Urea Denaturation of Active-Site Spin-Labeled α-Chymotrypsin†

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ABSTRACT: α-Chymotrypsin, spin labeled with the DFP analog 4-hydroxy-2,2,6,6-tetramethylpiperidino-1-oxyl monoethylphosphorofluoridate ester (III), was studied in varying urea concentrations at pH 2 and 5.5. It was found that the concentration of urea necessary to unfold the labeled enzyme (as monitored by the increase in mobility of the covalently attached spin label) was pH dependent. The increased stability of the labeled enzyme at the higher pH correlates well with results obtained by other methods. This is in contrast to the unfolding behavior of a similar spin-labeled DFP analog in guanidine hydrochloride (Morrisett, J. D., and Broomfield, C. A. (1971), *J. Amer. Chem. Soc. 93, 7297*) where no pH dependence was observed. A spin-labeled acyl-chymotrypsin, where the acyl group was 3-carbonyl-2,2,5,5-tetramethylpyrroline-1-oxyl (IV), exhibited unfolding behavior in urea at pH 2 similar to the phosphorofluoridate-labeled enzyme above III. Although labels III and IV gave weakly and strongly immobilized spectra, respectively, their different interactions with the enzyme imparted no apparent differences in stabilization of active-site conformation.

The urea-induced denaturation of α-CT[1] has been studied by a variety of physical techniques including viscometry (Harris, 1956), ultraviolet spectrophotometry (Martin and Bhatnagar, 1966), optical rotation (Neurath *et al.*, 1956), and fluorescence (Hopkins and Spikes, 1967). The changes in protein structure brought about by urea as observed by these above methods are usually difficult to assign to a particular region in the macromolecule. In the spin-label method a small conformationally sensitive reporter group is incorporated at a specific site; the observed spectral changes reflect conformational changes brought about in the *local vicinity* of the spin label (McConnell and McFarland, 1970; Smith, 1972). Changes in the rotational tumbling rate of the protein as a whole may also contribute to these spectral changes; however, this occurs only in a very specific range of spin-label macromolecule rotational tumbling times (McCalley *et al.*, 1972). Active-site-directed spin-labeled substrates (Berliner and McConnell, 1966, 1971; Kosman *et al.*, 1969) and inhibitors (Morrisett *et al.*, 1969; Wong *et al.*, 1972[2]) for α-CT have been reported.

Morrisett and Broomfield (1971) have recently studied the guanidine hydrochloride denaturation of α-CT labeled at Ser-195 with I and II (Figure 1) by both electron spin resonance (esr) and circular dichroism (CD). In part, their esr studies indicated that the concentration of guanidine hydrochloride required to unfold either I-CT or II-CT was essentially pH independent. The CD results, however, were highly pH dependent, with a significantly lower concentration required for denaturation at low pH than near neutral pH. Thus, the two techniques were monitoring different conformational changes in different parts of the enzyme.

We have examined the urea induced denaturation of α-CT labeled with the DFP analog III, and with the acyl intermediate IV from the α-CT catalyzed hydrolysis of the substrate V (Figure 1). Contrary to the unfolding phenomena in guanidine hydrochloride, a pH dependence was observed in urea for label III.

Materials and Methods

Chymotrypsin (lot CDI 8LK) was purchased from Worthington Biochemical Corp. Ultra pure urea (lot V3204) was from Mann Research Labs. The spin-label III (4-hydroxy-2,2,6,6-tetramethylpiperidino-1-oxyl monoethyl phosphorofluoridate ester) was purchased from SYVA, Palo Alto, Calif.

The spin-labeled substrate V was synthesized from the corresponding carboxylic acid, 3-carboxy-2,2,5,5-tetramethylpyrroline-1-oxyl (Rozantsev and Krinitskaya, 1965), and *p*-nitrophenol by the identical method for the pyrrolidinyl analog (Berliner and McConnell, 1966): mp 116–118.5 uncor; mass spectrum parent peak at 305. *Anal.* Calcd for $C_{15}H_{17}N_2O_5$: C, 58.99; H, 5.62; N, 9.18. Found: C, 58.87; H, 5.76; N, 9.37.

Esr measurements were made at 26 ± 2° on a Varian E-4 spectrometer at X-band frequency. Activity and protein concentration measurements were carried out on a Unicam SP 1800 spectrophotometer. An absorptivity of 5.0×10^4 M^{-1} cm^{-1} at 280 nm was used for protein concentration determinations (Laskowski, 1961). All pH measurements were made with a Radiometer pHM 26 meter.

Enzyme Derivatives. The enzyme was labeled with inhibitor III at pH 5.0 by the procedure of Morrisett and Broomfield (1971) and then dialyzed exhaustively at pH 2, 0.1 M NaCl, until all spectral contributions from unbound spin label disappeared. In a typical experiment a stock solution of III-CT was found to contain no activity with acetyltyrosine ethyl ester (Pierce Chemicals) by the method of Schwert and Takenaka (1955). A spin concentration measurement showed it to contain approximately 0.96 spin-label molecule per protein molecule by esr.

In a typical preparation of IV-CT, the enzyme (approximately 10 mg/ml) was reacted with a 2- to 5-fold excess of V in pH 5, 0.1 M sodium acetate. Due to solubility problems, it was necessary to add the substrate (from an acetonitrile stock solution) dropwise over a 5- to 10-min period. The final mixture contained 15% (v/v) acetonitrile. This was fol-

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[1] Abbreviations used are: esr, electron spin resonance; CD, circular dichroism; α-CT, α-chymotrypsin; I-CT, II-CT, III-CT, or IV-CT, α-chymotrypsin labeled with I, II, III, or IV, respectively; DFP, diisopropyl phosphofluoridate.

[2] To be published.

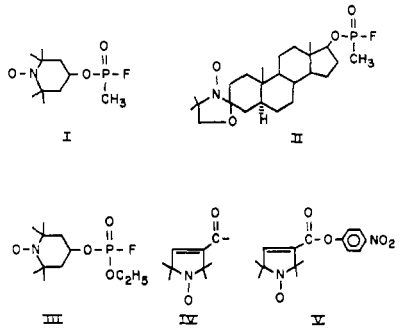

FIGURE 1: Structures of spin labels.

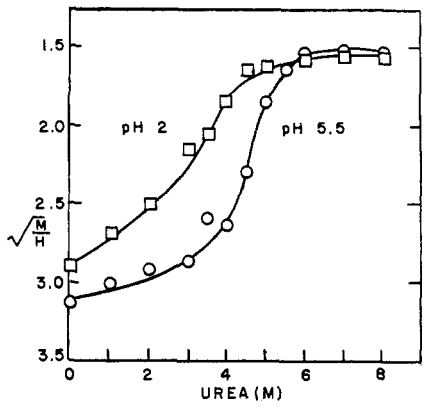

FIGURE 3: Change in the esr spectra of III-CT at pH 2 (□) or pH 5.5 (○) with increasing urea concentration.

lowed by exhaustive dialysis at pH 2, 0.1 M NaCl to remove all free unbound label. The resultant enzyme solution was occasionally as low as 12% acylated; however, it is important to note that the esr experiment detects only labeled enzyme.

Denaturation. An appropriate volume of a concentrated urea stock solution at an apparent pH of either 2 (0.1 M NaCl) or 5.5 (0.1 M sodium citrate) was added to a volume of spin-labeled enzyme stock solution (also at the same pH) to attain the desired final urea concentration. Esr spectra were taken repeatedly on each sample to ensure that the unfolding was complete (usually 8–15 min). For III-CT at pH 5.5, it was essential to measure all spectra within 60–120 min after exposure to this pH as a slow hydrolysis yields free spin label which adds spectral components superimposable with those measured for the urea-unfolded enzyme (Morrisett and Broomfield, 1971).

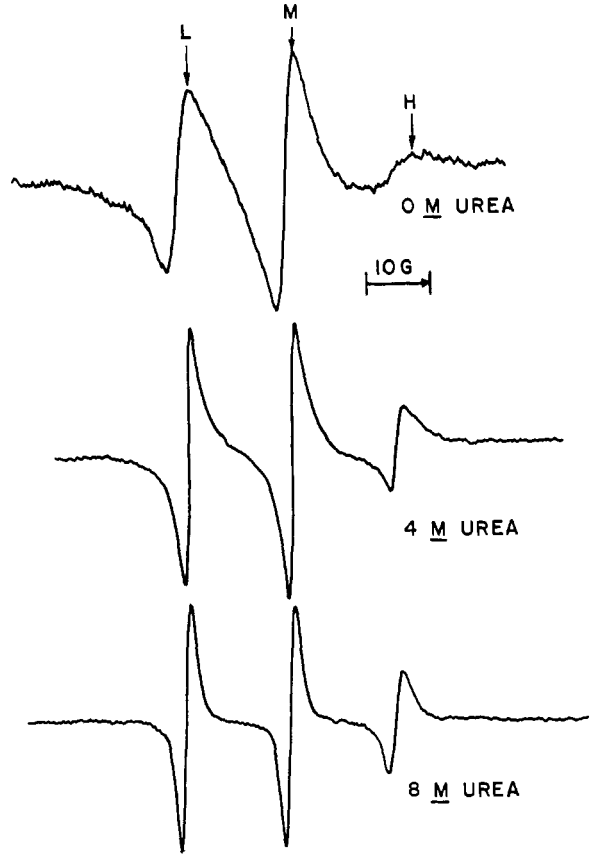

FIGURE 2: Esr spectra of α-chymotrypsin labeled with III at pH 2, 0.1 M NaCl at various urea concentrations.

Results

Figure 2 shows spectra at various urea concentrations, pH 2, for α-CT labeled with inhibitor III. This class of inhibitor has been shown to mimic DFP by stoichiometrically inactivating the active-site serine-195 of α-CT (Morrisett *et al.*, 1969). α-CT labeled with III exhibits a "weakly immobilized" spectrum indicating that the label has a moderate degree of motion with respect to the protein (Stone *et al.*, 1965).

As the urea concentration increases the three spectral lines narrow in width and increase in peak height. These changes signify an increase in the average motion of the spin label with respect to its environment, *i.e.*, a loosening (or unfolding) of tertiary structure surrounding the label. An empirical indicator of this unfolding has been monitored by the change in the ratio of peak heights of lines H to M in Figure 1 (Morrisett and Broomfield, 1971). A parameter more quantitatively related to the change in the molecular tumbling rate of the spin label is obtained by calculating the inverse square root of this ratio, $(M/H)^{1/2}$ (Keith *et al.*, 1970).

Figure 3 shows the change in $(M/H)^{1/2}$ with urea concentration for III-CT a pH 2 and 5.5. It should be noted that an obvious pH dependence is apparent for the extent of unfolding with varying urea concentration. At either pH, the spectra in 8 M urea are reversible to the 0 M urea spectrum after dialysis at pH 2.

The acyl-enzyme IV-CT yields spectra in various urea concentrations at pH 2 as shown in Figure 4. The spectrum in 0 M urea is "strongly immobilized," that is, indicative of spin label held rigidly at the active site of the enzyme (Berliner and McConnell, 1966). The very weak lines at L′ and H′ in this spectrum represent a small concentration of free nitroxide carboxylate resulting from very slow deacylation of IV-CT at this pH. As the urea concentration is increased, new lines grow at L′, M′, and H′ representing a state of increased mobility of the still covalently bound acyl group. These changes are accompanied by a simultaneous decrease in the "native" lines B and W.

In order to compare the unfolding of IV-CT with III-CT at pH 2, the most sensitive indicator is the relative peak height of the high field line, H/M for III-CT and H′/M′ for IV-CT, respectively (Figure 5). We could not study IV-CT at pH 5.5 as deacylation becomes appreciable. However, it is interest-

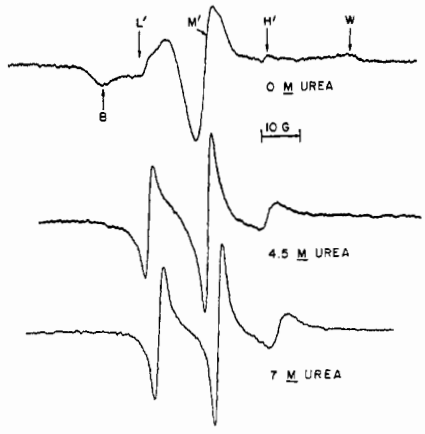

FIGURE 4: Esr spectra of α-chymotrypsin labeled with IV at pH 2 at various urea concentrations.

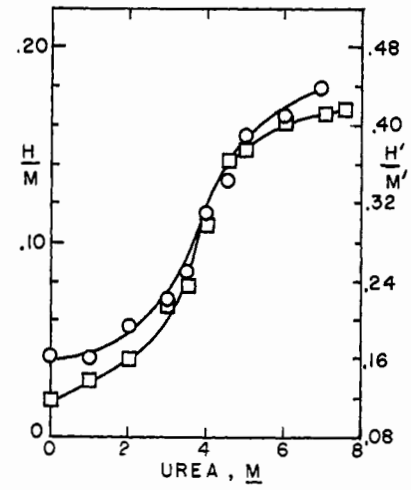

FIGURE 5: Comparison of changes in the esr spectra of III-CT (□) and IV-CT (○) in pH 2.0 to increasing urea concentration.

ing to note that a sample of IV-CT in pH 5.5, 8 M, yields a 0 M spectrum after dialysis at pH 2.

Discussion

The results presented here parallel those previously measured by other techniques. Martin and Bhatnagar (1966) showed by difference ultraviolet (uv) spectroscopy that DFP-CT is more stable to unfolding nearer to its functional pH optimum in experiments at pH 4 and 7. Figure 3 shows definite evidence that the spin-labeled DFP analog, III-CT, is more stable to urea induced unfolding at the higher pH. These results are certainly in contrast to the esr results in guanidine hydrochloride where no pH dependence was found. Yet the CD changes in guanidine hydrochloride displayed a similar pH dependence (Morrisett and Broomfield, 1971) to those studies in urea presented here and elsewhere (Martin and Bhatnagar, 1966). These combined results certainly exemplify the differences in the mechanism of unfolding of α-CT mediated by these two denaturants. Perhaps the conformationally linked effects of certain charged residues critical to active-site conformation are, in essence, obliterated by the relatively high concentration of associated guanidinium cations. This may be manifested in either specific ion or general ionic strength effects which would effectively "neutralize" all or certain electrostatic differences between the acid and pH 5.5 forms of the enzyme as monitored by active site conformation. The effects of general ionic strength effects on the pH dependence of chymotrypsin denaturation will be presented in future work.

Labels III and I differ slightly in structure by the substitution of an ethoxy for a methyl group. It is difficult to see how this difference could account for the differences discussed above since the esr spectra of I-CT or III-CT indicate that they are undergoing substantial rotational tumbling of similar magnitude. Thus both labels are sensing a large area of active-site structure and its changes. From the discussion to follow below, it is clear that all of the labels are sensing the same overall change.

The structurally different probe IV appears to reflect the same conformational transition in the active site at pH 2 as does III-CT (Figure 5). Although IV interacts more strongly with the protein, as evidenced by its complete immobilization, no marked (acyl group) "stabilization" is apparent from the esr data. This is to be compared with the derivative II-CT of Morrisett and Broomfield (1971) which exhibited spin-label immobilization similar to that of IV-CT (Figure 4), but *also* increased stability towards guanidine hydrochloride over the other derivative, I-CT. In our studies, however, whereas the enzyme–acyl group complex (IV-CT) is tightly bound, it apparently does not stabilize the active site structure any more than does the phosphorylated derivative, III-CT, in terms of the concentration of urea necessary to unfold the enzyme.[3]

It should also be noted that both labels III and IV contain bulky dimethyl groups. These features probably exclude either label from binding in the specificity pocket (tosyl hole) of the enzyme. The crystallographic data indicate that this pocket is, at most, 3.5–4.0 Å thick (Steitz *et al.*, 1970). Previous studies on the protection of α-chymotrypsin to urea denaturation have involved the effects of substrates and inhibitors on the rate of unfolding in strong urea solutions (Friedberg *et al.*, 1969; Hopkins and Spikes, 1967). In part they found that those derivatives capable of binding (covalently or noncovalently) to the active site were effective in decreasing the rate of unfolding. It would be of interest to test the effects of aromatic spin-labeled derivatives (Wong *et al.*, 1972)[2] believed to bind in this pocket on both the equilibrium between the the native and unfolded state(s) and on the rates of unfolding.

Acknowledgments

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[3] On the other hand, conformational stabilization by groups bound at the active site may not be very appreciable at pH 2.

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