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Thermal stability of the three domains of streptokinase studied by circular dichroism and nuclear magnetic resonance

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Abstract

Streptococcus equisimilis streptokinase (SK) is a single-chain protein of 414 residues that is used extensively in the clinical treatment of acute myocardial infarction due to its ability to activate human plasminogen (Plg). The mechanism by which this occurs is poorly understood due to the lack of structural details concerning both molecules and their complex. We reported recently (Parrado J et al., 1996, *Protein Sci* 5:693–704) that SK is composed of three structural domains (A, B, and C) with a C-terminal tail that is relatively unstructured. Here, we report thermal unfolding experiments, monitored by CD and NMR, using samples of intact SK, five isolated SK fragments, and two two-chain noncovalent complexes between complementary fragments of the protein. These experiments have allowed the unfolding processes of specific domains of the protein to be monitored and their relative stabilities and interdomain interactions to be characterized. Results demonstrate that SK can exist in a number of partially unfolded states, in which individual domains of the protein behave as single cooperative units. Domain B unfolds cooperatively in the first thermal transition at approximately 46 °C and its stability is largely independent of the presence of the other domains. The high-temperature transition in intact SK (at approximately 63 °C) corresponds to the unfolding of both domains A and C. Thermal stability of domain C is significantly increased by its isolation from the rest of the chain. By contrast, cleavage of the Phe 63–Ala 64 peptide bond within domain A causes thermal destabilization of this domain. The two resulting domain portions (A1 and A2) adopt unstructured conformations when separated. A1 binds with high affinity to all fragments that contain the A2 portion, with a concomitant restoration of the native-like fold of domain A. This result demonstrates that the mechanism whereby A1 stimulates the plasminogen activator activities of complementary SK fragments is the reconstitution of the native-like structure of domain A.

Keywords: circular dichroism; domains; fibrinolysis; NMR; protein fragments; streptokinase; thermal stability

Streptokinase is a bacterial exoprotein from *Streptococcus equisimilis* that indirectly causes the activation of human plasminogen. SK and Plg form an avid 1:1 stoichiometric complex with the subsequent appearance of a serine protease active center from within the Plg moiety; this complex acts, in turn, as an activator of other Plg molecules (McClintock & Bell, 1971). SK is in widespread clinical use to treat acute myocardial infarction as a consequence of its function as an activator of vascular fibrinolysis (Martin, 1982; ISIS-3, 1992). An understanding of the molecular mechanism of Plg activation by SK has, however, been precluded by the lack of high-resolution structural information about these proteins and their complexes.

SK has been described as a flexible multidomain protein (Radek & Castellino, 1989; Pautov et al., 1990; Damaschun et al., 1992). A recent NMR study has indicated that SK is composed of at least three domains that have independent stability (Teuten et al., 1993). Proteins that participate in fibrinolysis almost invariably are of multidomain character. Moreover, it is becoming apparent that the

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Abbreviations: SK, *Streptococcus equisimilis* streptokinase; Plg, human plasminogen; SK fragments: A1 (residues 1–63), B (residues 147–287), B′ (residues 147–292), C (residues 288–380), B-C (residues 147–380), A2-B (residues 64–292), A2-B-C (residues 64–380); DSC, differential scanning calorimetry; T_m, mean temperature of an unfolding transition.

regulation of proteolytic function within these processes is frequently mediated by multiple intramolecular domain–domain interactions (Marshall et al., 1994; Bakker et al., 1995). A better understanding of the domain organization and of interdomain interactions within SK may not only illuminate understanding of SK function, but also the general folding properties of these types of multidomain proteins.

A well-established method of studying the domain organization of proteins is the characterization of the resistance to proteolysis of particular regions of their sequences, together with measurement of the structural and functional integrity of protein fragments generated by these means. Several fragments and modified forms of SK have been isolated previously (Brockway & Castellino, 1974; Siefring & Castellino, 1976; Jackson et al., 1986; Malke et al., 1987; Misselwitz et al., 1992; Rodríguez et al., 1994, 1995; Shi et al., 1994; Reed et al., 1995; Young et al., 1995). A number of SK fragments remain folded when isolated in solution and a minority maintain an ability to cause Plg activation, although, in most cases, this occurs at substantially reduced rates when compared with native SK. We have recently conducted a study of SK in which proteolytic fragments corresponding to different parts of the SK sequence have been isolated and characterized by structural and functional means (Parrado et al., 1996). All fragments were shown to conserve native-like structures in solution according to CD and NMR measurements, with the exception of a fragment containing residues 1–63, which was found to be substantially unstructured. This study indicates that intact SK is composed of three structurally independent domains and a less structured C-terminal tail. The N-terminal domain (domain A; approximately residues 1–145) was found to strongly regulate the functions of the remaining two domains. The second (domain B; approximately residues 146–290) and the third (domain C; approximately residues 291–380) domains appear to be essential for plasminogen activator activity (Rodríguez et al., 1995; Parrado et al., 1996). The SK C-terminal tail (approximately residues 381–414) is less structured, relatively more susceptible to proteolysis, and unnecessary for full SK activity.

The study of structural cooperativity within protein fragments, for example, by monitoring unfolding of proteins by heat or chemical denaturants, can provide further information to establish the nature of protein domains. A variety of techniques has been used to investigate these processes, including differential scanning calorimetry, CD, fluorescence spectroscopy, and NMR spectrometry (Novokhatny et al., 1991; Teuten et al., 1991, 1993; Vysotchin et al., 1993; Nowak et al., 1994; Kurochkin et al., 1995). A previous DSC study with intact SK reported two major thermal transitions occurring at very similar temperatures under several conditions (Welfle et al., 1992). A number of proteolytic SK fragments were later studied by DSC (Misselwitz et al., 1992), although complete assignment of the unfolding transitions to specific regions of SK was not possible because of the lack of fragments representing all regions of the molecule. Unfolding by temperature and guanidine hydrochloride of the intact protein followed by NMR showed that three regions of SK experience independent unfolding (Teuten et al., 1993), but the results could not be correlated to the domain organization of the protein.

By contrast with other methods, the use of NMR to follow unfolding processes allows detection of effects on individual residues, providing detailed information about the molecular processes occurring during the disruption of tertiary structure. We have recently presented the one-dimensional (1D) ^{1}H NMR spectra of isolated SK fragments in D_2O at pD 7.0, compared these with the spectrum of intact SK (Parrado et al., 1996), and assigned a number of resonances in the intact protein to different domains. These resonances serve as specific probes of unfolding processes involving individual domains as well as allowing monitoring of the disruption of domain–domain interactions.

The SK fragments studied in this work are described in Table 1 in terms of their sequences and molecular masses. To facilitate clarity, we introduce a notation for the fragments in terms of the domains they correspond to in the intact protein, namely, A, B, and/or C. A subset of fragments contains only an N-terminal or a C-terminal portion of domain A; these are referred to as A1 and A2, respectively. Fragments B and B′ differ by five residues at their C terminus. Table 1 also includes the equivalent notation used previously (Parrado et al., 1996).

In the present study, several isolated SK fragments and two noncovalent complexes between complementary fragments are used to analyze the unfolding and thermostability of streptokinase domains, using CD and NMR. Domain A reconstitution promoted by association of complementary fragments is described and its functional relevance interpreted. The results provide further insight into the structural and functional organization of this biomedically important protein.

Results

Stability of streptokinase domains B and C

Thermal unfolding of SK fragments was monitored by CD and 1D ^{1}H NMR using samples in 20 mM phosphate buffer, pH 7.0. The temperature profiles for far-UV CD experiments using various SK fragments and intact SK are shown in Figure 1. The reversibility of unfolding processes under the CD conditions was found to be almost complete in all cases, with only slight losses (less than 10%) of amplitude for refolding transitions, whereas in NMR experiments using higher sample concentrations, refolding of SK and some of the fragments (particularly fragments C and A2-B) and intact SK was incomplete. Fitting of the CD profiles to two-state or three-state unfolding models was performed for each fragment in order to estimate the apparent thermodynamic parameters of the observed transitions (Table 2).

Table 1. *Sequences and calculated molecular masses of SK fragments studied in this work, obtained by chymotryptic digestion of SK*[a]

Fragment notation		Sequence	Molecular mass
A-B-C-t[b]	(Intact SK)	I1–K414	47,286.8
A1	(SK7)	I1–F63	6,765.6
A2-B[c]	(SK26)	A64–L292	26,172.3
A2-B-C	(SK36)	A64–Y380	36,407.8
B	(SK16)	K147–F287	16,371.4
B′	(SK17)	K147–L292	16,980.0
B-C	(SK27)	K147–Y380	27,215.5
C	(SK11)	D288–Y380	10,862.1

[a]Notation used in Parrado et al. (1996) is shown in parentheses.
[b]Intact SK is composed of three domains A, B, and C, and a C-terminal tail (t) (Parrado et al., 1996).
[c]This fragment is accompanied by some proportion of the fragment A64–L287.

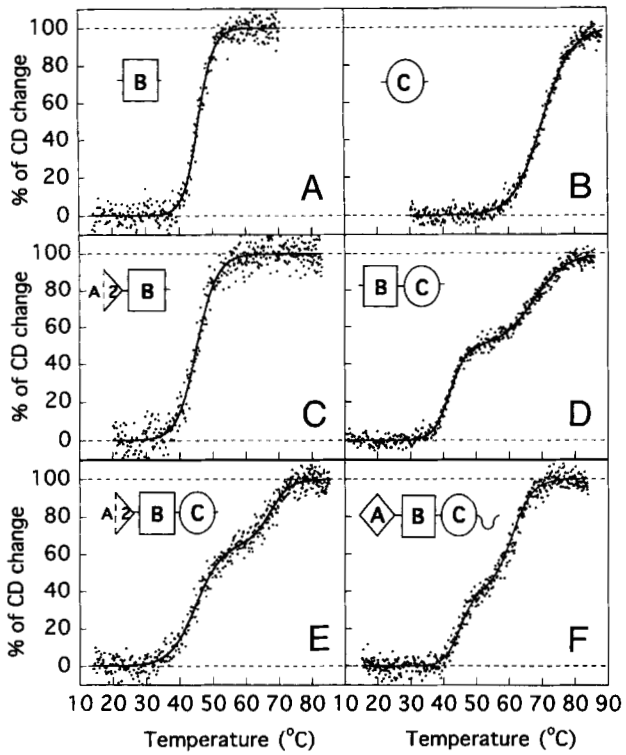

Fig. 1. Thermal unfolding of SK and SK fragments at pH 7.0 monitored by far UV CD. **A:** Fragment B. **B:** Fragment C. **C:** Fragment A2-B. **D:** Fragment B-C. **E:** Fragment A2-B-C. **F:** Intact SK. Schematic pictures of each molecule are shown for clarity. The percentage changes in CD signal with temperature have been calculated using the change in CD signal at 200 nm. Dots represent experimental values. Lines represent best fits using the equations of two-state or three-state unfolding models.

Table 2. *Apparent increments of enthalpy of unfolding, ΔH, and mean temperatures (T_m), for the two thermal transitions (1) and (2) observed by CD for the unfolding of native SK and SK fragments at pH 7.0*[a]

Fragment	$T_m(1)$ (°C)	$\Delta H(1)$ (kJ/mol)	$T_m(2)$ (°C)	$\Delta H(2)$ (kJ/mol)
Intact SK	44.8 ± 0.3	367 ± 31	61.4 ± 0.2	315 ± 18
A2-B-C	45.0 ± 0.3	209 ± 12	67.5 ± 0.4	333 ± 37
B-C	42.2 ± 0.2	320 ± 16	67.0 ± 0.3	194 ± 11
A2-B	45.3 ± 0.3	275 ± 17	—	—
B	46.0 ± 0.2	365 ± 18	—	—
C	—	—	72.3 ± 0.5	195 ± 8

[a] Values were obtained from the fitting of CD thermal profiles according to two-state and three-state unfolding models.

Fragment B exhibits a single two-state unfolding transition with a mean temperature (T_m) of 46.0 ± 0.2 °C when monitored by far-UV CD (Fig. 1A; Table 2). Figure 2 shows the temperature dependence of the NMR spectrum of fragment B′. All signals that are shifted away from their random coil positions show the same temperature dependence, reflecting the cooperative disruption of tertiary contacts. The intensity of these resonances decreases with increasing temperature, indicating slow chemical exchange between the folded and the unfolded states of this fragment. The temperature dependence of the integrated areas of several resolved resonances indicates a mean temperature of unfolding of 48.8 ± 0.7 °C, close to the value obtained from the CD experiments for fragment B (Table 2).

According to far-UV CD measurements, thermal unfolding of fragment C occurs via a single transition at 72.3 ± 0.5 °C (Fig. 1B; Table 2), consistent with the disruption of the secondary structure of a single domain. Figure 3 shows the thermal unfolding of fragment C as investigated by NMR. The unfolding transition observed by NMR occurs at approximately 70 °C, in agreement with the CD data. It was noted that resonances close to 6.6 ppm, attributable to aromatic protons that are shifted upfield in the folded fragment, have a temperature dependence somewhat different from that of the upfield shifted methyl resonances present between 0.5 and −0.1 ppm and also different from that of the resonances between 5.0 ppm and 5.7 ppm; the latter resonances are likely to arise from C_α protons in β structure. The aromatic resonances remain sharp during the unfolding transition and are evident even at temperatures above 70 °C, whereas other resolved resonances become noticeably broadened even at temperatures significantly below 70 °C. This is likely to be an indication of incompletely cooperative unfolding behavior of the isolated fragment C structure. This is consistent with the relatively low value of the apparent enthalpy of unfolding obtained from the CD unfolding profile for fragment C (Table 2), compared with those measured for compact globular

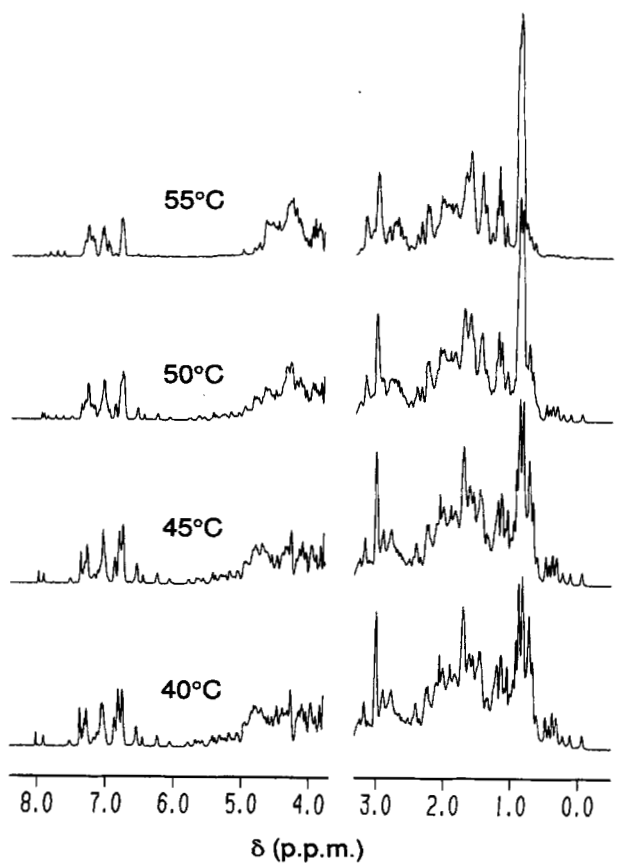

Fig. 2. Temperature dependence of the 500-MHz 1D NMR spectra of fragment B′ in D_2O at pD 7.0. Temperatures are indicated adjacent to each spectrum.

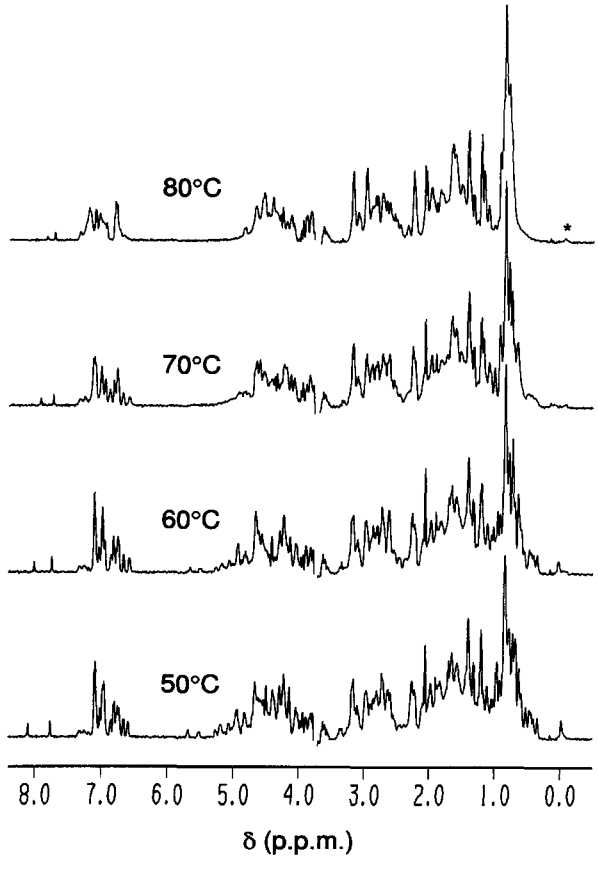

Fig. 3. Temperature dependence of the 500-MHz 1D NMR spectra of fragment C in D_2O at pD 7.0. Temperatures are indicated adjacent to each spectrum. Asterisk indicates an impurity in the sample.

proteins of the same size that follow a two-state unfolding behavior (Privalov, 1979).

The thermal unfolding of fragment B-C monitored by CD yields a profile that is indicative of a two-step transition (Fig. 1D), with the presence of an unfolding intermediate at approximately 55 °C. Temperatures for midpoints of the two transitions were found to be a few degrees lower than the temperatures observed for the isolated fragments B and C (Table 2). Thermal unfolding experiments monitored by NMR also show two unfolding transitions, consistent with CD results. NMR spectra of B-C at 40 °C, 55 °C, and 80 °C are shown in Figure 4, representing the fragment in the fully folded, intermediate, and unfolded states, respectively. The spectrum of the folded state of fragment B-C is similar to the sum of the spectra of isolated fragments B′ and C, although with broader resonances (Parrado et al., 1996). A subset of resonances disappears in a first transition with a mean temperature of approximately 45 °C, as a consequence of the unfolding of one of the two domains of fragment B-C. Comparison of the spectra with those shown in Figure 2 shows that these resonances are from residues within domain B; this indicates that domain B unfolds in this first transition, giving rise to a partially unfolded fragment in which domain C remains well structured. The higher temperature transition is similar to that observed in the unfolding of the isolated fragment C except that the T_m is somewhat lower (67 °C instead of 72 °C).

CD unfolding experiments demonstrate that fragment A2-B displays a single unfolding transition at a very similar temperature to that of fragment B (Fig. 1C). This is consistent with the observation by NMR that there are no additional resolved resonances in the spectrum of this fragment compared with that of fragment B′ (see Figs. 2, 6). The broader lines in the A2-B spectrum relative to that of B′ can be attributed to its larger size and the fact that the A2 portion of the fragment (residues 64–146) appears to adopt an unstructured conformation (Parrado et al., 1996).

According to CD measurements, fragment A2-B-C unfolds in a two-step process similar to that of fragment B-C, yet with a slightly higher mean temperature for the first transition (Fig. 1E; Table 2). The temperature dependence of the NMR spectrum of fragment A2-B-C appears very similar to that of fragment B-C, indicating that the presence of the A2 portion of SK sequence does not influence significantly the unfolding properties of the remainder of the fragment, as already suggested for fragment A2-B. It therefore appears that A2 does not possess a fixed tertiary structure when within either the isolated A2-B or A2-B-C fragments; this indicates that the A1 sequence is an essential requirement for the structural integrity of domain A. CD and NMR experiments also show that isolated fragment A1 is unstructured in solution at neutral pH. Several resonances in the spectrum of native intact SK that are not present in the spectra of any of the folded fragments—A2-B-C, A2-B, B-C, B′, and C—can be observed by comparing Figures 2, 3, 4, and 8; these are likely to correspond to residues of domain A when in its structured form.

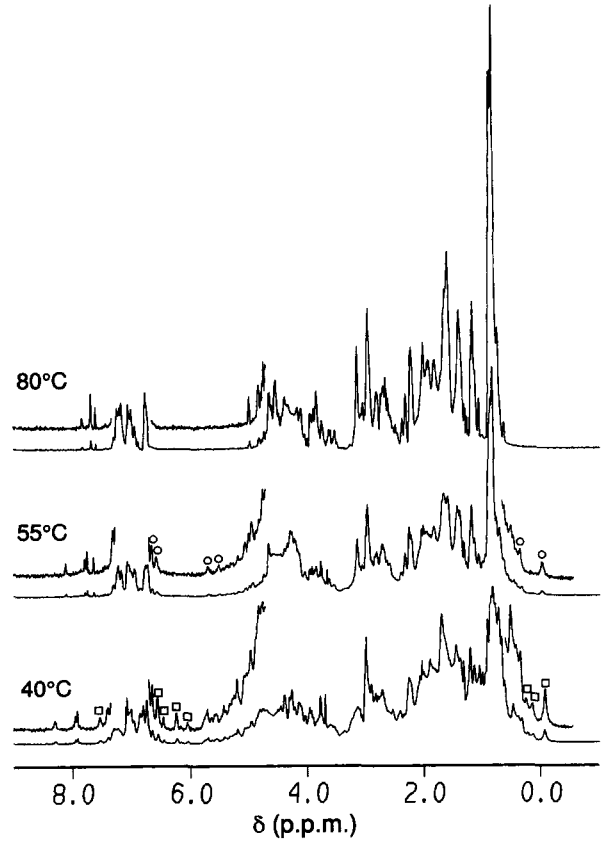

Fig. 4. One-dimensional NMR spectra at 500 MHz of fragment B-C in D_2O at pD 7.0 at three different temperatures indicated adjacent to each spectrum. Regions of the spectra with well-resolved resonances have been amplified. Resonances marked with squares and circles have been identified as belonging to domains B and C, respectively, as discussed in the text.

Refolding of streptokinase domain A from unstructured fragments

Having determined that fragments A1 and A2 are both unstructured in isolation, it is of interest to investigate whether their co-addition results in the reconstitution of the structure of domain A. To this end purified fragment A1 was added in a 5:1 molar excess to samples of A2-B-C, A2-B, and B-C at approximately 6 μM concentration, in 0.1 M phosphate buffer, pH 7.0, at room temperature. After incubation for 5 min, the samples were analyzed by gel filtration to monitor complex formation (Fig. 5). In control experiments with individual fragments, fragment A1 was found to elute anomalously: several peaks of different apparent molecular masses were observed, one of which appears to correspond to an aggregated state of the fragment. Fragments A1 and B-C were found not to interact with significant affinity, even after several hours of incubation, in accordance with the similarity of the elution profiles of fragment B-C in presence and absence of fragment A1. Analysis of peak fractions by SDS-PAGE shows only the presence of fragment B-C in the major peak arising from the elution of a mixture of fragments A1 and B-C. By contrast, the elution profile of fragment A2-B-C is clearly modified by the addition of A1; a significant increase in the area of the major peak is produced by the binding of fragment A1 to A2-B-C. The same result was obtained with the addition of A1 to A2-B, although here the relative increase in peak area is more pronounced. SDS-PAGE of samples from the major peak of both elution profiles reveals the presence of fragment A1 co-eluting with A2-B-C and with A2-B. These results indicate that each of these two fragments form high-affinity noncovalent complexes with fragment A1. The apparent mass of the A1:A2-B-C complex, calculated using molecular mass standards, is not significantly different from that of isolated A2-B-C (both around 55 k), despite the 7 k increase in mass caused by the binding. Moreover, the apparent mass of A1:A2-B complex is clearly reduced compared with isolated A2-B (from 48 k to 43 k). This indicates that the binding of fragment A1 to A2-B or A2-B-C increases the degree of compactness of the resulting complexes when compared with the isolated fragments A2-B and A2-B-C.

Following the addition of a 3:1 molar excess of isolated A1 to A2-B or A2-B-C in deuterated phosphate buffer, pD 7.0, at room temperature, dramatic changes in their 1D-NMR spectra are evident. This experiment is shown for fragment A2-B in Figure 6, where the NMR spectra of fragments A1 and A2-B and the spectrum of the two-chain complex A1:A2-B are shown. Resonances of intact SK assigned to domain A (see Parrado et al., 1996) become visible as a consequence of the interaction between A1 and A2-B.

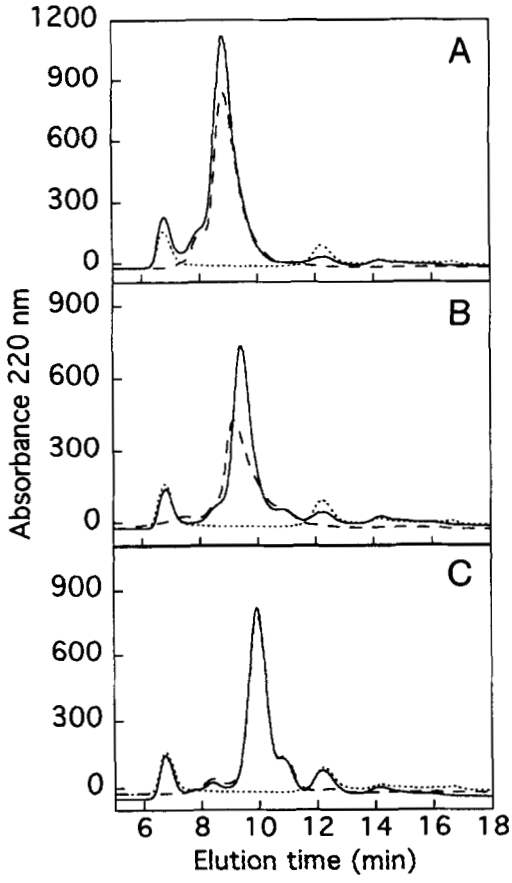

Fig. 5. Gel filtration profiles obtained in a Superdex 75-HR column for mixtures of fragment A1 with either fragment (**A**) A2-B-C, (**B**) A2-B, or (**C**) B-C in a 5:1 molar ratio (solid lines). Dotted and dashed lines correspond to control experiments with isolated A1 and with the larger fragments, respectively.

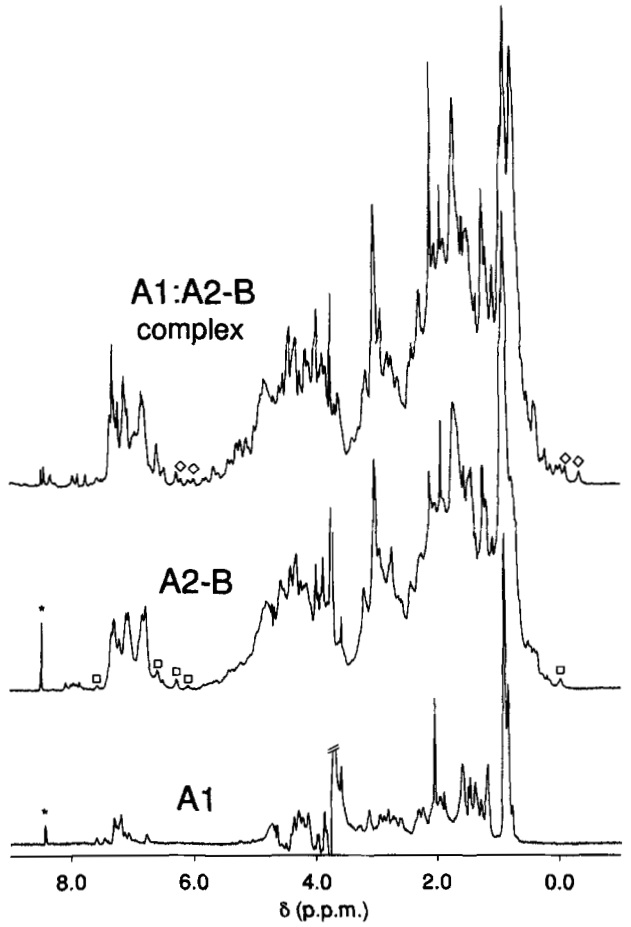

Fig. 6. One-dimensional NMR spectra at 500 MHz in D_2O, pD 7.0, at 35 °C, of samples of fragments A1, A2-B, and A1:A2-B two-chain complex. Asterisk indicates an impurity in the sample. Resonances marked with diamonds and squares have been identified as belonging to domains A and B, respectively, as discussed in the text.

Additionally, several H2 resonances of His residues between 7.6 ppm and 8.5 ppm can be seen to be narrower and better resolved in the A1:A2-B spectrum compared with the spectrum of isolated A2-B. These observations indicate clearly that binding of A1 to A2-B restores the native structure of SK domain A. Similarly, for fragment A2-B-C virtually all resolved resonances present in intact SK are recovered as a consequence of the specific binding of A1 to A2-B-C. These results are consistent with an increase in compactness of each complex as a consequence of the refolding of domain A.

Thermal stability of the two-chain domain A

Thermal unfolding experiments monitored by NMR were performed with the two-chain complexes A1:A2-B and A1:A2-B-C prepared as above. Figure 7 shows the NMR spectra at three different temperatures for each of these complexes. In the A1:A2-B spectrum, a number of resonances can be identified clearly as belonging to either domain A or B. The two domains unfold at similar temperatures, approximately 50 °C, with no native-like resonances present above this thermal transition. The initial spectrum was not recovered in this case after cooling of the NMR sample and visible aggregation of the sample was observed. In the case of the complex A1:A2-B-C, two unfolding transitions are observed. The first transition at approximately 50 °C involves resonances from both domains A and B, whereas domain C unfolds in a second transition close to 70 °C. In both two-fragment complexes, domain A is clearly less thermally stable than it is in intact SK (see below) because in the latter case resonances belonging to domain A disappear at higher temperatures (around 60 °C). This is likely to be a consequence of the 63–64 peptide bond cleavage within domain A. Decreases of unfolding temperatures produced by proteolytic cleavage have been observed for other proteins such as triosephosphate isomerase (Sun et al., 1993) and lysozyme (De Laureto et al., 1995).

Thermal unfolding of three-domain intact streptokinase

A two-step unfolding profile is indicated by CD studies of the thermal unfolding of intact SK (Fig. 1F). This is consistent with previous studies that used differential scanning calorimetry (Radek & Castellino, 1989; Welfle et al., 1992). Figure 8 shows the same process monitored by NMR (see also Teuten et al., 1993). Interpretation of these data is complicated by the relatively large number of resonances and their overlap. In addition, for SK the different transitions are closer in temperature than for the SK fragments, as observed previously by CD unfolding experiments. A number of well-resolved resonances, however, disappear in a first unfolding transition at approximately 50 °C; for example, in the aromatic region of the NMR spectrum, upfield-shifted resonances at

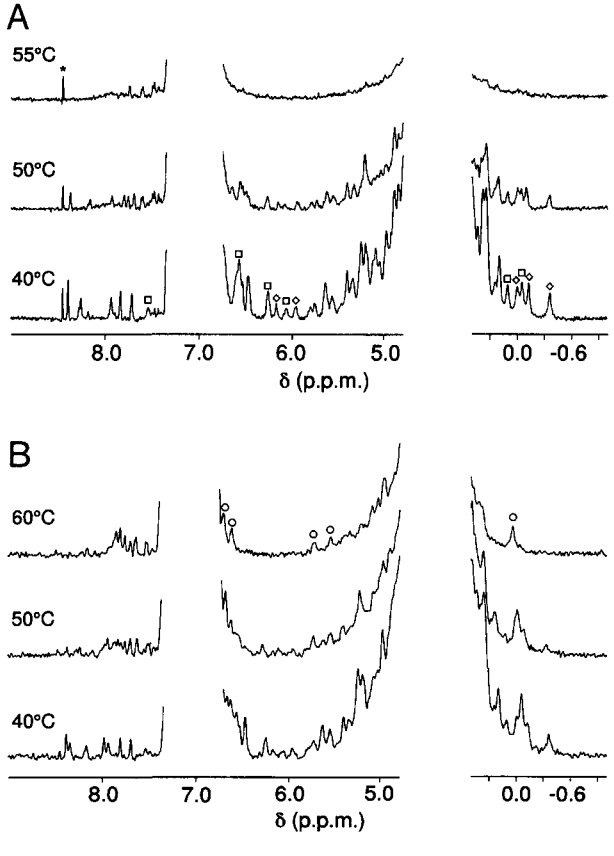

Fig. 7. One-dimensional NMR spectra at 500 MHz of the two-chain complexes (**A**) A1:A2-B and (**B**) A1:A2-B-C in D_2O at pD 7.0 at three different temperatures indicated adjacent to the spectra. Only regions of the spectra with resolved resonances are shown. Asterisk indicates an impurity in the sample. Resonances marked with diamonds, squares, and circles are identified as belonging to domains A, B, and C, respectively.

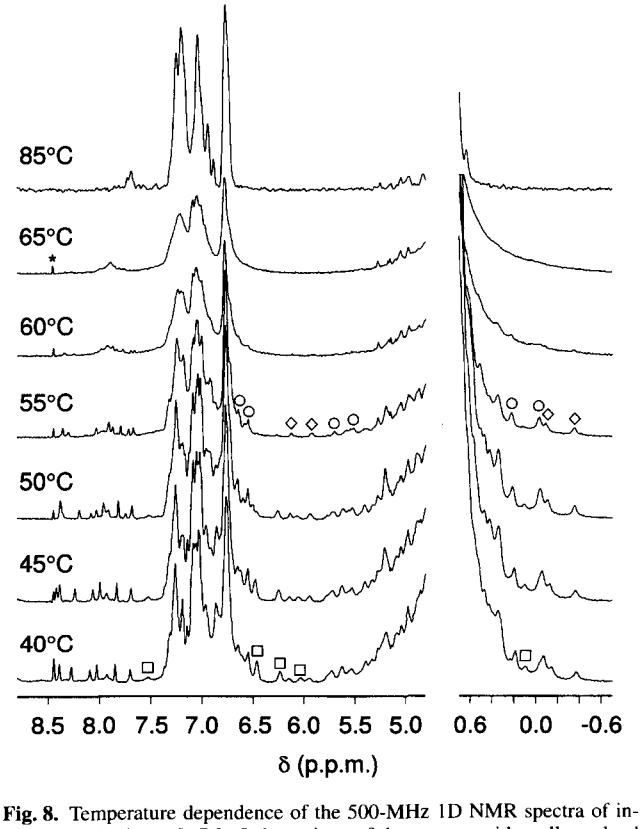

Fig. 8. Temperature dependence of the 500-MHz 1D NMR spectra of intact SK in D_2O at pD 7.0. Only regions of the spectra with well resolved resonances are shown. Temperatures are indicated adjacent to each spectrum. Asterisk indicates an impurity in the sample. Resolved resonances marked with diamonds, squares, and circles are identified as belonging to domains A, B, and C, respectively.

6.05 ppm, 6.24 ppm, and 6.58 ppm, identified as belonging to a phenylalanine residue from domain B of SK (Teuten et al., 1993; Parrado et al., 1996), disappear in this transition. Additionally, a broad, downfield-shifted resonance at 7.54 ppm, likely to arise from an aromatic group, and a resolved resonance from a methyl group at 0.1 ppm, also vanish in this transition. Each of these resonances has been identified as arising from residues in domain B (Parrado et al., 1996). A number of peaks in the spectrum disappear at higher temperatures, close to 60 °C. Some of these are present in the spectrum of fragment C, for example, two resonances close to 6.6 ppm from one or more aromatic residues, two resonances from methyl groups at −0.05 ppm and 0.2 ppm, and two resonances at 5.5 ppm and 5.7 ppm from C_α protons; this part of the protein unfolds in the high temperature transition, as observed for fragments B-C and A2-B-C. Other resonances also disappear in this transition, such as two well-resolved resonances arising from methyl groups at −0.4 ppm and −0.15 ppm and also two resonances at 5.9 ppm and 6.1 ppm (Fig. 8). It has been proposed previously (Parrado et al., 1996) and demonstrated here that these signals correspond to domain A, which is not present in a folded conformation in any of the isolated fragments.

At high temperatures, a few sharp resonances remain in the NMR spectrum of SK between 5.0 and 5.3 ppm, even up to 85 °C; resonances between 4.8 and 5.0 ppm have been observed also in the NMR spectra of some of the fragments at high temperatures (e.g., Figs. 2, 4). The origin of these relatively downfield resonances is unclear, although it is likely that they correspond to residual β structure within the SK chain. This is consistent with the presence of significant ellipticity around 220 nm in the far-UV CD spectrum of intact SK and SK fragments at high temperatures observed by us and by others (Radek & Castellino, 1989; Welfle et al., 1992). Additionally, the majority of resonances in the NMR spectrum of SK at 65 °C are very broad. At higher temperatures (85 °C) resonances become sharper, and the spectrum resembles more than expected for an unfolded polypeptide chain. By contrast, none of the SK fragments shows this behavior at high temperature. This change between 65 °C and 85 °C is not, however, reflected in any change in the CD spectrum of SK, either in the far-UV or the near-UV ranges, although it appears to involve heat absorption according to the presence of an endothermic peak at 80 °C in DSC measurements (results not shown). Low recovery of native-like resonances was observed in the NMR spectrum upon cooling of the heated samples of SK; resonances were much broader than those of the unheated SK. No visible aggregation of the protein occurred during these experiments. The nature of the processes influencing SK structure at high temperatures is currently under investigation.

Discussion

This work presents the characterization of a variety of fragments and fragment complexes containing different domains of SK using thermal unfolding experiments. The observation of differences in the stability properties of a domain in the presence or absence of other domains constitutes a common tool to probe domain–domain interactions. The thermal unfolding of the isolated fragment B′ occurs entirely cooperatively. In the NMR experiments, simultaneous loss of all resolved resonances representing the tertiary structure is observed, with a T_m coincident with that determined for the unfolding transition by CD. The observed transition for B′ accounts for all resonances that disappear at the low temperature transition observed in the NMR spectra of fragments B-C, A2-B-C, and intact SK. The unfolding temperatures of domain B in the different SK fragments are very similar, indicating that its stability is largely insensitive to the presence or absence of domains A or C, indicating relatively weak interactions between either of these domains and domain B.

In contrast to this, isolated fragment C undergoes thermal unfolding in a less cooperative manner. Additionally, the temperature of unfolding of domain C varies in an unusual manner in that it increases progressively when measured using intact SK, fragments A2-B-C, B-C, and C (Table 2). It would appear that this domain is thermally stabilized by its isolation from the remainder of the chain. This result is contrary to what is generally observed for the unfolding of proteins with interacting domains. Theoretical models generally account for pairwise domain–domain interactions by using a favorable interface free energy that goes to zero when one of the domains unfolds (Brandts et al., 1989; Freire et al., 1992). These models have been successful in explaining the unfolding properties of several proteins with domains communicating by pairwise interactions. They predict that increasing interdomain interactions produce an increase in the unfolding temperature (T_m) of the less stable domain, whereas the T_m of the most stable domain remains unchanged. In the case of SK domains, these models appear not to be valid and other factors are likely to affect their unfolding properties.

A first explanation for this unusual change of stability of domain C is that possible differences between the thermodynamic properties of the unfolded chain for isolated fragment C, fragment B-C, and intact SK could modify the energetics of unfolding of domain C. In support of this, there is evidence of residual secondary structure at high temperature in most of the fragments, and particularly in intact SK, according to their CD spectra. In addition, the NMR spectrum of full-length SK at 65 °C, after the tertiary structure of all the domains has been disrupted, does not correspond to a fully unfolded state; the spectrum contains very broad signals that could indicate the presence of rapid conformational exchange of the SK chain and/or aggregation of the protein. This behavior is not observed in any of the fragments. A second possible explanation is that isolation of the domains by proteolysis produces a folded domain C, but one with greater extent of disorder than the domain when within the intact protein. Two-dimensional NMR spectra of isolated fragment C at 35 °C suggest the presence of some disordered regions (not shown). Local unfolding in the isolated domain would simultaneously decrease the enthalpic content and increase the entropic content of the folded conformation of the domain. If the entropic contribution is greater than the enthalpic effect, a net increase of the unfolding temperature would result. Experimental evidence to examine these tentative explanations will be sought from detailed thermodynamic and structural studies of SK fragments.

The high temperature transition of intact SK at approximately 60 °C involves the unfolding of domains A and C. These domains have similar stabilities in the full-length protein under our experimental conditions. This result poses the question of whether the unfolding of domains A and C is independent or is coupled as a result of interactions between them. The latter case would necessarily result from a nonlinear spatial arrangement of SK domains with its sequence. Unfortunately, the N-terminal domain of SK (domain A) has not been isolated in intact form from our proteolytic mixtures. Nevertheless, domain A refolds into a native-like conformation not only in the A1:A2-B-C noncovalent two-chain complex, but also in the A1:A2-B two-chain complex lacking do-

main C. This indicates that domain C is not required for the reconstitution and the structural integrity of domain A. Similarly, domain C is folded in a native-like conformation in fragment B-C and also in its isolated form (fragment C). The fact that the temperature of unfolding of the two-chain domain A is the same in the noncovalent complexes A1:A2-B and A1:A2-B-C, each in presence and absence of domain C, supports the idea of independent unfolding of the domains. Unfolding experiments of SK followed by NMR using guanidine deuterochloride (Teuten et al., 1993) provide additional evidence for this: the N-terminal part of SK (domain A) becomes extensively unstructured between 1 M and 1.5 M of denaturant, whereas resonances assigned in this work to domain C still remain in the spectrum of SK. All this evidence suggests that there is little or no interaction between the terminal (A and C) domains of the protein.

These results demonstrate, therefore, that SK is composed of three domains that show a high degree of independent behavior. Nevertheless, a certain degree of interaction between adjacent domains has been proposed on the basis of the extent of line broadening of resonances observed in the NMR spectra of fragment B-C or intact SK relative to the corresponding resonances in the spectra of isolated domains B and C (Parrado et al., 1996). This is also observed for resonances corresponding to domain B in the spectrum of the two-domain complex A1:A2-B when compared with the spectrum of isolated fragment B′ (see Figs. 2, 6). In addition, small shifts of some resonances resulting from residues in domain C occur on its isolation from larger fragments or on the unfolding of domain B in fragment B-C and intact SK. For instance, during the thermal unfolding of fragment B-C followed by NMR, the lowest-field His H2 resonance (assigned to His 358 within domain C by comparison of the spectra of fragments B′, C, and B-C) shows a significant transition occurring simultaneously with the unfolding of domain B (not shown). This perturbation of the environment of surface residues associated with the unfolding of the adjacent domain is consistent with a limited degree of interdomain interaction.

Several previous studies have indicated the importance of approximately the first 60 residues at the N terminus of SK in the regulation of its Plg activator activity (Siefring & Castellino, 1976; Shi et al., 1994; Young et al., 1995; Parrado et al., 1996). These N-terminal peptides (similar to fragment A1) remain noncovalently bound to the rest of the SK chain in proteolytic preparations (Siefring & Castellino, 1976; Misselwitz et al., 1992; Parrado et al., 1996) and are capable of producing a large increase in the activity of other SK fragments (Shi et al., 1994; Parrado et al., 1996). Isolated fragment A1 has been described as being mainly unstructured at neutral pH, under conditions where it was capable of potentiating the activator activity of fragment A2-B-C up to 100-fold (Parrado et al., 1996). It has also been suggested that the 64–146 region (A2) of isolated A2-B-C and A2-B is mainly unstructured at neutral pH due to the absence of tertiary contacts essential for the folding of this part of domain A of SK (Parrado et al., 1996). In the present work, the 63-residue N-terminal peptide A1, which possesses no ordered tertiary fold when isolated in solution, has been found to reestablish the necessary tertiary contacts with the remainder of domain A of SK on their co-addition, causing its refolding to a native-like conformation. Complementation between protein fragments and their refolding into a native-like conformation is not a frequently observed phenomenon, although it has been documented previously for a number of proteins; for instance, chymotrypsin inhibitor-2 (De Prat Gay & Fersht, 1994) and thioredoxin (Tasayco & Chao, 1995). It is demonstrated here that the potentiation effect of SK fragment A1 on the plasminogen-activator activity on its addition to fragment A2-B-C, with virtually full recovery of native SK activity (Parrado et al., 1996), is caused by the reconstitution of domain A.

Materials and methods

Protein samples

Streptokinase was isolated from culture filtrates of *S. equisimilis* at the Biological Pilot Plant of SmithKline Beecham Pharmaceuticals, Gronau, Germany, using methods described in U.K. patent application no. 8824496.7. The purity of this material was judged by electrophoretic methods to be greater than 95%. SK fragments were obtained by chymotryptic digestion of SK and purified in a two-step chromatographic method as described previously (Parrado et al., 1996). Purified fragments were lyophilized and stored at −20 °C. All reagents used were of analytical grade.

Samples for CD measurements were prepared by dissolving lyophilized material in water and applying the resulting solution to a Sephadex G-25 PD-10 column (Pharmacia Biotech) preequilibrated with buffer. All measurements were made in 20 mM phosphate buffer, pH 7.0. SK and SK fragment concentrations were estimated from absorption measurements at 280 nm, using absorption coefficients $e^{0.1\%}$(280 nm) calculated from their amino acid sequence as before (Parrado et al., 1996). NMR samples were prepared by dissolving lyophilized protein in deuterated 10 mM phosphate buffer, pD 7.0. Samples contained 0.01 mM 1,4 dioxan as an internal chemical-shift reference, which was taken to resonate 3.741 ppm downfield of tetramethylsilane.

CD measurements

Far-UV CD measurements were performed in a Jasco 720 spectropolarimeter equipped with thermostatic cell holder, using cells with a pathlength of 1 mm. Sample concentrations varied between 0.1 and 0.2 mg/mL. Thermal unfolding of SK and SK fragments was monitored at 200 nm, measuring the appearance of the negative CD band typical of polypeptides in a disordered conformation. Maximal CD signal changes with temperature were observed at this wavelength for all fragments. In a typical experiment, the temperature of the sample was raised continuously by means of an external water bath, circulating water through the cell holder. The temperature inside the cell was measured continuously using a calibrated thermocouple. A similar cooling experiment was performed to monitor the refolding process. Fragment B was used in CD studies of the thermal unfolding experiments of the central domain of SK, whereas fragment B′, with five residues more (Table 1), was used in equivalent NMR experiments. No differences in behavior of these fragments could be detected by CD.

Gel filtration experiments

Samples of fragments were loaded on a 1-cm × 30-cm Superdex 75HR (Pharmacia/LKB) column attached to a Gilson HPLC instrument equipped with an automated sample injector. The buffer used was 0.1 M phosphate, pH 7.0, and the flow rate was 1 mL/min. The column was calibrated with gel filtration molecular mass standards (Sigma, UK and Biorad, UK) to estimate apparent molecular masses of eluting species. Elution profiles were recorded

by monitoring absorbance at 220 nm. Samples from the eluting peaks were collected and analyzed by SDS-PAGE according to Shägger and von Jagow (1987), using both Coomassie blue and silver staining of gels.

NMR measurements

One-dimensional NMR spectra were obtained using a 500-MHz Omega/GE instrument at the Oxford Centre for Molecular Sciences. Sample concentrations varied between 0.1 and 0.5 mM and 500–1,000 scans were acquired for adequate signal-to-noise ratios. Data points (8,000) were acquired with quadrature detection and a spectral width of 6,000 Hz, using a pulse angle of 60 degrees. Prior to Fourier transformation, spectra were resolution-enhanced by Lorentz–Gauss multiplication. For thermal unfolding experiments, NMR spectra were obtained at several temperatures in the range where unfolding was observed during CD experiments. The NMR probe temperature was calibrated using methanol and ethylene glycol standards.

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