of extrapolation of the data to zero protein concentration. All values are corrected to the density and viscosity of water at 20°. A partial specific volume of 0.738 ml/g (Taylor and Botts, 1968) was used for both native and altered streptokinase. The solvent was 0.05 M Tris-HCl (pH 8.0).

Molecular weight of native and altered streptokinase were carried out by the meniscus depletion technique of Yphantis (1964), utilizing Rayleigh optics. Rotor speeds ranging from 28,000 to 32,000 rpm and column heights of 3 mm were used. All protein solutions were dialyzed against 0.05 M Tris-HCl (pH 8.0) and adjusted with the final dialysate to 0.15–0.20 mg/ml prior to analysis. The temperature of all experiments was 20°. The results of at least five fringes at each speed were averaged in order to obtain a molecular weight.

Polyacrylamide gel electrophoresis on each streptokinase were carried out at pH 9.5 (Davis, 1964) and in sodium dodecyl sulfate (Weber and Osborn, 1969).

Amino Acid Analysis. The amino acid compositions of each streptokinase were determined by hydrolyzing 0.5-mg samples of the protein in 6 N HCl for 24, 48, and 27 hr at 110°. Analyses were performed on a Beckman Model 117 amino acid analyzer. Duplicate samples were analyzed at each time interval.

The tryptophan content of each streptokinase was determined by the method of Edelhoch (1967) and the total half-cysteine was determined as cysteic acid by subjecting 6 N HCl hydrolysates of samples previously treated with performic acid to amino acid analysis (Moore, 1963).

The total amide content of each streptokinase was determined by the carbodiimide method of Hoare and Kohsland (1967). Our procedures have been described in detail earlier (Sodetz *et al.*, 1972).

End-Terminal Analysis. Quantitative amino-terminal amino acid analyses on each streptokinase were performed as described by Stark (1967). Protein concentrations were determined at the cyclization step by removing an aliquot and hydrolyzing it in 6 N HCl at 110° for 24 hr. Amino acid analyses were performed as described above.

Carboxyl-terminal analyses were performed by hydrozinolysis as described previously for native streptokinase by Morgan and Henschen (1969).

Automatic Edman degradations of each purified streptokinase were performed with a Beckman Model 890B sequencer. The program was structured to the basic Quadrol methodology (Edman and Begg, 1967) with modifications as recommended by Hermodson *et al.* (1972). In particular, nitrogen flushing was incorporated into the fine vacuum system, a single acid cleavage was employed, and the program was initiated with the cleavage rather than the coupling reaction.

Lyophilized streptokinase samples (4–6 mg) were dissolved in 0.3–0.4 ml of anhydrous trifluoroacetic acid and dried in the sequencer cup without delay. Edman degradation was then initiated. The butyl chloride extracts from each degradation cycle, which were stored at 7° in the sequencer fraction collector, were externally evaporated at 25° under a stream of nitrogen gas. The thiazolinone to phenylthiohydantoin conversion was then affected (Edman and Begg, 1967; Hermodson *et al.*, 1972). The amino acid phenylthiohydantoin derivatives were identified with the aid of a Beckman GC-45 gas chromatograph on a 2 mm × 4 ft glass column packed with SP-400 (10% on Supelcoport, 100–120 mesh, Supelco, Inc.). Nitrogen was employed as the carrier gas. The temperature program used (Pisano *et al.*, 1972) permitted unambiguous assignment of the phenylthiohydantoin derivatives, except for discriminations between the threnonine and glycine, and between the threonine and proline derivatives. In these cases silica gel thin-layer chromatography, employing solvent XM, was used for assignments (Iragami and Murakami, 1972). Solutions of authentic amino acid phenylthiohydantoin derivatives (Pierce Chemical Co.) were used to calibrate the flame response of the gas chromatography for purposes of calculating the molar responses of the observed peaks.

Circular dichroism studies were performed with a Cary 60 spectropolarimeter with optical rotatory dispersion and

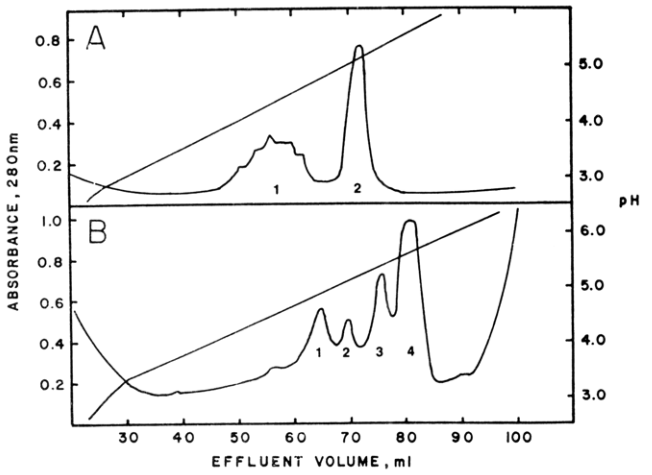

FIGURE 1: Isoelectric focusing profiles of native and modified streptokinase. (A) Native streptokinase (two salt cuts) focused at 4° for 68 hr on a pH 3–6 gradient. The absorbance at 280 nm and the experimental pH gradient are shown on the graph. Fractions of 1 ml were collected. Peak 2 contained all the streptokinase activity. (B) Plasminogen activator complex (1:1 streptokinase–human plasminogen, 5-min incubation) focused in 6 M urea for 68 hr at 4° with a pH 3–6 gradient. The absorbance at 280 nm and the experimental pH gradient are indicated on the graph. Fractions of 1 ml were collected. All the streptokinase activity was found in peak 4.

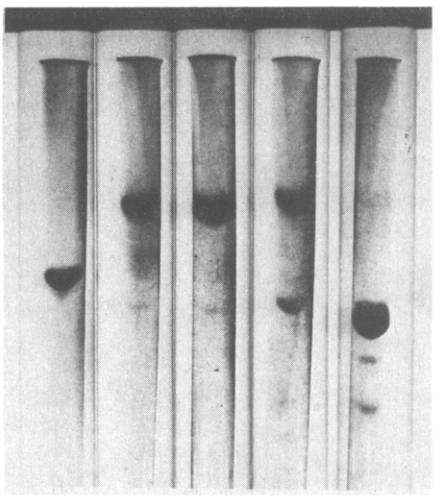

FIGURE 2: Sodium dodecyl sulfate gel analysis of the fractions obtained from Figure 1. Left to right: peak 2 from Figure 1 A; peak 1 from Figure 1 B; peak 2 from Figure 1 B; peak 3 from Figure 1B; peak 4 from Figure 1 B.

circular dichroism capabilities. In these runs 1-, 5-, and 10-mm cells were interchanged such that the optical density of the proteins did not exceed 1.0 at any wavelength. Ellipticity $[\theta]$ values were recorded directly from the instrument and converted to molecular ellipticity $[\theta]$, expressed in deg cm^2 per dmol of amino acid according to the relationship: $[\theta] = M_0\theta/10lc$, where M_0 is the mean residue weight of the protein, l is the path length of the cell in cm, and c is the concentration of protein in g/ml.

Enzyme Assays. In general, these assays consisted of incubating the desired levels of plasminogen and native or modified streptokinase for certain time periods and adding an aliquot of the mixture, equivalent to 30–40 μg of the initial plasminogen, to L-Tos-Arg-OMe (final concentration 0.01 M). After 10-min incubation, the remaining Tos-Arg-OMe was analyzed as previously described (Brockway and Castellino, 1971). Specific details of these assays are given in their appropriate places in the text.

Bovine fibrin plate assays were carried out as described by Astrup and Müllertz (1952) as modified by Tomar and Taylor (1971). The fibrin plates were kindly donated to us by Dr. Fletcher B. Taylor, Jr. The quantities of materials assayed in each well of the plates are given in the appropriate figure legend.

Materials. All amino acid analyzer reagents as well as all sequenator reagents were purchased from the Pierce Chemical Co. Tos-Arg-OMe was a product of the Cyclo Chemical Co. Polyacrylamide gel electrophoresis supplies were purchased from Canalco Inc. Hydrazine (97%) was obtained from Matheson, Coleman & Bell. Other materials were of the highest available purity.

Results

Figure 1 shows the isoelectric focusing profile obtained at the final stage of the native streptokinase purification. The value of the isoelectric point obtained from these data is 5.2. This value differs from the previously reported number of 4.7, obtained by other techniques (De Renzo *et al.*, 1967a,b).

The molecular weight of our native streptokinase, obtained by sedimentation equilibrium ultracentrifugation was 44,000 ± 2000 and the $s^0_{20,w}$ was 3.03 ± 0.07 S. Sodium dodecyl sulfate and pH 9.5 polyacrylamide gels of the native streptokinase showed no evidence of heterogeneity in the preparation. The molecular weight of sodium dodecyl sulfate denatured streptokinase, obtained by sodium dodecyl sulfate gel electrophoresis using bovine serum albumin, catalase, ovalbumin, pepsin, and trypsin as standards, was 45,000 ± 3000. Figure 1 also shows the isoelectric focusing profile obtained for the purification of streptokinase from the activator complex. Since the pH gradient in this experiment was in the range of 3–6, all the plasmin has migrated into the electrode solution. Although several peaks are present, the sodium dodecyl sulfate gels in Figure 2 show that only one of these bands could be derived from streptokinase. The other bands possess molecular weights which are too large. These other species could originate as either undigested plasminogen or as the small amount of serum albumin added to stabilize the streptokinase. Characterization of some physical properties of this streptokinase indicate a molecular weight of 36,000 ± 2000 by sedimentation equilibrium ultracentrifugation and an $s^0_{20,w}$ value of 2.82 ± 0.06 S. Sodium dodecyl sulfate and pH 9.5 polyacrylamide gels of this protein also showed a high degree of homogeneity in this preparation. The molecular weight of this material, obtained by sodium dodecyl sulfate gel electrophoresis, is 35,000 ± 2500. Since the size and charge properties of native streptokinase and the streptokinase isolated from the activator complex are significantly different, it is clear that an alteration in the structure of streptokinase accompanies its incorporation into the human plasminogen activator complex.

Figure 3 shows the time course of the degradation of streptokinase in human plasminogen activator complex, as monitored by unreduced sodium dodecyl sulfate gels. It can be seen that streptokinase is quickly degraded to a species of lower molecular weight (35,000) which is stable from 30 sec to 10 min. After this time more extensive degradation of streptokinase occurs and many other bands are present. More rapid degradation of streptokinase is also evident at higher incubation temperatures. Thus the altered streptokinase isolated above at short time periods appears to possess the significance required for these studies. The third, lowest molecular weight

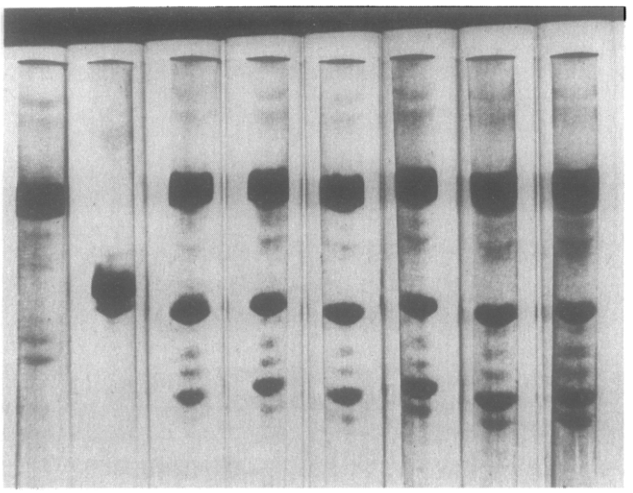

FIGURE 3: Effect of time of incubation of 1:1 mixtures of streptokinase and human plasminogen on the species produced at 30°. The gels shown are unreduced sodium dodecyl sulfate gels. Left to right: native human plasminogen; native streptokinase; incubation of the two proteins for 15 sec; 30 sec; 1 min; 2 min; 5 min; 10 min.

material present in the gels of Figure 3 is likely peptide material derived from plasminogen or streptokinase.

Further studies were designed in order to more fully characterize the nature of the above described alteration produced in streptokinase. Amino-terminal end-group analysis indicated that native streptokinase contained 0.92 mol of isoleucine/mol of protein at the amino terminus. Carboxyl-terminal end group analysis, by hydrazinolysis, demonstrated that lysine was the carboxyl-terminal amino acid of native streptokinase. Although the same carboxyl-terminal amino acid was found to be present in altered streptokinase, serine (0.65 mol/mol of protein) was found as the new amino-terminal amino acid. Although the quantitation is lower than desired in this case, results such as these are not uncommon for amino-terminal serine (Stark, 1967).

The amino acid compositions of native and altered streptokinase are compared in Table I. All values represent the average of 24-, 48-, and 72-hr hydrolysis times, except serine and threonine which have been extrapolated to zero hydrolysis time and valine, leucine, and isoleucine which are given as the 72-hr hydrolysis times. Values for tryptophan and total half-cysteine were determined in separate experiments. The total free carboxyl groups of the two proteins were determined by incorporation of glycine into these residues. The values

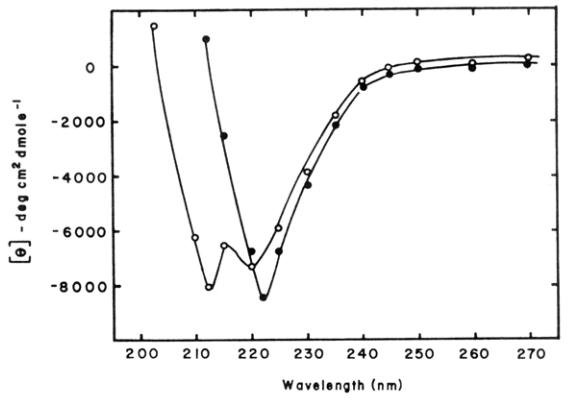

FIGURE 4: Circular dichrosim spectra of native and altered streptokinase. Curve 1 (○) is a plot of molar ellipticity (θ) *vs.* wavelength for native streptokinase. Curve 2 (●) is the same plot for altered streptokinase.

TABLE I: Amino Acid Compositions of Native and Altered Streptokinase.

Amino Acid	Amount (mol/mol) Present in[a]	
	Native Streptokinase	Altered Streptokinase
Lysine	29	26
Histidine	9	8
Arginine	18	14
Aspartic acid	57	47
Threonine[b]	26	20
Serine[b]	24	19
Glutamic acid	42	35
Proline	21	15
Glycine	20	17
Alanine	21	19
Valine[c]	20	17
Methionine	4	4
Isoleucine[c]	20	17
Leucine[c]	35	29
Tyrosine	20	16
Phenylalanine	14	12
Cysteic acid[d]	0	0
Tryptophan[d]	2	2
Amide[d]	34	

[a] Rounded to the nearest integer. [b] Extrapolated to zero hydrolysis time. [c] These values represent the 72-hr hydrolysis time. [d] Determined separately as described in the text.

reported here were obtained at reaction time periods which suggested that glycine incorporation was complete.

Table II gives the sequential amino acid release from the amino-terminal region of each streptokinase preparation along with the absolute molar values of the amino acid phenylthiohydantoin derivatives. In the case of native streptokinase, the value for Trp-6 is low but this is not an extraordinary finding for this particular residue. Arginine-10 was identified qualitatively by a spot test and the absolute molar value of Ser-12 is not given, due to unreliable quantitation by gas chromatography. In the case of altered streptokinase, residue 6 could not be identified and the absolute values of Ser-1 and -12 are not given for the same reasons as explained above.

Circular dichroism analysis of native and altered streptokinase are compared in Figure 4. There is clearly a shift to a more random or a less helical conformation in the altered streptokinase. It could be argued that the conformational shift in altered streptokinase resulted from the presence and subsequent removal of urea in the purification process. However, addition and removal of urea from native streptokinase did not seem to effect the circular dichroism spectra. An analogous argument could be made for altered streptokinase, but we realize that this aspect was not rigorously proven. Since it is not possible to purify altered streptokinase without denaturing the activator complex, an analogy such as this is the best that can be done under the circumstances.

We next decided to assay our preparation of altered streptokinase as a potential direct plasminogen activator. We first determined that native and altered streptokinase were refractive in activity toward synthetic ester substrates, such as Tos-Arg-OMe and to reagents, such as diisopropyl fluorophosphate. We then tested both streptokinase preparations as activators of human plasminogen in order to compare the

TABLE II: Automatic Sequential Amino Acid Release from the Amino Terminus of Native and Altered Streptokinase.

	Native Streptokinase[a]		Altered Streptokinase[d]	
Step	Residue	nmol	Residue	nmol
1	Isoleucine	100	Serine	*c*
2	Alanine	120	Lysine	13[b]
3	Glycine	70	Proline	44
4	Proline	60	Phenylalanine	42
5	Glutamic acid	49	Alanine	48
6	Tryptophan	15[b]	*e*	
7	Leucine	54	Aspartic acid	28
8	Leucine	58	Serine	*c*
9	Aspartic acid	34	Glycine	19[b]
10	Arginine	*c*	Alanine	38
11	Proline	26	Methionine	19
12	Serine	*c*	Serine	*c*

[a] 120 nmol taken for analysis. [b] Quantitation is ordinarily low. [c] Not quantitated. [d] 100 nmol taken for analysis. [e] Could not be identified.

potency of each preparation. The first experiments were designed to demonstrate whether altered streptokinase formed plasminogen activator with human plasminogen at the same rate as native streptokinase. These assays were performed at 1:1 ratios of both native and altered streptokinase with plasminogen, conditions under which plasminogen activator activity dominates (Zybler *et al.*, 1959; Blatt *et al.*, 1964; Ling *et al.*, 1965, 1967). These assays were performed by incubating 0.03 ml of 1.31×10^{-5} M plasminogen in 0.05 M Tris-HCl (pH 8.0), 0.015 ml of 2.63×10^{-5} M native streptokinase in the same buffer, and 0.15 ml of this buffer for various times in separate tubes. At the desired times 0.05 ml of 0.05 M Tos-Arg-OMe was added and the reaction was allowed to proceed for 10 min. At this time the remaining Tos-Arg-OMe was assayed as described previously (Brockway and Castellino, 1971). Table III lists the results obtained and shows that a parallel in the development and loss of esterase activity in the plasminogen activator occurs with both native or altered streptokinase. In order to conclusively demonstrate that the esterase activity observed was in fact due to plasminogen activator production, we tested the above preparations for their capacity to activate bovine plasminogen. This species of plasminogen is claimed to be insensitive to activation by streptokinase but is supposedly sensitive to activation by altered streptokinase (Taylor and Beisswenger, 1973). We attempted to verify these results in the following experiment. Here stock solutions of bovine plasminogen (3.3 mg/ml), native streptokinase (1.2 mg/ml), altered streptokinase (0.52 mg/ml), and human plasminogen (1.2 mg/ml), all in 0.05 M Tris-HCl–0.01 M L-lysine (pH 8.0) were employed. The preincubation mixtures consisted of 0.010 ml of bovine plasminogen, 0.2 ml of buffer, and 0.015 ml of native streptokinase for 10 and 30 min. At this time 0.025 ml of 0.1 M Tos-Arg-OMe was added and the solution was incubated for an additional 10 min. The remaining Tos-Arg-OMe was assayed as described above. The stoichiometry of native streptokinase to bovine plasminogen in this experiment was roughly 1:1. The results of this experiment are given in Table IV and show that no plasmin activity is generated. Other experiments were performed at ratios of streptokinase–bovine plasminogen (1:10) and again, as shown in Table IV, no bovine plasmin activity resulted. The same experiments were performed with altered streptokinase by preincubating 0.01 ml of bovine plasminogen, 0.185 ml of buffer, and 0.03 ml of altered streptokinase for 10 and 30 min. All other steps were as above. In this case there was approximately a 1:1 molar ratio of altered streptokinase to bovine plasminogen. Again, as shown in Table IV, no bovine plasmin activity results. The same conclusions can be drawn from other data in Table IV at 1:10 ratios of altered streptokinase to bovine plasminogen. Thus, it appears that altered streptokinase does not, in itself, activate bovine plasminogen under the conditions used in these assays. The next experiments were designed to test our conclusions concerning the data of Table III which indicate that mixtures of native or altered streptokinase with human plasminogen can form plasminogen activator and thereby activate bovine plasminogen. These experiments were carried out by incubating 0.005 ml of human plasminogen with 0.005 ml of native streptokinase (1 → 2 dilution of the stock solution) or 0.005 ml of altered streptokinase in a total volume of 0.215 ml for

TABLE III: Development of Activator Esterolytic Activity with Time in Solutions of Native and Altered Streptokinase and Human Plasminogen (1:1 Protein Ratio).

	Resulting Act.[a]	
Time of Incubn (min)	SK[b]	SK′[c]
2	5.94	6.06
5	5.48	5.74
7.5	5.45	5.46
10	5.37	5.06
14	5.15	4.78

[a] Expressed as μmol of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of human plasminogen originally added. [b] Refers to native streptokinase. [c] Refers to altered streptokinase.

TABLE IV: Test of the Activation of Bovine Plasminogen with Native and Altered Streptokinase.

Condns of Incubn of Bovine Plasminogen	Incubn Time (min)	Resulting Act.[a]
1SK[b]:1BPg[c]	10	0
	30	0
1SK[b]:10BPg[c]	10	0
	30	0
1SK′[d]:1BPg	10	0
	30	0
1SK′[d]:10BPg	10	0
	30	0
33 μg of BPg with activator prepared with native SK[e]	10	6.3
33 μg of BPg with activator prepared with altered SK[e]	10	6.5

[a] Expressed as μmol of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of bovine plasminogen originally added. [b] Refers to native streptokinase. [c] Refers to bovine plasminogen. [d] Refers to altered streptokinase. [e] Described in text.

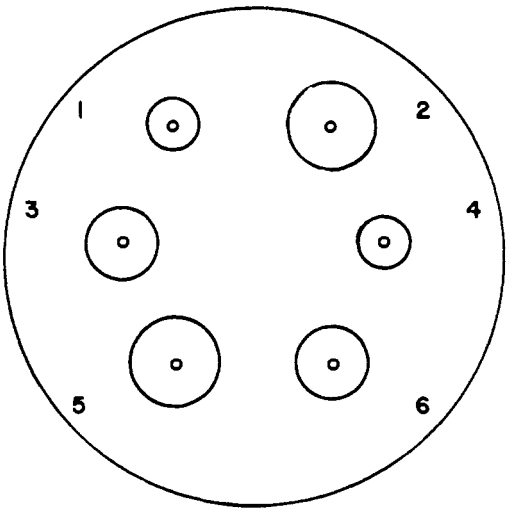

FIGURE 5: Bovine fibrin plate analysis of the activation properties of altered streptokinase. This plate is a tracing of the actual fibrin plate indicating the rings of lysis in each of the wells. The plate was developed for 12 hr at room temperature. The samples were as follows: well 1, 3.9 μg of altered streptokinase; well 2, 3.9 μg of altered streptokinase + 0.026 μg of human plasminogen; well 3, 3.9 μg of altered streptokinase + 0.005 μg of human plasminogen; well 4, 3.9 μg of altered streptokinase which was previously treated with diisopropyl fluorophosphate. The excess reagent was dialyzed out prior to placement of this sample on the plate; well 5, as in well 2 except that diisopropyl fluorophosphate treated altered streptokinase was used; well 6, as in well 3 except that diisopropyl fluorophosphate treated altered streptokinase was used.

2 min. These conditions were shown in Table III to yield maximum levels of activator esterase activity. At this time 0.010 ml of bovine plasminogen was added for 10 min. Following this, 0.025 ml of 0.1 M Tos-Arg-OMe was added for an additional 10 min and the remaining Tos-Arg-OMe was assayed as above. The results, also given in Table IV, show high levels of bovine plasmin activity in each case. The low levels of activator present in each assay give only insignificant esterase activity in themselves. All other conceivable controls were run. These results fortify the data of Table III and show that either streptokinase can form plasminogen activator in the presence of human plasminogen. Although native and altered streptokinase were unable to activate bovine plasminogen directly in solution, the results of Figure 5 show that it is possible to observe a small ring of lysis with altered streptokinase in bovine fibrin plates. However, this ring of lysis is greatly enhanced upon addition of a small amount of human plasminogen to the well containing altered streptokinase. The amounts of human plasminogen added are certainly in the range that Taylor and Beisswenger reported as possible contaminants of their modified streptokinase. This indicates that the modified streptokinase still is able to form a plasminogen activator complex in the presence of human plasminogen. It is further demonstrated in Figure 5 that pretreatment of the modified streptokinase with diisopropyl fluorophosphate does not change the above results. We cannot explain the apparent activation of bovine plasminogen in the fibrin plates by modified streptokinase except to postulate that the agar plate or some component therein can somehow lead to an artifactual result or can stabilize an event that does not occur in solution. On the other hand, the fibrin plate method may be exceedingly sensitive to small activation effects. However, since the activator activity of the modified streptokinase is greatly enhanced upon addition of small amounts of human plasminogen, we feel that the small and apparent independent activity of the modified streptokinase is not meaningful in terms of a final mechanism.

TABLE V: Development of Plasmin Esterolytic Activity with Time in Solutions of Native and Altered Streptokinase and Human Plasminogen (1SK:20HPg Protein Ratios).

Time of Incubn (min)	Resulting Act.[a] SK[b]	SK[c]
2	5.56	4.10
5	5.15	5.50
7.5	4.88	6.02
10	4.66	5.98
15	4.32	5.48
20	3.83	4.94

[a] Expressed as μmol of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of plasminogen originally added. [b] Refers to native streptokinase. [c] Refers to altered streptokinase.

Finally, the ability to both native and altered streptokinase to form human plasmin from human plasminogen was tested. These assays were performed at 1:20 molar ratios of each streptokinase to human plasminogen, conditions under which plasmin activity dominates. The assays were performed in the same manner as described above except for adjustments in the amounts of streptokinase added. The results of this experiment are given in Table V. It appears to take slightly longer for human plasmin activity to develop when altered streptokinase is employed, compared to native streptokinase. Otherwise, we feel that both native and altered streptokinase behave in similar fashions.

Discussion

The nature of the physical and chemical alterations produced in streptokinase, as a result of its incorporation into the human plasminogen activator complex, have been elucidated in this manuscript. Native streptokinase is a single chain protein of mol wt 44,000. The protein possesses an amino-terminal isoleucine residue and a carboxyl-terminal lysine residue. The only noteworthy items concerning its chemical structure are that it appears to be devoid of any cystine or cysteine residues and it possesses a relatively low isoelectric point of 5.2. However, this value is higher than the previously reported value of 4.7 for native streptokinase (De Renzo *et al.*, 1967a,b). From calculations on data of the type presented in Figure 3, we obtain roughly 12% of helical structure in native streptokinase. This result is in agreement with the value of 12% reported by Taylor and Botts (1968). Several different groups of investigators have reported amino acid compositions of native streptokinase. Our values, rereported in Table I, most closely agree with the results of Morgan and Henschen (1969). A significant consideration in these amino acid compositions is the value obtained for methionine. Morgan and Henschen (1969), as well as the present manuscript, obtain values of 4 mol of methionine/mol of streptokinase. Taylor and Botts (1968) reported that there was essentially no methionine present in streptokinase whereas De Renzo *et al.* (1967), reported values of 3 mol of methionine/mol of streptokinase. Consideration of what value is the correct one is very important to those contemplating amino acid sequence studies on streptokinase. In this regard, Morgan and Henschen (1969) have obtained five cyanogen

bromide peptides from native streptokinase which account for all of the amino acid present in this protein. This fact seems to establish the number of 4 mol/mol as the correct value for methionine.

Isolation and characterization of streptokinase from the streptokinase–human plasmin activator complex shows that native streptokinase must lose an amino-terminal peptide(s) of total mol wt 8000 as a result of its incorporation into the complex. Consideration of the amino-terminal amino acid sequences of both native and altered streptokinase in conjunction with data obtained by others allows additional insight to be made concerning the chemical structure of this molecule. Morgan and Henschen (1969) have isolated five cyanogen bromide peptides from digests of native streptokinase. One peptide, which they labeled fragment 3, appears to be the amino-terminal peptide. This peptide possesses a molecular weight of 8950. Significantly, our analysis of the amino-terminal sequence of altered streptokinase demonstrates the presence of methionine in position 11. A simple calculation on the predicted size of the peptide lost from the amino terminus of streptokinase to yield altered streptokinase can be made from the sequence information and the cyanogen bromide data and the value is approximately 7800. This value is entirely consistent with our physical data. Further, analysis of our amino-terminal amino acid sequence of altered streptokinase shows other consistencies with the data of Morgan and Henschen (1969). For example, since the amino acid residue following methionine in altered streptokinase is serine, it follows that one of the cyanogen bromide peptides of Morgan and Henschen must possess an amino-terminal serine residue. Fragment 1 (F1), according to their terminology, fills this requirement. The cyanogen bromide peptides can than be alligned from the amino terminus as: F3-F1-(F2,F5)-F4. Fragment 4 (F4) was placed at the carboxyl terminus since it did not contain homoserine. Only fragment 2 (F2) and fragment 5 (F5) require proper placement in sequence. Analysis of the amino acid composition of F3 (Morgan and Henschen, 1969) and the fact that the first 11 residues of altered streptokinase originate from the carboxyl terminus of F3 allows one to calculate the number of amino acid residues which must be lost in the transition of native to altered streptokinase. Some examples indicate that 10 aspartic acid, 7 leucine, and 6 glutamic acid residues should be lost. The data in Table I show that actually 10 aspartic acid, 6 leucine, and 7 glutamic acid residues are in fact lost. Considerations such as these show the internal consistency of our data on the structure of native and altered streptokinase.

Finally, and most importantly, it is necessary to consider the kinetic comparisons of native and altered streptokinase in terms of the mechanism of activation of plasminogen to plasmin. It had been assumed for a long time that the active site in the streptokinase–human plasmin activator complex resided in the plasmin moiety of the complex. Many kinetic and physical studies were in accord with this view. Later, it became known that, although the complex of streptokinase and human plasmin is an effective plasminogen activator, the true activator of human plasminogen was a complex of streptokinase and human plasminogen, which contained an active site. This active site is thought to exist in the plasminogen portion of the complex (Reddy and Markus, 1972). These ideas have been reinforced for the streptokinase-mediated activation of rabbit plasminogen (Schick and Castellino, 1973). However, Taylor and Beisswenger (1973) reported the separation of an altered streptokinase, isolated from the human plasminogen activator complex, which contained the activator active site. We have very carefully attempted the isolation of this streptokinase from highly active plasminogen activator complexes. The altered streptokinase which we obtain in a highly purified form, and which we have extensively characterized in this report, has no independent plasminogen activator activity in purified systems but does have slight activity in bovine fibrin plates. However, this preparation has full plasminogen activator activity toward bovine plasminogen when trace amounts of proactivator (human plasminogen) are added. We feel that both native and this particular form of altered streptokinase carry out exactly the same functions (although perhaps at dissimilar rates) and that the amino-terminal peptide(s) lost in streptokinase during its incorporation into the plasminogen activator complex is of little consequence in ascribing a function for this protein. It is, of course, possible that Taylor and Beisswenger (1973) have isolated a different altered streptokinase than ours, but this cannot be determined at this time due to the lack of physical and chemical measurements reported for their protein. In this same context, other altered forms of streptokinase have been isolated from the human plasminogen activator complex. Summaria *et al.* (1971) isolated streptokinase fragments which had $s^0_{20,w}$ values between 0.8 and 2.0 S. These fragments were found to be bound to the complex. When dissociated from the activator complex, the streptokinase fragments had neither streptokinase activity nor were they able to form activator when human plasminogen was added back to the system. These data, coupled with the data of Taylor and Beisswenger and with the data presented in this manuscript, serve to illustrate that several types of fragmented streptokinase can exist in the activator complex and can have different properties. The streptokinase fragment which we are describing is one that appears very early, if not initially, in the activator complex and is present when the plasminogen activator complex possesses its greatest activity. An additional possibility is that the streptokinase purified by Taylor and Beisswenger from Varidase and the streptokinase purified in this manuscript from Kabikinase may be different. Further work on the system will provide the answers to these questions.

Additionally, caution must be exercised in accounting for plasminogen activation merely by observing lysis in fibrin plates. The need for other supporting evidence such as Tos-Arg-OMe assays and behavior on sodium dodecyl sulfate gels cannot be overemphasized.

Acknowledgments

We thank Dr. Howard Steinman for performing the automatic amino acid sequence analyses in native and altered streptokinase.

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Molecular Weight, Subunit Structure, and Amino Acid Composition of the Branched Chain Amino Acid Aminotransferase of *Salmonella typhimurium*†

Elizabeth L. Lipscomb, H. Robert Horton, and Frank B. Armstrong*

ABSTRACT: A molecular weight of 183,000 ± 5% and a sedimentation coefficient of 9.8 S ± 2% were obtained for the branched chain amino acid aminotransferase of *Salmonella typhimurium* by the use of ultracentrifugation techniques. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed a single monomeric species with a molecular weight of 31,500 ± 10%; threonine was identified as its N-terminal amino acid. It is concluded that the native form of the enzyme is a hexamer composed of seemingly identical subunits. The amino acid composition was also determined. Results of *p*-mercuribenzoate titration studies imply that two-thirds of the sulfhydryl groups (possibly two of the three cysteinyl residues calculated per subunit) are shielded in the native form and that the enzyme contains no disulfide bridges. However, it appears that a sulfhydryl group is not required for enzymatic activity.

The availability of highly purified branched chain amino acid aminotransferase (L-leucine:2-oxoglutarate aminotransferase, EC 2.6.1.6; trivial name: transaminase B) of *Salmonella typhimurium*, crystallized by Coleman and Armstrong (1971), made feasible for the first time a number of studies permitting the physical and chemical characterization of this enzyme. The following report is primarily concerned with the determination of the $s_{20,w}$ value at infinite dilution, the molecular weight, subunit structure, and the amino acid composition of this aminotransferase. Information regarding aggregation properties of the enzyme, identification of its N-terminal amino acid residue, and the effects of thiols and sulfhydryl reagents on the enzyme are also included.

Experimental Section

Enzyme Purification and Assay. The crystallization procedure and assay for transaminase B were as previously described by Coleman and Armstrong (1971). Enzyme preparations with specific activities of 3500–4000 μmol of isoleucine transaminated per hr per mg of protein were used in this study. Protein was determined by the method of Lowry *et al.* (1951).

† From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27607. *Received December 10, 1973*. This investigation was supported in part by National Institutes of Health Research Grant GM-14184-06 and is a contribution from the Schools of Agriculture and Life Sciences and Physical and Mathematical Sciences. Paper No. 4233 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C.