Resistance of Soybean Trypsin Inhibitor (Kunitz) to Denaturation by Guanidinium Chloride*

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Viscosity measurements in 6 M guanidinium chloride (Gdm·Cl) (pH 5.2, 25°C) suggest that Kunitz soybean trypsin inhibitor (STI) undergoes a slow unfolding which requires over 2 weeks to reach completion. The reduced viscosity increased during this time from an initial value of 3.5 ml/g to a final value of 16 to 17 ml/g. At pH 7 and 25°C, over 4 weeks were required to reach the same final state. Gel chromatography of STI in 6 M Gdm·Cl yielded two peaks; one of equivalent hydrodynamic radius (Rg) equal to 38 Å and a second of Rg = 24 Å. Over a time interval of days, the quantity of STI of Rg = 38 Å progressively increased at the expense of material of Rg = 24 Å. Material from both peaks had the same molecular weight and amino acid composition as native STI, and after renaturing in dilute buffer, both totally inhibited trypsin at a molar ratio of 1:1. Exposure of native STI to 6 M Gdm·Cl also produced a slow disappearance of the CD absorption bands of STI in the near-ultraviolet to ultimately yield a featureless spectrum. Lower pH, by globular proteins and are characteristic of the spectra of disulfide cross-linked randomly coiled polypeptide.

In several respects, soybean trypsin inhibitor (Kunitz) is a rather eccentric water-soluble protein. Hydrodynamic measurements in 6 M guanidinium chloride (Gdm·Cl) suggest that Kunitz soybean trypsin inhibitor (STI) undergoes a slow unfolding which requires over 2 weeks to reach completion. The reduced viscosity increased during this time from an initial value of 3.5 ml/g to a final value of 16 to 17 ml/g. At pH 5.2 and 25°C, over 4 weeks were required to reach the same final state. Gel chromatography of STI in 6 M Gdm·Cl yielded two peaks; one of equivalent hydrodynamic radius (Rg) equal to 38 Å and a second of Rg = 24 Å. Over a time interval of days, the quantity of STI of Rg = 38 Å progressively increased at the expense of material of Rg = 24 Å. Material from both peaks had the same molecular weight and amino acid composition as native STI, and after renaturing in dilute buffer, both totally inhibited trypsin at a molar ratio of 1:1. Exposure of native STI to 6 M Gdm·Cl also produced a slow disappearance of the CD absorption bands of STI in the near-ultraviolet to ultimately yield a featureless spectrum. Lower pH, by globular proteins and are characteristic of the spectra of disulfide cross-linked randomly coiled polypeptide.

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hobited 1.8 mg of trypsin by the p-toluenesulfonyl-l-arginine methyl
liter assay. The ST1 preparation stabilized five times exhibited no
minor gel filtration components under denaturing conditions while
small amounts of other proteins (some with antitryptic activity)
were apparent in the twice crystallized ST1 (see Fig. 2).

Hydrodynamic Measurements — Viscosities were measured in size
75 Cannon-Manzini semimicroviscometers with nominal flow times
for water of 150 s. Flow times were measured with a Hewlett-
Packard autoviscometer at a temperature of 25.00 ± 0.005°.

Gel chromatography in 6 M GdmCl was performed on a 6% agarose column. Elution positions from the column were calibrated
with respect to equivalent hydrodynamic radii in the usual fashion
(11).

Spectral Measurements — CD measurements were made on a Cary
6 r spectropolarimeter equipped with a model 6002 CD attachment.
The CD was calibrated to the ORD (12) with doubly sublimed d-tolu-
camphorsulfonic acid. Spectra were measured on ST1 solutions of
about 1 mg/ml concentration. ST1 concentrations were estimated
determined by absorption using an E1% 1 cm of 10.6 ml g-1 cm-1 (13). A
value of 0.11 was used for the mean residue molecular weight of ST1
and was calculated from its amino acid sequence (6).

Ultraviolet absorption measurements were performed on a Cary
14 spectrophotometer. Difference spectral measurements between
ST1 in 8 M GdmCl and native ST1 employed a technique of solvent
matching similar to that of Herzovitz and Laskowski (14).

Other Physical Measurements — Molecular weights were estimated
by sedimentation equilibrium according to the long column meniscus
depletion method of Chervenka (15). Disc gel electrophoresis was at
pH 9 (16), at pH 3 in 8 M urea (17), or in SDS (18).

RESULTS

Hydrodynamic Properties of ST1 in Gdm · Cl — Most native
globular proteins exhibit negative CD ellipticity bands at 209
and 222 nm. Upon unfolding of the polypeptide these two
bands disappear and a single negative band at 200 nm is
produced. As mentioned earlier, the far-ultraviolet CD spec-
trum of native STI closely resembles that of an unfolded
polypeptide (2). This peculiar feature of ST1 rules out the use
of CD in the far-ultraviolet region as an initial means to detect
and interpret any changes which might occur in the organiza-
tion of the STI polypeptide backbone upon its exposure to
concentrated Gdm . Cl. We instead employed viscosity meas-
urements in the preliminary investigations. These viscosity
experiments were performed on the twice crystallized prepara-
tion of STI.

Fig. 1 shows the change in the reduced viscosity of ST1 in 6
M GdmCl, pH 5.2, as a function of time. The viscosity rapidly
increased from 3 ml/g to 7 ml/g, and then slowly increased over
a 2-week period to a final range of values which fell between 16
and 17 ml/g. When this experiment was performed at pH 7,
over 4 weeks were required for completion of the transition.

From the initial rapid increase in viscosity which was ob-
served upon placing ST1 in 6 M Gdm· Cl, it was first suspected
that a partially unfolded intermediate of STI was rapidly
formed. Later gel chromatographic (Fig. 2) and CD measure-
ments suggested instead that this initial, rapid viscosity in-
crease was a result of rapid unfolding by protein contaminants
in the twice crystallized ST1 preparation.

The length of time required for the transition undergone by
ST1 in 6 M Gdm· Cl as observed by viscosity suggested that gel
chromatography in 6 M Gdm· Cl could be employed to corrobo-
rate and perhaps extend the interpretation of the viscosity res-
ults. ST1 was incubated at 25° in concentrated Gdm· Cl, and
 aliquots were removed at intervals of several days and sub-
jected to gel chromatography in 6 M Gdm· Cl on a calibrated
6% agarose column. Typical results are illustrated in Fig. 2.
Initially, a zone which eluted at a position corresponding to an
Rf of 24 A was the major component (Fig. 2A), with a species of
Rf = 38 A also present in limited amounts. Longer incubation
of ST1 in the denaturant before subjection to gel chromato-
graphy produced an increase in the amount of 38-A material and a
concomitant decrease of the 24-A zone (cf. Fig. 2, B and C).

Subjection of ST1 in concentrated Gdm· Cl to 70° for 15 min

![Fig. 1. Change in the reduced viscosity of ST1 in 6 M Gdm· Cl, pH 5.2 and 25°. The concentration of ST1 in this solution was 3.2 mg/ml. Instrumentation and methodology are given under "Experimental Procedures."](image-url)
yielded only the 38-Å species (Fig. 2D). Minor components present in twice crystallized STI (Fig. 2D) were totally absent in material crystallized five times, and only the 24- and/or the 38-Å protein species were observed. Protein from each chromatographic peak had the same amino acid composition as native STI and exhibited the same sodium dodecyl sulfate-gel electrophoretic mobility as STI exposed directly to sodium dodecyl sulfate. The molecular weight of each chromatographic species as determined by sedimentation equilibrium in 6 M Gdm·Cl without reduction was 21,000 (STI molecular weight from its amino acid sequence is 20,100). The sedimentation equilibrium results rule out the possibility that the 38-Å chromatographic species might be a dimer of STI formed through disulfide interchange, and support the conclusion that the increase in the hydrodynamic volume of STI results from an unfolding of its polypeptide chain. Removal by dialysis of the Gdm·Cl from either the 38-Å or the 24-Å chromatographic species yielded a soluble product of $M_r = 22,000$ as estimated by sedimentation equilibrium. In dilute buffer, each chromatographic species inhibited trypsin activity 100% when preincubated with trypsin on a gram per g basis (1.2 mol of STI/mol of trypsin). Samples of STI which were heated to 70° in 6 M Gdm·Cl to produce only the 38-Å chromatographic species of 38-Å radius from Gdm·Cl gel chromatography and for STI heated at 70° for 15 min in 6 M Gdm·Cl.

When STI from either the 24-Å or the 38-Å gel chromatographic zones or STI which had been heated in 6 M Gdm·Cl was returned to dilute buffer, its CD spectrum returned to that exhibited by the native state. When refolded STI was re-exposed to 6 M Gdm·Cl, it again underwent the slow unfolding as monitored by CD and gel chromatography.

Difference spectra were obtained for STI soon after its exposure to 6 M Gdm·Cl and again after its CD spectrum ceased to change. In each case, native STI served as the reference. For the sample in Gdm·Cl for only a short period of time (<4 days), a red shift was observed in its absorption spectrum. While STI which has completed its CD spectral change in 6 M Gdm·Cl exhibited the blue-shifted absorption spectrum which is typical of denatured proteins (9), STI heated in 6 M Gdm·Cl or STI from the 38-Å gel chromatographic zone also exhibited the blue-shifted absorption spectrum.

Spectral Measurements—Fig. 3, Curve 1, is the CD spectrum we observed for native STI and which agrees with published spectra (2, 5). Upon exposure of STI to 6 M Gdm·Cl, the broad, negative ellipticity band between 290 and 250 nm exhibits a slight increase in amplitude (Fig. 3A, Curve 2), while the positive ellipticity band at 226 nm undergoes a 3-fold increase in amplitude (Fig. 3B, Curve 2). STI from the gel chromatographic elution zone which corresponds to an $R_c$ of 24 Å possesses a CD spectrum similar to Curve 2 of Fig. 3.

As the time of incubation in the denaturant increases, the amplitudes of the ellipticity bands at 275 nm and 226 nm decrease. Curve 3 of Fig. 3 illustrates one of these intermediate spectra. This sample had been in 6 M Gdm·Cl, pH 7.35 and 25°, for about 15 days. Changes in the spectrum continue until the final state, as illustrated by Curve 4, Fig. 3, is reached. CD spectra identical with Curve 4 are also observed for the STI species of 38-Å radius from Gdm·Cl gel chromatography and for STI heated at 70° for 15 min in 6 M Gdm·Cl.

The presence of reducing agent enhanced the rate of unfolding of STI in 6 M Gdm·Cl. In the presence of 0.1 M 2-mercaptoethanol at pH 8.5 and 25°, the unfolding of STI was complete in about 12 h. Carboxymethylation of this product and subsequent gel chromatography in 6 M Gdm·Cl yielded a single

![Fig. 3. Ultraviolet CD spectra of native ST1 and of ST1 in 6 M Gdm·Cl. Five times crystallized STI was utilized at a concentration of 0.778 mg/ml. Curve 1, native ST1 in 0.1 M KCl, pH 7.35. Curve 2, ST1 in 6 M Gdm·Cl, pH 7.35, at 25° for ~4 h. Curve 3, ST1 in 6 M Gdm·Cl, pH 7.35, at 25° for 15 days. Curve 4, ST1 in 6 M Gdm·Cl, pH 7.35, at 70° for 15 min then returned to 25°. A, near-ultraviolet CD spectra. Cell path length was 1.0 cm. B, far-ultraviolet CD spectra. Cell path length was 2.0 mm.](http://www.jbc.org/)

[Downloaded from http://www.jbc.org on August 29, 2017]
Because of the atypically slow rate of unfolding of STI, it was possible to employ gel chromatography to corroborate the viscosity results. Gel chromatography was utilized to effect both a function of time (Fig. 2). The final state observed by gel chromatography is also consistent with that of a disulfide cross-linked polypeptide chain, since combination of the estimated hydrodynamic radius, 38 Å, and the molecular weight, 21,000, yields an estimate of 17 ml/g for the intrinsic viscosity of this chromatographic species (estimated by Equation 23-5 of Ref. 20). The early appearing gel chromatographic species observed in these experiments, \( R_e = 24 \) Å, most probably represents a reaction zone which is composed of native STI together with small amounts of the unfolded state. The small fraction of native STI which would unfold while the sample is passing through the gel column would be sufficient to shift the elution position of this zone from that expected for the native state, \( R_e = 21 \) Å, to a position of apparent \( R_e = 24 \) Å. The CD spectrum of material from the zone of \( R_e = 24 \) Å (Fig. 3, Curve 2) would argue against the possibility that this zone represents a rapidly formed intermediate state of less organized structure which is on the pathway between the native and unfolded states of STI.

Spectral measurements in the near-ultraviolet region confirm our interpretation of the hydrodynamic data. The CD ellipticity bands generated by the asymmetric environments of the aromatic amino acid side chains should be effaced upon a complete unfolding of the polypeptide chain. This is what we observed for STI; the amplitude of its near-ultraviolet CD bands diminish concomitantly with an increase in the hydrodynamic size of the molecule. The final state of unfolding, as observed hydrodynamically, is characterized by a featureless CD spectrum and an ultraviolet difference spectrum typical of structureless polypeptides.

It is worthwhile to note that because the unfolding of STI is so slow, it is possible to observe directly the effect of concentrated Gdm-Cl on the absorption intensity of the native protein. The allowance for this effect must generally be made by extrapolation of physical parameters from experimental points outside the denaturation transition zone (e.g., Figs. 1 and 2 of Ref. 21).

A limited examination of the refolding of STI emphasizes two additional points. First, when returned to dilute buffer, disulfide cross-linked, unfolded STI rapidly refolds to a conformation whose biological and physical properties are indistinguishable from native STI. Second, the unfolding behavior of STI is an intrinsic property of the protein since the re-unfolding of STI (purified in the unfolded state; then refolded) proceeds as slowly as it did the first time the protein was unfolded.

Our experimental data are not sufficiently detailed to permit identification of the amino acid side chain(s) responsible for the 226 nm ellipticity band of native STI. The absorption intensity of this band is initially greatly enhanced when STI is placed in the concentrated denaturant (cf. Curve 2, Fig. 3B), and investigations by others strongly suggest that this band is generated by one or more tyrosines in the molecule. Based upon their study of the effects of chemical modification of tyrosyl and tryptophanyl residues of STI on its CD spectrum, Baba et al. concluded that the tyrosyl residues give rise to the 230 nm band (24). Bowman-Birk soybean trypsin inhibitor which possesses no tryptophanyl residues (24) also exhibits a CD spectrum qualitatively nearly identical with STI (24). Kay demonstrated for this soybean trypsin inhibitor that the CD band in the 230 nm region is the \( L_a \) CD band of the tyrosyl side chains (24).
Resistance of STI to Denaturation by Guanidinium Chloride

In agreement with observations by Kunitz (25) or Wu and Scheraga (26) for STI in dilute buffer and by Edelhoch and Steiner (1) for STI in concentrated urea, we observe that the rate of STI unfolding in 6 M Gdm·Cl is increased at lower pH and/or higher temperatures. Both disulfide bonds are located on the surface of STI (7); their presence apparently furnishes considerable structural stabilization to the native molecule, since the rate of unfolding of STI is increased manyfold under reducing conditions.

Thus, STI may be added to a relatively short list of water-soluble globular proteins which have been demonstrated to be unusually stable to the effects of denaturants such as Gdm·Cl. Among these proteins are included a protease inhibitor (24), the subtilisin enzymes (27, 28), and uricase (29). The exceptional conformational stability of these proteins may be a manifestation of domains within the molecules which are intrinsically quite stable and which, in turn, limit solvent accessibility primarily to the peripheral regions of the globular structures. Certainly the hydrogen isotope exchange properties of STI (30) and the subtilisin enzymes (31) are interpretable in terms of this model (30).

It is not surprising that broadly specific proteases, as potentially their own substrates, might possess a more stable native structure that would preclude, or diminish, self-inactivation. Similarly, those protease inhibitors which are themselves proteins, would require exceptional preservation of native structural integrity. Otherwise, sites on the inhibitor other than the protease-binding site would be readily available for proteolytic attack and subsequent inhibitor inactivation.

REFERENCES
