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

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Most proteins exposed to short wavelength ultraviolet light show a characteristic fluorescence due to their tryptophan residues. A change in the immediate environment of the tryptophan residues can alter both the quantum efficiency of fluorescence and the wavelength of the emission peak. Thus differences in fluorescence spectra of proteins in the native and denatured state are sensitive monitors of the conformational state of proteins (Steiner et al., 1964). We have previously found that chymotrypsin shows a progressive increase in its rate of denaturation in 8 M urea, as measured by fluorescence, during photodynamic inactivation (Hopkins and Spikes, 1965).

This communication is concerned with the effects of high concentrations of urea on the fluorescence of native chymotrypsin (CT), native chymotrypsinogen (CTG), diisopropylchymotrypsin (DIP-CT), cinnamoyl-chymotrypsin (Cin-CT), and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated chymotrypsin (TPCK-CT). DIP-CT is an inactive form of chymotrypsin produced by treatment with DFP (diisopropyl-fluorophosphate); in the reaction the serine residue of the active site is converted to DIP-serine (Oosterbaan and van Adrichem, 1958). Cin-CT is a covalently bonded enzyme-substrate intermediate produced by reacting CT with cinnamoylimidazole (Schonbaum et al., 1961). The cinnamoyl group is attached to the serine residue of the active site (Noller and Bernhard, 1965). TPCK-CT is an inactive derivative of

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CT; the TPCK group is attached to the histidine residue of the active site (Schoellmann and Shaw, 1963).

When buffered solutions of these proteins were excited with 290 nm light, fluorescence with a peak at 341 nm was emitted. At 20° C, solutions of CT made 8 M in urea displayed a time-dependent increase in fluorescence and a red shift in the emission peak to 356 nm (see Fig. 1). The fluorescence transition of DIP-CT in 8 M urea at 20° C was slower, and the final extent of change was smaller (see Fig. 1); CTG behaved in the same way. As the temperature was increased, these latter systems showed further increases in both intensity and red shift of the fluorescence peak until, at approximately 30° C, the changes were of the same magnitude as those for CT in 8 M urea at 20° C. At higher temperatures the transition rates of DIP-CT and CTG in 8 M urea were faster, but no further increases in fluorescence intensity and red shift were observed (see 60° C data in Fig. 1).

The rate of appearance of the 356 nm emission peak in 8 M urea could be

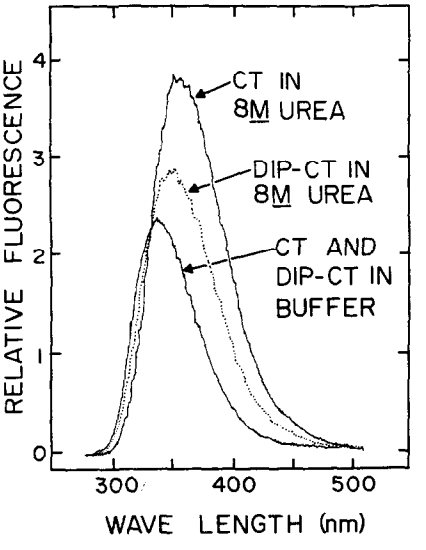

Figure 1. Curves showing the equilibrium fluorescence emission spectra of chymotrypsin (CT), and DIP-chymotrypsin (DIP-CT) in buffer and in buffered 8 M urea. All spectra were measured at 20° C. If the DIP-CT in 8 M urea is heated to 60° C briefly before measurement, its spectrum becomes identical to that of CT in 8 M urea. The solutions contained 0.5 mg/ml protein in 0.125 M phosphate buffer at pH 8.2. Spectra were measured with an Aminco-Bowman spectrophotofluorometer fitted with a Corning 0-54 filter to eliminate possible interference by scattered 290 nm exciting light.

followed (see Fig. 2). The fluorescence transitions were apparent first order for all proteins studied. Typical rate constants (k) are shown in Table I.

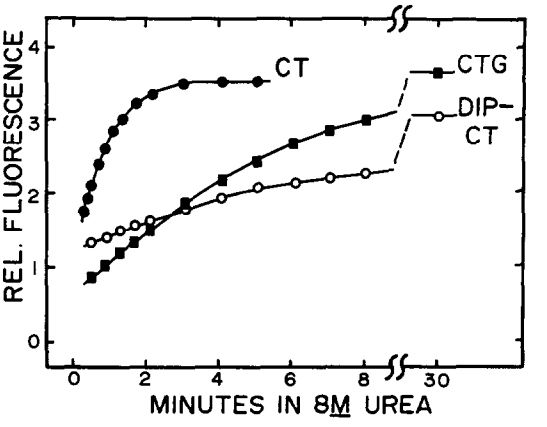

Figure 2. The time-course of the fluorescence transitions of chymotrypsin (CT), chymotrypsinogen (CTG), and DIP-chymotrypsin (DIP-CT) in 8 M urea at 28° C. The solutions contained 0.5 mg/ml protein in 0.125 M sodium phosphate buffer at pH 7.2. The fluorescence was measured at 356 nm using 290 nm exciting light.

Table I

First Order Fluorescence Transition Rate Constants of Chymotrypsin and Some Derivatives in 8 M Urea*

	CT	CTG	DIP-CT	Cin-CT	TPCK-CT
$k(min^{-1})$	1.2	0.16	0.12	0.12	<0.01

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

The fluorescence transition of CT was studied as a function of pH and urea concentration. As shown in Fig. 3, the transition rate was slowest in the pH range of 6-7. A similar pH profile was observed by Martin (1964), who examined the denaturation of CT in 8 M urea by measuring changes in the absorption spectrum of the protein. We found that the transition rate was also critically dependent on the urea concentration. While no changes in the fluorescence pattern were observed at urea concentrations below 4.0 M at 30° C, the transition rate increased rapidly as the urea concentration was increased from 6.0 M to 8.0 M.

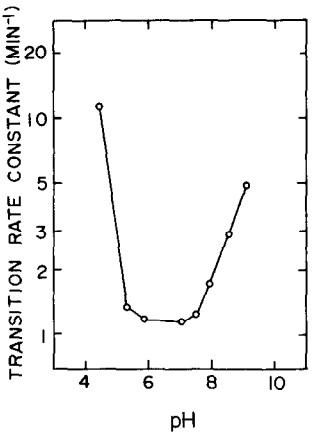

Figure 3. The effect of pH on the first order rate constant for the fluorescence transition of chymotrypsin (CT) in 8 M urea at 28° C. The solution contained 0.5 mg/ml protein in 0.125 M sodium phosphate buffer.

The rate of the fluorescence transition was very temperature sensitive. Using absolute reaction rate theory, the kinetics of denaturation in 8 M urea were calculated in order to gain further information on the differences in stability of CT and DIP-CT toward urea denaturation. The results are given in Table II.

Table II

Kinetics of Denaturation of CT and DIP-CT in 8 M Urea*

	E (kcal/mole)	$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta F^{\ddagger}$ (30°, kcal/mole)	$\Delta S^{\ddagger}$ (E.U.)
CT	49	48	20	94
DIP-CT	31	30	21	27

* Solvent: 0.25 M phosphate, pH 7.3. Temperature ranges: CT, 23°-33° C; DIP-CT, 28°-40° C

Our data confirm the conclusions of Martin and Bhatnagar (1966) that DIP-CT and CTG are more stable than CT to urea. Furthermore, our data show that Cin-CT and TPCK-CT are also more stable in 8 M urea than CT since the rates of denaturation, as measured by tryptophan fluorescence, are much slower. The demonstration that the

derivative forms of CT show decreased fluorescence transition rates suggests that these forms may be thermodynamically more stable. If so, the increased stability could be attributed to either a different conformation of the derivative or an improved local binding resulting from the attachment of the derivative group to the protein. The difference in activation entropies for the denaturation of CT and DIP-CT in 8 M urea (see Table II) are consistent with a difference in conformation but not necessarily consistent with the conclusion that DIP-CT is more compactly folded than CT. It has been shown that the interpretation of thermodynamic data for protein-unfolding reactions is often difficult and equivocal (Brandts, 1964, 1967). Nevertheless, our data are consistent with current models of enzyme mechanism (Yapel et al., 1966; Lumry and Eyring, 1954; Koshland, 1958) which propose important changes in conformation of the enzyme during the course of catalytic hydrolysis of the substrate. Our data further suggest that some substrates tend to protect the enzyme against inactivation by inducing conformational changes which result in a more stable form of the enzyme.

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