DENATURATION OF PROTEINS IN 8M UREA AS MONITORED BY TRYPTOPHAN FLUORESCENCE: TRYPSIN, TRYPSINOGEN AND SOME DERIVATIVES*

by

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Tryptophan residues in proteins fluoresce in a broad band with a maximum at approximately 340 nm when excited with 290 nm ultraviolet light. Since the intensity of fluorescence as well as the location of the emission peak is changed when the immediate environment around the fluorescing residues is altered, protein fluorescence has been used as a sensitive monitor of conformational changes in the protein (Steiner et al, 1964). We previously reported that the denaturation of chymotrypsin, chymotrypsinogen and some chymotrypsin derivatives in 8 M urea is a time-dependent reaction which can be conveniently followed by measurement of the protein fluorescence (Hopkins and Spikes, 1967). The rate of denaturation examined in this way follows apparent first-order kinetics and the calculated rate constant is a measure of the stability of the protein to 8 M urea.

The present paper describes similar studies using trypsin, trypsinogen (TG), diisopropyl-fluorophosphate treated trypsin (DIP-T) and 1-chloro-2-

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tosylamido-7-amino-2-heptanone treated trypsin (TLCK-T). DIP-T is an inactive form of trypsin produced by treatment with diisopropylfluorophosphate; the inhibitor reacts with a serine residue located in the active site region of the enzyme (Dixon et al., 1958). TLCK-T is an inactive derivative of trypsin in which the reagent has reacted with a histidine residue located in the active site (Shaw et al., 1965).

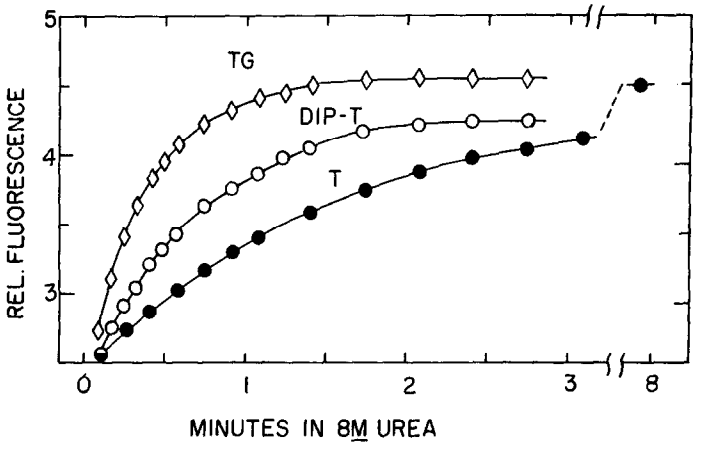

Figure 1. The time-course of the fluorescence transitions of trypsin(T), trypsinogen (TG), and DIP-trypsin (DIP-T) in 8 M urea at 25.6°C. The solutions contained 0.5 mg/ml protein and were buffered with 0.25 M sodium phosphate, pH 7.2. The fluorescence was measured at 356 nm using 290 nm exciting light.

Like the chymotrypsin family, the above proteins in buffered solutions displayed a red-shift in the emission peak from 341 nm to 356 nm and an increase in fluorescent intensity when the solution contains 8 M urea. No shift in fluorescence was observed in 5 M urea at pH 7.2 and 30°C. The rate of the fluorescence shift increased rapidly as the urea concentration was increased from 6 M to 8 M. The urea denaturation of trypsin, DIP-T, and TG was followed by measuring the rate of appearance of the 356 nm emission peak as shown in Fig. 1. The denaturation reactions were all apparent first-order and rate constants (k) for the denaturation of

trypsin, TG, DIP-T, and TLCK-T in 8 M urea were calculated. A_S may be seen in Table I, TG and DIP-T are denatured more rapidly than trypsin. These results are in sharp contrast with studies on the denaturation of the chymotrypsin family in 8 M urea where the active enzyme is denatured more rapidly than the zymogen and the diisopropylphosphate derivative (Hopkins and Spikes, 1967).

Table I

First-Order Fluorescence Transition Rate Constants of Trypsin, Trypsinogen and Some Derivatives in 8 M Urea.

	T	TG	DIP-T	TLCK-T
$k(min^{-1})$	1.1	4.5	3.1	>100

Solvent: 0.25 M phosphate, pH 7.2, temperature 28°C

Using differential spectrophotometry at 293 nm, Delaage and Lazdunski (1967) observed a first-order denaturation of trypsin and DIP-T in 7.4 M urea at 20°C under pH conditions slightly higher than ours. It is somewhat difficult to compare our data with theirs because of differences in pH and urea concentration, but our rate constants for trypsin and DIP-T at 20°C are the same order of magnitude as theirs, and the relative rates of trypsin to DIP-T are in excellent agreement.

The stability of trypsin to denaturation in 8 M urea was examined during the course of inactivation of the enzyme with 1-chloro-2-tosylamido-7-amino-2-heptanone (TLCK).

When trypsin was placed in an excess of TLCK there was an immediate three-fold increase in the rate of its fluorescence transition in 8 M urea (see Fig. (2), Curve 1) even though the enzymatic activity with benzoyl-L-arginine-ethylester was not affected at this time. The identity of this more easily denatured intermediate trypsin derivative is not known.

With increasing time of exposure to TLCK, trypsin activity was progressively lost and the rate of the fluorescence transition in 8 M urea increased as shown in Fig. (2).

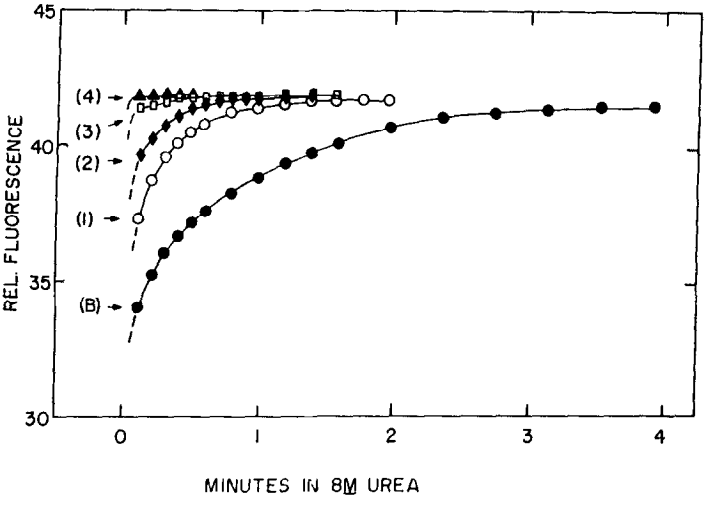

Figure 2. Fluorescence transitions of trypsin in 8 M urea at 27°C before (B) and after increasing time of incubation (1-4) with TLCK reagent. Trypsin (120uM) in 0.1 M Tris buffer, pH 7.7 was incubated at 27°C with 1 mM TLCK for up to one hour. The enzyme activity of aliquots taken during the course of reaction was assayed with benzoyl-L-arginine-ethylester. Percent remaining enzyme activities relative to untreated trypsin incubated under similar conditions were (1), 99 percent; (2), 59 percent; (3), 17 percent; and (4), 1 percent. Fluorescence intensities were corrected for absorption by TLCK reagent.

Trypsin was most resistant to urea denaturation at pH 7, with the denaturation rates increasing rapidly with pH changes to lower and higher values (see Fig. (3)). In addition, the rate of fluorescence transition was very sensitive to temperature. Using absolute rate theory, the thermodynamic parameters of denaturation in 8 M urea were calculated for T, TG, and DIP-T (see Table II). The kinetics of DIP-T denaturation in 8 M urea as a function of temperature were complicated. The Arrhenius plot was linear up to 26°C ($\Delta H^{\ddagger}$ = 16 kcal/mole) but, at higher temperatures the slope deviated upward from linearity ($\Delta H^{\ddagger} >$ 16 kcal/mole).

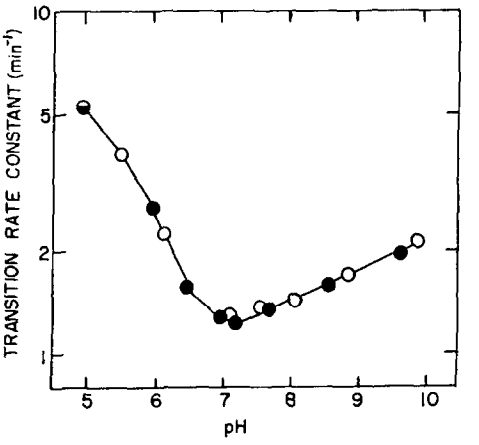

Figure 3. The effect of pH on the first-order constant for the fluorescence transition of trypsin in 8 M urea at 28°C. The solutions contained 0.5 mg/ml protein and were buffered with 0.25 M sodium phosphate.

Table II

Thermodynamics of Denaturation of T, TG, and DIP-T in 8 M urea

	$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta F^{\ddagger}$ (kcal/mole, 25°)	$\Delta S^{\ddagger}$ (E.U.)
T	28 ± 1	20.2 ± 0.1	28 ± 3
TG	23 ± 1	19.3 ± 0.1	-13 ± 3
DIP-T	16 ± 1	19.6 ± 0.1	-12 ± 3

Solvent: 0.25 M phosphate, pH 7.2. Temperature ranges: T, 22° - 36°C; TG, 21° - 30°C; DIP-T, 19° - 26°C.

The data show that TG, DIP-T and TLCK-T are denatured at higher rates in 8 M urea than the active enzyme. Relative to trypsin, the decreased stabilities of DIP-T and TLCK-T could be due either to changes in conformation or to altered local binding resulting from the attachment of the inhibitor group to the protein. One might conclude that the large differences in the entropies of activation of trypsin, TG and DIP-T favor the interpretation that the altered denaturation rates are due to changes in conformation. This conclusion must be accepted with caution, however, because

entropy is not always a measure of changes in protein conformation (Lumry and Biltonen, in press).

The above data on trypsin, TG, DIP-T and TLCK-T extend our previous urea-denaturation studies on derivatives of another closely related proteolytic enzyme, chymotrypsin, as monitored by tryptophan fluorescence. Both chymotrypsin and trypsin show maximal stabilities to 8 M urea in the pH region of 7, although the details of the effects of pH on the first-order fluorescence transition rates are different. Also, the rates of denaturation of these two proteolytic enzymes in 8 M urea are similar. Despite these similarities, and those demonstrated in catalytic function and amino acid composition, the stabilities of the zymogens and diisopropyl-derivatives of these two enzymes in 8 M urea appear to be quite different. While our data are consistent with current models of enzyme mechanism (Yapel et al., 1966; Lumry and Eyring, 1954; Koshland, 1958) which include changes in enzyme conformation during the catalytic process, they suggest that the conformational changes involved in the catalytic mechanism of trypsin may be quite different from those of chymotrypsin.

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