Structural Transitions of Soybean Trypsin Inhibitor

II. THE DENATURED STATE IN UREA

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(Received for publication, August 1, 1962)

In the companion paper of this series data have been presented on the properties of soybean trypsin inhibitor in water (1). In the present paper the studies have been extended to the effect of high levels of urea upon this protein.

Few processes in biochemistry have been the subject of so many publications as the action of concentrated urea solutions upon proteins. Nevertheless, there is no agreement in detail as to either precisely what happens or the mechanism of action. Current thinking has swung sharply away from the traditional picture that urea acts by virtue of its competitive rupture of intramolecular hydrogen bonds (2-4). As to the physical state of proteins in urea, many investigators have accepted the view that their state approaches that of a completely unorganized random coil, restrained only by primary cross-linkages, such as -S-S- bridges (5).

However, in three cases for which this question has been examined carefully, fluorescence polarization and other data have revealed the existence of definite residual structure in 9 M urea (6-8). In all three cases perturbations of the residual refractory structure can be produced at extremes of pH or at elevated temperatures.

It is the purpose of the present paper to examine the properties of soybean trypsin inhibitor in concentrated urea, with particular regard to the presence of residual structure and the dependence of the latter upon pH and temperature.

EXPERIMENTAL PROCEDURE

The details of the various physical measurements have been described in the preceding paper (1). Urea was recrystallized once from ethanol before use.

The solubility determinations were made according to the procedure of Kunitz (9) with some minor modifications. The reaction was stopped and denatured protein simultaneously precipitated by the addition of 7 volumes of an acetate buffer which resulted in a final pH of 4.6 and ionic strength of 0.27. The solution was then allowed to stand at room temperature for about 2 hours to allow complete precipitation of denatured protein. The concentration of native STI1 was determined from the absorbancy of the supernatant after removal of the precipitate by centrifugation.

RESULTS

Properties of Soybean Trypsin Inhibitor in Urea

Fluorescence Polarization—At 25° and neutral pH the relaxation time of STI in 9.0 M urea is reduced from that found in water under the same conditions. Assuming a value of 0.8 × 10−4 for the excited lifetime (6), ρ2 is equal to 0.7 × 10−8 (±10%). A value of 2.5 × 10−4 was found in 0.30 M KCl at pH 6.6 (1). Although substantial, the relative decrease is less than has been observed in the cases of several other proteins (6, 7). The value of ρ2 is much too large to be consistent with a completely disorganized, randomly coiled configuration under these conditions.

Examination of the thermal dependence of polarization reveals that 1/P + 1/3 varies linearly with T/μ up to about 40°. At higher temperatures, a definite upward curvature is present (Fig. 1). This is consistent with, and suggests, a thermal rupture of weak linkages at elevated temperatures, resulting in an enhanced molecular flexibility. This is in harmony with other evidence which indicates that further molecular changes occur at high temperatures in 9 M urea.

The molecular state of STI in urea appears to be fairly refractory to acid pH with regard to its internal rigidity. Thus, essentially no change in polarization accompanies a shift in pH from neutrality to 2.8. A marginal decrease (2%) occurs after standing at pH 2.8 for 2 hours.

In contrast, important changes in polarization occur at alkaline pH values. The polarization at 25° decreases above pH 9 (Fig. 2) and time-dependent changes become evident above pH 12. The alkaline pH-dependence of polarization in 9 M urea shows changes comparable in magnitude to those observed in aqueous media (1).

Viscosity—The decrease in relaxation time observed in the presence of 9 M urea suggests that internal degrees of rotational freedom have been introduced as a result of the action of this medium. This would be expected to accompany any major unfolding of the coiled polypeptide backbone. It is, of course, also possible that the decrease in relaxation time may arise in part from a transition to a state of greater molecular symmetry.

To confirm the result obtained by fluorescence polarization and to differentiate between the above possibilities, the intrinsic viscosity of STI was determined in water and in 9 M urea (Fig. 3). A significant increase occurs. The combined results of polarization and viscosity studies therefore suggest strongly that an expansion of the molecular domain occurs in 9 M urea, accompanied by an important loss of internal rigidity.

The low magnitude of the increase in viscosity, in comparison with those observed in the cases of other proteins, may reflect the existence of regions whose tertiary structure is refractory to urea, or the presence of a high degree of cross-linking by S—S bridges.

In any event it is clear that the molecular state of STI in 9 M
Urea does not approach the limiting case of a random, completely unorganized, coil. Considerable residual structure remains. This is lost only under more drastic conditions, as will be discussed in subsequent sections.

**Ultraviolet Fluorescence**—The addition of urea to ST1 results in a progressive enhancement in the intensity of tryptophan fluorescence at 350 mp (Fig. 4). The relative intensity varies almost linearly up to urea levels of 9 M. This kind of variation is, in fact, not dissimilar to that anticipated for an inert solvent effect. Teale (10) has described similar behavior for several proteins in mixtures of water and organic solvents of lower polarity.

The acid pH dependence of the ultraviolet fluorescence of ST1 in 8 M urea is essentially similar to that found in water, except for a small alkaline displacement of the zone of transition (Fig. 5). Apparently, whatever molecular events are responsible for the exaltation observed in water at acid pH persist in the
presence of high levels of urea. However, the change in relaxation time observed in water appears to be suppressed under these conditions since very little change in relaxation time was observed between pH 4.4 and neutrality in 8 M urea and 0.30 M KCl.

The alkaline branch of the pH profile of the fluorescence intensity in 9 M urea shows a progressive quenching, which begins at about pH 9.5. Immediate back titration from pH values as high as 12.0 results in almost complete coincidence with the forward branch (Fig. 6). Comparison of the reversible portion of the curve in urea with that in water reveals an alkaline displacement of the former amounting to about 0.9 pH unit. This almost certainly reflects the intrinsic solvent effect of urea upon the phenolic ionization (11).

At about pH 11.5, slow increases in intensity with time begin to be perceptible. These become rapid above pH 12 and will be discussed in later sections.

Spectrophotometric Titration of Tyrosyl Residues—The ionization of the tyrosines of ST1, as monitored by the increase in absorbancy at 295 mp, is also shown in Fig. 6. The data, when properly normalized, are almost coincident with the fluorescence-quenching profile. Both are displaced by about 0.9 pH unit to the alkaline of the corresponding curves in water. This is in harmony with expectations if quenching arises from radiationless exchange between tryptophan and ionized tryosine.

The observation that the effect of urea upon the reversible part of the ionization curve of tyrosine can be accounted for by a purely solvent action (11) suggests that no important change in the environment of the tyrosines has been produced by the action of urea at neutral pH.

Above pH 12, time effects occur which parallel those observed for the tryptophan fluorescence.

Ultraviolet Difference Spectra—The difference spectra of ST1 produced by concentrated urea solutions at pH 7.0 does not result in a blue shift, as found with many other proteins (12-14). Instead, a strong red shift occurred with peaks at 280, 286, and 292 mp (Fig. 7). Moreover, the magnitude of the increase in absorbancy varied linearly with the concentration of urea up to values of 9 M (Fig. 8).

The increase in absorbancy in urea is in the direction expected for the effect of an increase in refractive index of the solvent on the chromophores exposed to the solvent (15, 16). In fact, the data show no evidence of any transition resulting from the loss of intramolecular interactions.

In contrast to concentrated urea solutions, mixtures of ethylene glycol and water are considered to be without significant influence on the structure of proteins (15, 17). As illustrated in Fig. 7, the difference spectrum produced by 53% ethylene glycol (by volume) is quite comparable to that induced by 8.9 M urea. A linear increase in absorbancy was observed also with increasing concentrations of ethylene glycol (Fig. 8).

Optical Rotation—The specific levorotation of ST1 was essentially unchanged from its value in water when the urea concen-
Denaturation was increased to 8.7 M at neutrality, after corrections were made for the refraction of the solvent. Moreover, the enhancement in specific rotation observed in water at pH values below 6 (1) occurs to approximately the same extent in 8.7 M urea (Fig. 5).

Rates of Denaturation

**Influence of pH**—Under the conditions of the previous section, the various properties of STI that were evaluated showed no time dependence and were reversible. However, as the pH approaches ~12, slow changes in all the molecular properties of STI become evident. The rate increases with pH and by pH 13 is quite rapid.

Denaturation is here given its classical meaning, namely the loss in solubility at the isoelectric point of the protein. STI loses its characteristic solubility properties near pH 12 at rates which are readily measurable at 25°. The rate of denaturation follows first order kinetics, as evidenced by the linear variation of the logarithm of the fraction of soluble material with time (Fig. 9). The rate constants at several pH values are plotted in Fig. 10. It can be seen that the rate of denaturation in 9 M urea in strong alkali increases with the 0.96th power of the hydroxyl ion concentration.

The reduced specific viscosity of STI in 9 M urea shows no significant change with pH at 25° until the pH is raised to the zone where denaturation occurs. Between pH 12 and 13, the rate of increase in viscosity may be followed readily and the process has been found to conform to first order kinetics. The reduced specific viscosity at zero time did not change from its value at neutrality. The first order velocity constants obtained by viscometry, at three pH values, are in complete agreement with those found by loss in solubility (Fig. 10). When the pH of a solution of STI in 9 M urea was reduced to 2.85, only a very slow increase was observed which extrapolated at zero time to the value found at neutral pH.

In contrast, a significant fall in polarization occurs before the first measurement can be made (Fig. 2). Subsequently, a time-dependent decrease occurs which obeys first order kinetics. The rate constants obtained from polarization of fluorescence data at pH 12.53 and 12.85 in 9 M urea agree with those obtained by solubility and viscosity (Fig. 10).

As noted in the previous section, the fluorescence of the tryptophan residues in STI is markedly quenched between pH 10 and 12, without apparent time effects. However, at more alkaline pH values, time-dependent increases in fluorescence intensity occurred, the rates of which obeyed first order kinetics. The net increase in intensity at pH 12.8 was approximately equal to the loss encountered between neutrality and this pH at zero time. As can be seen in Fig. 10, the rates obtained by fluorescence agreed with those of the other methods studied.

It has been shown that a fraction of the tyrosyl residues in STI may be titrated reversibly in 9 M urea. At pH values above ~12, time effects became significant and an appreciable hysteresis was found between the forward and reverse titration curves. If the absorbancy as a function of time is observed at a constant pH above 12, a rate curve is obtained which reveals the rate of ionization of those tyrosyl residues which have not been titrated at zero time. The rate must be followed to completion of the reaction, since the final value of the absorbancy shows a significant increase between pH 12 and 13. For slow rates, the reaction curves were heated briefly at 50° to obtain an accurate final value of the absorbancy within a reasonable time. The rate data were found to follow first order kinetics (Fig. 11) and the velocity constants agreed with those reported above (Fig. 10).

The specific rotation of STI in 9 M urea is essentially the same as in water between pH values 2 and 11. At pH 13.0 in 9 M urea, an increase in specific rotation occurred, the rate of which followed first order kinetics. The total increase in levorotation was of about the same magnitude as that which occurs in acid between pH 6 and 2. The velocity constant fell fairly close to the curve determined by the other methods (Fig. 10). The small discrepancy probably results from the failure to control the temperature rigorously at 25°, as was done in the other experiments.

Since strong alkali can lead to fission of primary linkages and concomitant changes in molecular size, the hydrolysis of STI was evaluated by several procedures. It was found that no increase in absorbancy of the supernatant remaining after precipitation with pH 4.6 buffer occurred if fully denatured STI was allowed to stand at pH 12.85 for 15 hours at 25°. Appreciable
hydrolysis would have led to the formation of soluble, low molecular weight polypeptides. In addition, the viscosity did not decline within 1 hour after it had reached a maximal value at pH 12.85. Similarly, light-scattering measurements at pH 13 in 9 M urea showed only a trivial decrease (~7%) in reduced intensity after 2 hours, whereas the denaturation was complete within 15 minutes.

Effect of Temperature: 1. Alkaline pH: The effect of temperature on the rate of denaturation in 9 M urea at pH 12.85 was determined by spectrophotometry at 295 nm. At 18°C, the rate was too slow for convenient measurement. At somewhat higher temperatures, the rates increased rapidly and the data fit first order kinetics. The first order velocity constants showed a linear dependence on reciprocal temperature (Fig. 12). A heat of activation of 44 kcal per mole was calculated from the slope, by application of the theory of absolute reaction rates (18). Other thermodynamic activation parameters are reported in Table I.

2. Neutral pH: The influence of temperature on the rate of denaturation at neutral pH was investigated by several independent methods. Rates were determined at three temperatures by flow time measurements in a viscometer. The data fit first order kinetics (Fig. 11) and the velocity constants are reported in Table I and Fig. 12. The kinetics of the transition were also followed by the increase in tryptophan fluorescence (Fig. 11). The velocity constant computed from a first order plot of the data was in good agreement with that obtained by viscosity. As will be reported in the next section, the difference spectrum of thermally denatured ST1 shows major blue shifts for the principal peaks of both the tryptophanyl and tyrosyl residues. The rate of change in absorption at 298 nm was determined at 55°C (Fig. 11). The velocity constant was in complete accord with those found by tryptophan fluorescence and viscosity. As is evident in Fig. 12, the temperature dependence of rate was the same at neutral pH as in strong alkali, although the free energy of activation was greater at neutrality. The possibility of the complication of these results by aggregation arising through disulfide exchange under these conditions must of course be considered. Light-scattering measurements for a 1% ST1 solution in 9 M urea and 0.1 M KCl (pH 7) showed only a marginal (3%) decrease in reduced intensity after 15 minutes at 55°C (measured at 25°C). Thus it is unlikely that aggregation is an important factor under these conditions.

It is worthy of mention that, as Table I shows, the entropy of activation is higher at alkaline pH than at neutral pH, although the difference is not great. Speculation on this point is of limited value without much more detailed information as to the state of the molecule under these conditions. One obvious explanation is that the initial states at zero time are different for the two conditions of alkaline pH at room temperature and neutral pH at elevated temperature. The apparent entropy of activation is a complicated function of the state of ionization, the hydration, and the molecular configuration of the initial and activated states. Since all three of these may change with pH it is not

![Fig. 11. First order velocity plot: △, absorption at 295 nm, pH 12.80, temperature = 25°C; ○, difference absorption at 298 nm, pH 7.0, temperature = 50.0°C; ●, fluorescence at 350 nm, pH = 7.0, temperature = 55.0°C. All solutions contained 9 M urea (0.01 M phosphate and 0.027 M NaCl).](http://www.jbc.org/content/248/5/935/f1.large.jpg)

![Fig. 12. Effect of temperature on the first order velocity constant, k (in reciprocal minutes), of ST1 denaturation at (a) ●, pH 12.80, as determined by absorption at 295 nm and (b) ○, at pH 7.0, as determined by viscosity; △, by fluorescence intensity at 350 nm; □, and by difference spectrometry at 298 nm. All solutions contained 0 M urea (0.01 M phosphate and 0.027 M NaCl). Top abscissa, ○; bottom abscissa, other symbols.](http://www.jbc.org/content/248/5/935/f2.large.jpg)

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* Kilocalories per mole.
† Calories per mole per degree.

Table I

Activation parameters of denaturation of soybean trypsin inhibitor

All solutions contained 9.0 M urea, 0.01 M phosphate, and 0.027 M NaCl. Data at high pH were obtained by absorption measurements at 295 nm. Data at neutrality obtained by viscosimetry.
surprising to find that the entropy of activation is pH-dependent. It is, for example, possible that the activation step at alkaline pH (but presumably not at neutral pH) may involve an ionization of a "masked" tyrosine which would make an entropic contribution not present at neutral pH.

**Properties of Thermally Denatured Soybean Trypsin Inhibitor**

As reported in the foregoing section, ST1 is denatured at neutrality in 9 M urea at temperatures between 40 and 60°. Major changes occur in the molecular properties of ST1 on denaturation which are not reversed on cooling to 25°. Therefore the molecular parameters of denatured ST1 may be measured without disturbance from any equilibrium between native and denatured forms. Thermally denatured ST1 was prepared by heating at 60° for 20 minutes.

**Polarization of Fluorescence**—The dependence of the polarization of ST1 on temperature is shown in Fig. 1. In contrast to the data on undenatured ST1 a linear relation was obtained throughout the temperature range investigated. It is evident from the data in Fig. 1 that a considerable decrease in relaxation time occurred with thermal denaturation. It should be noted, however, that the polarization still shows a significant dependence on temperature, indicating the existence of residual structural elements. A value for $P_{n0}$ of $0.3 \times 10^{-6}$ was computed from the data.

**Viscosity**—In harmony with the enhanced flexibility revealed by polarization measurements, a major increase occurred also in the viscosity of thermally denatured ST1 in 9 M urea. An intrinsic viscosity of 0.15 was found at pH 7.0 (Fig. 3). The final reduced specific viscosity of ST1 in 9 M urea, determined from the rate measurements in alkali (pH $>12$), was close to 0.28 at 1.2% protein. Evidently a further increase in the molecular domain of ST1 occurs in strong alkali, possibly because of the increased charge.

**Spectrometric Titration of Tyrosyl Residues**—The ionization of the tyrosyl residues, as measured spectrophotometrically at 295 m$_u$, is shown in Fig. 13. In contrast to the titration curve of undenatured ST1 in the same solvent, no time effects were observed and the data were completely reversible from pH 12.6. The titration data of undenatured ST1 are reproduced also in Fig. 13 and the marked reduction in the apparent pK of this group is evident. Moreover, the titration curve of denatured ST1 follows, almost, that of a univalent ion, indicating that the electrostatic work term has been reduced to a negligible value. The midpoint of the curve occurs close to pH 11.0 which agrees very well with the apparent pK of the phenolic hydroxyl group in 9 M urea (11). If a value of 2550 (19) is assumed for the increase in molar absorbancy at 295 m$_u$ for ionized tyrosine, then five tyrosines per mole are present in ST1.

**Fluorescence**—In contrast to the profound quenching of ST1 fluorescence that occurs in 9 M urea, a reduction of only about 10% takes place with thermally denatured ST1 in the same solvent. However, the pH dependence parallels the tyrosine ionization curve, again suggesting that the principal mechanism of quenching occurs through radiationless energy transfer between tryptophan and ionized tyrosyl residues. Complete reversibility was found in the fluorescence curve (Fig. 13).

**Difference Spectra**—The difference spectrum of thermally denatured ST1 in 9 M urea relative to unheated ST1 in the same solvent is illustrated in Fig. 14. The spectrum was very different from that observed in water at low pH (1). The absorption at the characteristic peaks, 279 and 285 m$_u$ of the tyrosyl residues, and 291 m$_u$ of the tryptophan groups, all show strong decreases or blue shifts that are several times greater than the changes which occur at acid pH in water. The tryptophanyl residue now shows both a blue shift (291 m$_u$) and a red shift (298.5 m$_u$). This dual behavior of the tryptophan group often characterizes the absorption changes where profound molecular unfolding accompanies the denaturation, as with pepsin and lysozyme (20, 21). In the preceding section, the rate of decrease of absorption at 298 m$_u$ was used to measure the rate of thermal denaturation.

**DISCUSSION**

A most salient feature of the results cited in the preceding sections is the remarkable resistance displayed by several properties of soybean trypsin inhibitor at neutral pH and 25°, to the...
action of such a powerful denaturant as urea. High levels of
urea produce only a relatively minor change in the internal
rigidity and shape of this protein. Moreover, the nature of the
influence of urea upon the difference spectra and tryptophan
fluorescence of ST1 is such as to suggest that the changes in
chromophore environment are largely of the kind often referred
to as solvent effects. Indeed, the difference spectra induced by
high levels of urea are very similar to those produced by the
addition of the largely indifferent solvent, ethylene glycol.

The molecular changes that occur between neutrality and
pH ~11.5 appear to involve only minor structural perturbations
which are completely reversible. Of all the properties examined,
only the polarization of fluorescence shows an instantaneous
change in its properties. Similar results have been found with
ST1 in 0.30 M KCl (1). With bovine thyroglobulin and rabbit
antibodies (6, 7), the polarization changes precede and anticipate
those changes in molecular form measurable by solubility,
optical rotation, viscosity, etc. It seems not unlikely that
fluorescence polarization measurements are particularly re-

cursive to microscopic changes in structure, such as increased
torsional motions of polypeptide segments which, although bound
to the protein, are not completely fixed.

Above pH 12, time effects become increasingly important.
Between pH 12 and 13, the rates change from a fairly slow to a
rapid reaction and show almost a first order dependence on the
concentration of hydroxyl ion. The reaction has been found to
obey unimolecular kinetics when followed by six different tech-
niques, as enumerated earlier. Because the various methods
respond to different molecular properties, it would appear (since
the rates are the same by all the methods) that the reaction
occurs between essentially two well defined states of the mole-
cule, i.e. an initial and final one. Intermediate states, of suffi-
cient stability to be observable by any of the methods evaluated,
do not appear to exist. Of interest is the fact that the initial
state of ST1 in 9 M urea is significantly different from its state
in aqueous media, as indicated by changes in relaxation time,
viscosity, and reactivity of the tyrosyl groups to iodination. Moreover, this state appears to be largely invariant to pH be-
tween 6 and 11.5, apart from the minor decrease in polarization
observed in alkali above pH ~10.

The denatured state of ST1 in 9 M urea shows a marked de-
crease in rigidity and increase in effective molecular volume
in comparison with the initial state in the same solvent. The pro-
nounced increase in viscosity can only result from a major fur-
ther unfolding of the polypeptide chains. Although a consider-
sable decrease in relaxation time occurs, the denatured form of
ST1 appears to retain some internal rigidity since it still possesses
a nontrivial polarization (and relaxation time). The depend-
ence of this residual structure upon disulfide bridges remains to
be assessed.

Consistent with the extensive molecular disorganization, re-
vealed by measurements which depend on the overall dimensions
of the molecule, are the changes in those properties which respond
to variations in the local environment of specific residues. The
difference spectra of thermally denatured ST1 reflect a profound
change in the absorption of the tryptophanyl and tyrosyl chromo-
phores. The absorption changes correspond to a strong blue
shift in the spectra and are similar to those encountered in
several other denaturation reactions where extensive structural
loss occur. These pronounced blue shifts are in strong contrast
to the small red shift of the tryptophan band seen in aqueous
media on acidification of ST1 from neutrality. In addition,
only a very minor alkaline quenching of tryptophan fluorescence
occurs in the denatured state of ST1 when compared to the
native state. The reduction in radiationless energy transfer is
quite compatible with the increase in effective volume shown by
the denatured form of ST1, which should result in enhanced
separation of tryptophan and tyrosine groups.

Perhaps the major conclusion arising from the preceding re-
results is that the molecular state of ST1 is by no means reduced
to that of an unorganized polypeptide at high levels of urea.
A definite residual structure survives the action of 9 M urea and
requires elevated temperatures or strongly alkaline conditions
for its dissolution.

A secondary finding is that the intensity of tryptophan fluo-
rescence can serve as a convenient and sensitive means of moni-
toring the rate of protein denaturation. Rates determined in
this way agree well with those found by other techniques.

SUMMARY

A relatively small change in the molecular domain of soybean
trypsin inhibitor occurs in 9 M urea, as evidenced by viscosity
and polarization of fluorescence measurements. Increasing con-
centrations of urea to 9 M produce only a red shift in the difference
absorption spectrum and a uniform increase in fluorescence inten-
sity of soybean trypsin inhibitor. At high temperatures or
at alkaline pH, a transition to a largely unorganized structure
occurs. In this change a blue shift develops in the difference
spectrum and major changes in viscosity and polarization of
fluorescence take place. Rates of denaturation have been mea-
sured by a number of unrelated procedures. The reaction follows
first order kinetics and the rate is the same for all methods under
a given set of environmental conditions. It has been suggested,
therefore, that the rate is determined by a single process between
an initial and final state.

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