Active Site Spin-Labeled α-Chymotrypsin. Guanidine Hydrochloride Denaturation Studies Using Electron Paramagnetic Resonance and Circular Dichroism

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Abstract: α-Chymotrypsin has been spin labeled at the active site serine residue by the *N*-oxyl-4′,4′-dimethyloxazolidine of 5α-androstan-3-one 17β-methylphosphonofluoridate (PCSL) and 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate (MCSL). The denaturation of each enzyme derivative as a function of guanidine hydrochloride concentration has been studied by epr and CD. The epr results indicate that (1) the concentration of denaturant required to bring about unfolding of the two derivatives is quite different, with CT-PCSL being the more stable, (2) for each of the two derivatives, the denaturant concentration required for unfolding is essentially pH independent, and (3) at pH 3.0, the mobility of the larger pentacyclic spin label actually decreases at low denaturant concentrations, then increases with further increase in concentration of guanidine hydrochloride. The CD results indicate that (1) in contrast to the epr results, the concentration of guanidine hydrochloride required to unfold each species is highly pH dependent with a much lower concentration required for denaturation at low pH than at neutral pH, (2) the spin-labeled derivatives denature at lower guanidine hydrochloride concentrations than the unlabeled enzyme, and (3) CT-PCSL is conformationally less stable than CT-MCSL. The implication of these observations is that the two techniques are actually monitoring conformational changes at separate parts of the enzyme molecule. It is suggested that tryptophan-141 is the chromophore whose perturbation gives rise to the CD transition, while the spin labels at serine-195 are reflecting structural changes in the active site region.

Since the introduction of the technique of spin labeling 6 years ago, this method has been used extensively to study a variety of biological systems. However, several problems immediately confront the investigator who wishes to use this tool to probe biological or biochemical systems. (1) The nitroxide free radical which serves as the reporter group is often quenched in biological oxidation–reduction systems and by thiols, thereby resulting in a decrease or loss of signal. (2) The spin label is sometimes bound nonspecifically at two or more conformationally different sites, thereby giving rise to a mixed epr spectrum containing mobile and immobile components. It is not uncommon for the mobile component to be the dominant one and sometimes it masks subtle changes of the immobile component which may be induced by environmental change of the system under study, *e.g.*, change in pH or addition of allosteric affectors. Much of the indiscriminant binding of spin probes to proteins observed during the early developmental stages of this technique has been precluded by the design and synthesis of labels which are highly specific for unique sites. (3) As is true with all reporter groups, the placing of spin labels in biochemical systems is always accompanied by the possibility that the label will perturb the system one wishes to examine. Thus, it is wise to use one or more additional methods to probe the system under study. (4) It is often difficult quantitatively to correlate changes in the epr spectra of spin-labeled systems with the actual conformational change occurring in that system.

We have been particularly interested in the last of these four general problems. Specifically, we have addressed ourselves to the problem of evaluating the spin-labeling method as a means of quantitatively monitoring the unfolding of proteins by denaturants.

For this evaluation α-chymotrypsin was chosen as a model. Several factors influenced our choice of this enzyme. (1) We had previously synthesized[1] an organophosphorus spin label which reacts specifically with the active site serine hydroxyl group of this esterase. (2) The chemistry of α-chymotrypsin has been studied extensively and today is well understood. (3) The three-dimensional structure of the enzyme has been determined by X-ray diffraction.[2] Such crystallographic data are of immeasurable value in the interpretation of experimental results.

This paper describes the preparation of two new active site spin-labeled derivatives of α-chymotrypsin and their behavior in guanidine hydrochloride as monitored by epr and CD. As an aid to interpretation of the resulting data, a limited number of viscosity and activity studies were also performed.

Experimental Section

Materials. Bovine α-chymotrypsin (three-times crystallized) was obtained from Worthington Biochemical Corp., lot CD1-6LD. For one series of experiments, the commercial material was purified by the method of Nakagawa and Bender.[3] Titration with *N-trans*-cinnamoylimidazole by the procedure of Schonbaum, *et al.*,[4] indicated that the commercially available enzyme was about 90% pure. However, there was no observable difference in the epr or ORD results obtained with purified material and that used as purchased. Sephadex G-25 was procured from Pharmacia Fine Chemicals, Inc. Guanidine carbonate from Fisher Scientific Co. was converted to the hydrochloride by the method of Kawahara and Tanford.[5] A 6 *M* solution has an absorbance <0.15 at 225 mμ. In some experiments sequanal grade material, lot number 01060-1 from Pierce Chemical Co., was used as received. 5α-Androstan-17β-ol-3-one was from Sigma Chemical Co. 1-Oxyl-

(1) J. D. Morrisett, C. A. Broomfield, and B. E. Hackley, Jr., *J. Biol. Chem.*, 244, 5758 (1969).
(2) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 46, 337 (1969).
(3) Y. Nakagawa and M. L. Bender, *Biochemistry*, 9, 259 (1970).
(4) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, 236, 2930 (1961).
(5) K. Kawahara and C. Tanford, *ibid.*, 241, 3228 (1966).

2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate (MCSL) was prepared by the method of Morrisett, *et al.*,[1] from the commercially available alcohol and methylphosphonodifluoridate. The difluoro compound may be prepared by the method of Dawson and Kennard[6] from the dichloro analog.[7] The *N*-oxyl-4′,4′-dimethyloxazolidine of 5α-androstan-3-one-17β-ol was synthesized according to the procedure of Hubbell and McConnell.[8]

Procedures. Electron paramagnetic resonance (epr) spectra were recorded on a Varian E-3 spectrometer operated at 9.5 GHz. Optical rotatory dispersion (ORD) and circular dichroism (CD) measurements were performed on a Cary 60 recording spectropolarimeter equipped with a Model 6001 CD accessory. All measurements were carried out at 27°. Protein concentration determinations and activity assays were carried out on a Cary 16 spectrophotometer equipped with a Model 1626 recorder interface and a Mosely Model 7100 B strip chart recorder. Infrared spectra were recorded on a Perkin-Elmer 221 spectrophotometer and mass spectra on a Hitachi Perkin-Elmer RMU-6E spectrometer. A Beckman research model pH meter with a Beckman 39030 combination electrode was used to determine pH. Viscosity measurements were made with an Ostwald-Cannon-Fenske viscosimeter having a flow time for water of 419 sec at 26.8°.

***N*-Oxyl-4′,4′-dimethyloxazolidine of 5α-Androstan-3-one 17β-Methylphosphonofluoridate (PCSL).** In 1.0 ml of benzene and 0.5 ml of triethylamine was suspended 156 mg (0.41 mmole) of the precursor alcohol. The stirred suspension was cooled to 5° on an ice bath and 0.41 ml (5 mmoles) of methylphosphonodifluoridate was added dropwise. After 20 min excess unreacted reagent was destroyed by the slow, careful addition of 1 ml of 5 *M* sodium acetate buffer, pH 4.5. A precipitate of triethylamine hydrofluoride formed immediately and was sedimented by centrifugation. The organic layer was decanted into a small vial. The benzene was evaporated, and the residue was dissolved in 0.5 ml of chloroform and applied to a 0.6 × 30 cm column of silica gel equilibrated with the same solvent. The column was developed with chloroform until the yellow band had descended 4–5 cm; then the band was eluted with 5% methanol–95% chloroform. Colored fractions were pooled, and the solvent was evaporated by a stream of nitrogen. The remaining yellow mass was broken up and dried *in vacuo* for 5 hr. The material was homogeneous by thin-layer chromatography on silica gel G (chloroform–methanol, 9:1, R_f 0.82), mp 143.5–145.0°. The infrared spectrum exhibited strong bands at 1465, 1280, 1050, and 835 cm^{-1}, confirming the CH_3–P, P=O, P–O–C, and P–F linkages, respectively. *Anal.* Calcd for for $C_{24}H_{40}NO_4PF$: C, 63.15; H, 8.77; N, 3.07; P, 6.79; mol wt, 456. Found: C, 63.2; H, 8.8; N, 3.0; P, 6.6. The mass spectrum exhibited a parent ion at *m/e* 456. The epr spectrum displayed the typical three hyperfine lines with a *g* value of 2.0057 ± 0.0003 in 10% aqueous acetone.

α-Chymotrypsin Spin Labeled with MSCL (CT-MCSL). The enzyme (125 mg) was dissolved in 5 ml of 0.1 *M* sodium acetate, pH 5.5 at 5°. To this slowly stirred solution was added 50 μl of a benzene solution 1.0 *M* in MCSL. After 2 hr, the activity[9] was less than 0.5% of the original activity. The reaction mixture was immediately applied to a 1.9 × 55 cm column of Sephadex G-25 equilibrated at 5° with either 0.1 *M* ammonium formate, pH 6.3, or 0.1 *M* KCl, pH 3.0. Protein in effluent fractions was determined by absorbance at 280 mμ, using the value $\epsilon_{280}^{1\%}$ 20.4.[10] Since the attachment of the nitroxide to the enzyme increased the absorbance by less than 0.1%, the same extinction value was used for both the native and modified chymotrypsin.

α-Chymotrypsin Spin Labeled with PCSL (CT-PCSL). Enzyme (125 mg, 5 μmoles) was dissolved in 4.5 ml of 0.1 *M* sodium acetate, pH 5.50 at 5°. To this stirred solution was added 4.56 mg (10 μmoles) of PCSL dissolved in 0.5 ml of acetone. After 12 hr the activity had diminished to about 3.4% of the original. To remove unreacted reagent, the reaction mixture was filtered through a sintered glass funnel, then passed through Sephadex as described for the preparation of CT-MCSL.

Determination of Labeling Reaction Stoichiometry. A 1.0-ml aliquot of chymotrypsin labeled with MCSL was diluted with an equal volume of 1.0 *M* sodium hydroxide and stirred at 50° for 24 hr. The amplitude of the center-field line of the epr spectrum of this sample was compared with the amplitude of a standard solution of the monocyclic alcohol obtained under identical spectrometer conditions. For these measurements, 100-μl capillaries were used to contain the samples. Such capillaries can be reproducibly oriented in the spectrometer cavity when the temperature controller accessory is used. Using the extinction value reported by Desnuelle,[10] it was calculated that 1.08 nitroxyl radicals were bound for each chymotrypsin molecule.

Preparation of Solutions in Guanidine Hydrochloride. There is a tendency at low pH and at certain guanidine hydrochloride concentrations for the spin-labeled α-chymotrypsin to aggregate. This is particularly true for the PCSL-CT. At the dilute concentrations required for the CD and esr studies, however, the rate of nucleation was sufficiently slow at 27° that the spectral data could be obtained before aggregation had proceeded to a significant extent. In order to accomplish this, solutions of guanidine hydrochloride were prepared at appropriate concentrations so that a stock solution of the chymotrypsin derivative could be added and mixed, and the spectrum scanned in the shortest possible time. Usually the epr or CD data were obtained within 30 min after mixing. At the end of each run the pH and A_{280} of each solution were measured.

Aggregation was a particularly serious problem in attempts to measure viscosity as a function of guanidine hydrochloride concentration. Since protein concentrations had to be much higher, nucleation rates were much greater, and for several concentrations of guanidine hydrochloride it was impossible to obtain meaningful viscosity data.

Results

α-Chymotrypsin reacts rapidly and specifically with either of the phosphonofluoridate spin labels (epr spectra of which are shown in Figure 1) so as to attach the label to the active site serine. The electron paramagnetic resonance spectra of the resulting products are shown in Figure 2. The spectrum of the monocyclic label (upper curve) attached to α-chymotrypsin reflects a significant degree of immobilization, with the high- and low-field lines being markedly diminished but still recognizable. A rotational correlation time of about 12 sec is estimated, based on the published results of studies on model systems.[11] Various parts of the spectrum are arbitrarily labeled a, b, c, d for convenience of reference. The spectrum of the pentacyclic label attached to α-chymotrypsin (Figure 2, lower curve), again arbitrarily labeled v, w, x, y, z for convenience of reference, is strongly immobilized. A rotational correlation time of 30 nsec was estimated,[12] indicating very little motional freedom of this radical relative to the macromolecule. However, this label is not maximally immobilized, as will be shown subsequently.

It was not immediately apparent why these labels should be immobilized to so great a degree. According to the three dimensional structure determined crystallographically by Blow, *et al.*,[13] the active site region of α-chymotrypsin lies in a shallow, almost superficial depression on the surface of the molecule. The depression is actually the hydrophobic pocket where the phenolic and phenyl side chains of tyrosine and phenylalanine are bound during the catalytic event.[14] Since the piperidinyl ring of MCSL has dimensions very similar to those of the side chains, it is logical to assume that this piperidinyl group can be bound at this same locus and in a similar fashion.[15] However, it would appear that the

(6) T. P. Dawson and K. C. Kennard, *J. Chem. Soc.*, 1671 (1957).
(7) A. M. Kinnear and E. A. Perren, *ibid.*, 3437 (1952).
(8) W. L. Hubbell and H. M. McConnell, *Proc. Nat. Acad. Sci. U. S.*, 63, 16 (1969).
(9) B. C. W. Hummell, *Can. J. Biochem. Physiol.*, 31, 1393 (1959).
(10) P. Desnuelle, *Enzymes*, 4, 93 (1960).
(11) I. C. P. Smith, "Biological Applications of Electron Spin Resonance Spectroscopy," J. R. Bolton, D. Borg, and H. Swartz, Ed., Wiley-Interscience, New York, N. Y., 1971.
(12) L. Stryer and O. H. Griffith, *Proc. Nat. Acad. Sci. U. S.*, 54, 1785 (1965).
(13) D. M. Blow, *Biochem. J.*, 112, 261 (1969).
(14) R. E. Dickerson and I. Geis, "The Structure and Action of Proteins," Harper & Row, New York, N. Y., 1969, Stereodrawings 12–14.

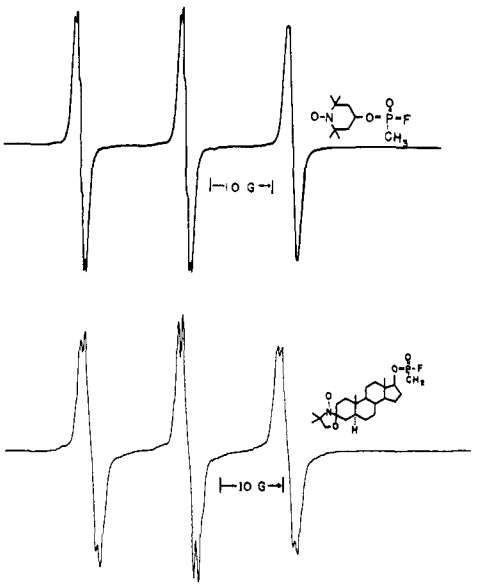

Figure 1. Epr spectra of two organophosphorus spin labels used in this study. Top: spectrum of 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate (monocyclic spin label, MCSL) in 0.1 *M* KCl, pH 3.0. Bottom: spectrum of the *N*-oxyl-4′,4′-dimethyloxazolidine of 5α-androstan-3-one 17β-methylphosphonofluoridate (pentacyclic spin label, PCSL) in 90% 0.1 *M* KCl, pH 3.0, 10% acetone.

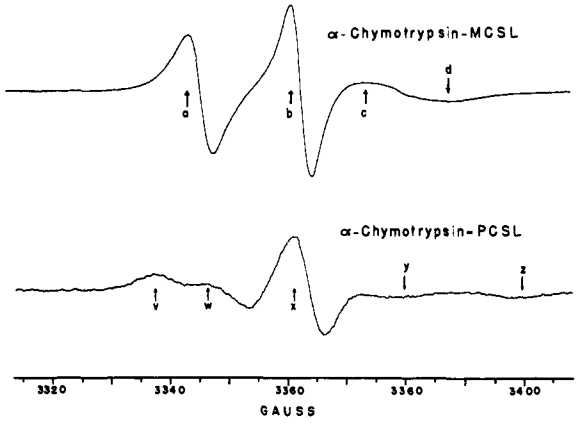

Figure 2. Epr spectra of α-chymotrypsin labeled with monocyclic spin label (top spectrum) and pentacyclic spin label (bottom spectrum), respectively. Each peak is labeled arbitrarily for convenience of reference (see text).

larger pentacyclic label would be sterically excluded from this hydrophobic pocket and thus would not be expected to be highly immobilized. Nevertheless, the epr spectrum indicates that, in fact, the larger label is more immobilized than the smaller.

A study of the change in mobility of the spin labels as the enzyme molecule was unfolded with guanidine chloride in two different pH regions enabled us to develop an explanation for this observation. The change in the epr spectrum of CT-MCSL as guanidine hydrochloride

(15) Evidence that this is indeed the case will be published in detail elsewhere. Suffice it to note that the spin-label alcohol, 1-oxyl-2,2,6,6-tetramethyl-4-piperidinol has been shown to be an inhibitor of α-chymotrypsin at concentrations greater than 10^{-4} *M*. Also, reaction of the MCSL with α-chymotrypsin is strongly inhibited by benzoyl tyrosine ethyl ester. Both of these pieces of evidence support the contention that the spin label binds in the hydrophobic pocket.

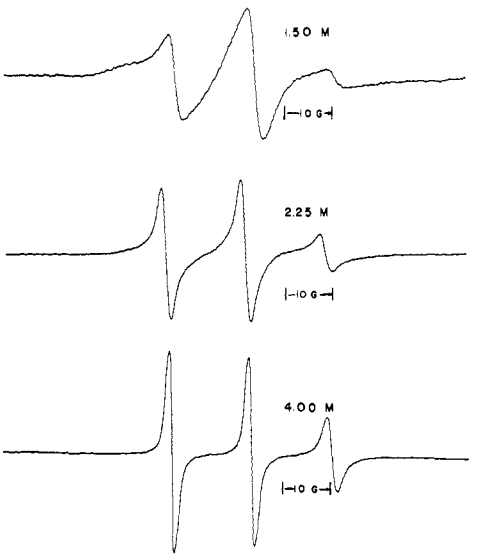

Figure 3. Change in epr spectrum of MCSL-chymotrypsin in solutions of progressively increasing guanidine hydrochloride concentration at pH 3.0. The height of the high-field resonance line (corresponding to that labeled "c" in Figure 2) relative to the center line was used as a measure of the amount the mobility of the label was changed by the guanidine hydrochloride.[16]

concentration is increased is shown in Figure 3. As expected, the freedom of motion of the spin label increases as the macromolecule is unfolded. This is reflected in a progressive increase in the ratio of the heights of the high-field resonance line (labeled c in Figure 2) to the center-field line (labeled b in Figure 2). The relative degree of mobility of the label may be expressed quantitatively in terms of this ratio.[16] The change in the resonance spectrum of the pentacyclic spin-labeled α-chymotrypsin is shown in Figure 4. Note that the amplitude of the high-field line is still significantly diminished even at high guanidine hydrochloride concentrations. For this reason the high-field resonance line was not considered sufficiently sensitive to serve as a quantitative indicator of the mobility of the enzyme-bound label. However, in this case the low-field line, labeled w in Figure 2, does change in a fashion which permits its use as a quantitative indicator of the degree of mobility of the enzyme-bound radical as guanidine hydrochloride concentration is increased.[16]

The change of mobility of the two labels on α-chymotrypsin with guanidine hydrochloride concentration is shown in Figure 5. There are three noteworthy features of this figure. (1) The concentration of guanidine hydrochloride required to bring about unfolding of the two derivatives is quite different, with CT-PCSL being the more stable. (2) For each of the two derivatives, the guanidine hydrochloride concentration required for unfolding is essentially pH independent. This is an extremely surprising observation. It is well known that protein denaturation is almost always pH dependent[17] and, therefore, a pH dependence was expected for the phenomenon being observed here. (3) The mobility of

(16) Correlation of the ratio c/b with relative mobility for the MCSL and the ratio w/x with the mobility for the PCSL is an empirical relationship. It is based on the observation that for the first derivative resonance spectrum of a nitroxide, the relative heights of the low-field and high-field resonance lines (corresponding to a and c in Figure 2) increase with decreasing viscosity of the environment. See ref 11.

(17) H. A. Scheraga, "Protein Structure," Academic Press, New York, N. Y., 1961, pp 81–128.

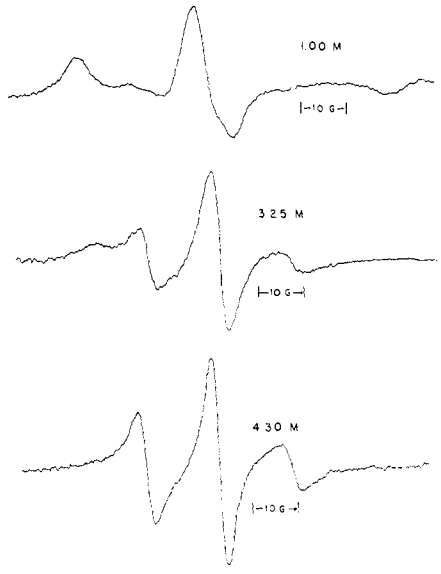

Figure 4. Change in epr spectrum of PCSL-chymotrypsin in solutions of progressively increasing guanidine hydrochloride concentration at pH 3.0. Since even at high guanidine concentrations this label has a significantly decreased mobility, the height of the low-field line labeled "w" in Figure 2 relative to the center-field line was used to measure mobility changes.[16] It is of particular interest to compare the top spectrum (1.00 *M*) with the lower spectrum of Figure 2.

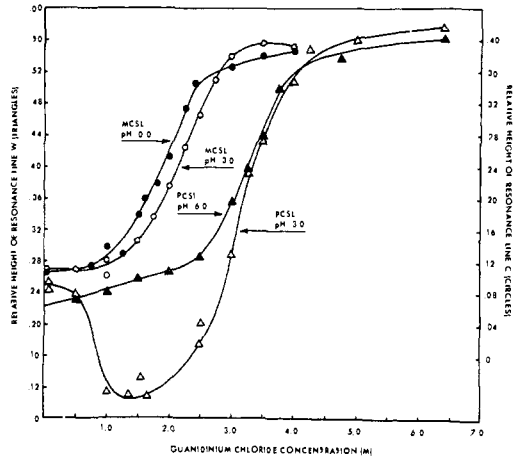

Figure 5. Change of electron spin resonance spectrum of spin-labeled α-chymotrypsins in solutions of increasing guanidine hydrochloride concentration: ○, MCSL-chymotrypsin at pH 3.0; ●, MCSL-chymotrypsin at pH 6.6; △, PCSL-chymotrypsin at pH 3.0; ▲, PCSL-chymotrypsin at pH 6.6.

the pentacyclic label actually decreased at low guanidine hydrochloride concentrations at pH 3.0 and then increased as the guanidine concentration was increased further.

Although close examination of the spectrum of CT-MCSL indicates a very small decrease in mobility at low guanidine hydrochloride concentrations, the change is almost negligible compared to that of the pentacyclic label.

These observations could not be interpreted correctly without additional data, so we found it necessary to study the denaturation phenomena by an independent technique. Optical rotatory dispersion and circular dichroism changes were each tested as possible indicators of denaturation. Similar results were obtained

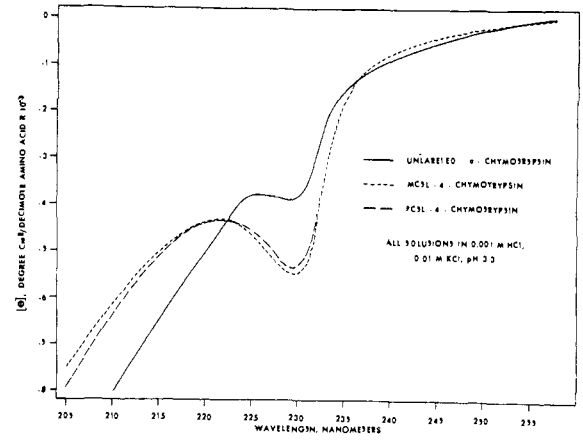

Figure 6. Circular dichroism spectra of: ——, α-chymotrypsin; – – –, MCSL-chymotrypsin; and — —, PCSL-chymotrypsin. All spectra were measured in 0.1 *M* KCl, pH 3.3.

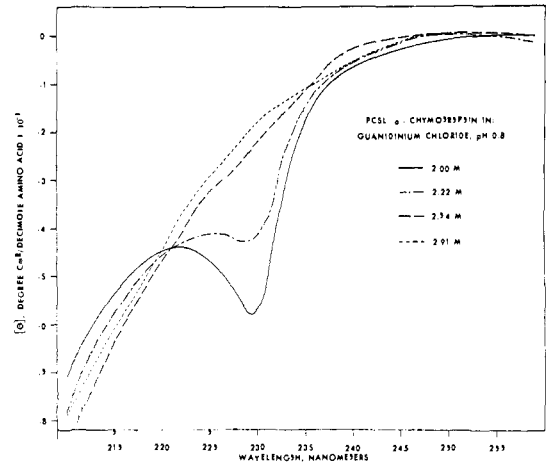

Figure 7. Change in the circular dichroism spectrum of PCSL-chymotrypsin as the concentration of guanidine hydrochloride is increased at 27° and pH 6.8: ——, 2.06 *M* guanidine; — · —, 2.22 *M* guanidine; — —, 2.74 *M* guanidine; – – –, 2.91 *M* guanidine. Under these conditions of temperature and pH, there is essentially no change in the spectrum below 2.0 *M* guanidine; above 2.9 *M* guanidine there is no further change. These spectral changes exemplify those seen in both derivatives (as well as the unlabeled enzyme) at both neutral and acidic pH.

with both methods. Circular dichroism was selected because the CD results were somewhat easier to quantitate.

The CD spectra of unlabeled α-chymotrypsin and both derivatives at low pH are shown in Figure 6. As with diisopropylphosphoryl chymotrypsin[18] and a number of other active-site-labeled derivatives (C. A. Broomfield and J. D. Morrisett, manuscript in preparation), the negative extremum at 230 nm is greater (*i.e.*, more negative) in these derivatives at low pH than in the unlabeled enzyme. When guanidine hydrochloride is added to solutions of either the labeled or unlabeled enzyme, the extremum at 230 nm decreases (becomes less negative) while the positive extremum at 222 nm remains essentially constant until very high concentrations of guanidine are reached. These changes are exemplified by the spectra in Figure 7, which are typical of the changes observed under all conditions tested. Thus, the difference in ellipticity at 230 nm and 222 nm can be used to

(18) M. Volini and P. Tobias, *J. Biol. Chem.*, 244, 5105 (1969).

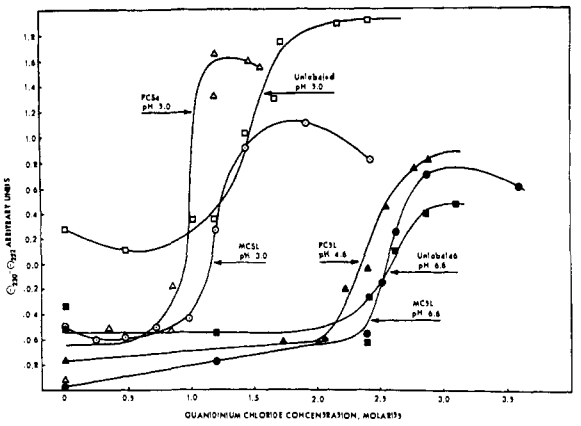

Figure 8. Change of circular dichroism spectra, as measured by the difference in ellipticity at 222 nm and 230 nm, with guanidine hydrochloride concentration: □, unlabeled α-chymotrypsin at pH 3.0; ■, unlabeled α-chymotrypsin at pH 6.6; ⊙, MCSL-chymotrypsin at pH 3.0; ●, MCSL-chymotrypsin at pH 6.6; Δ, PCSL-chymotrypsin at pH 3.0; ▲, PCSL-chymotrypsin at pH 6.6.

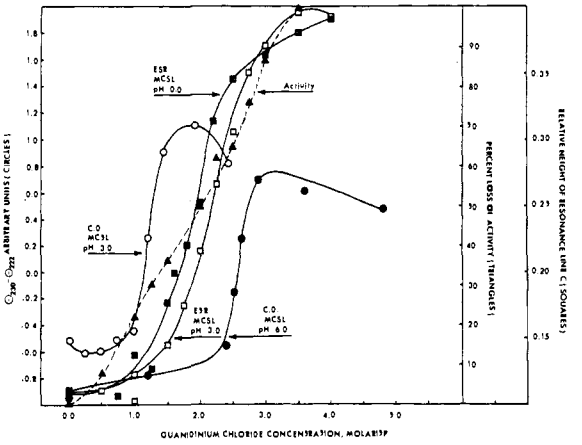

Figure 9. Changes in epr and CD spectra of MCSL-chymotrypsin in guanidine hydrochloride solutions at 27°: ○, CD changes at pH 3.0; ●, CD changes at pH 6.6; □, epr changes at pH 3.0; ■, epr changes at pH 6.6. For comparison, the loss of activity by α-chymotrypsin in guanidine hydrochloride at 27° and pH 6.8 is shown by the triangles and dashed curve.

quantitate the degree of unfolding as observed by circular dichroism.

The guanidine hydrochloride-induced denaturation of native CT, MCSL-CT, and PCSL-CT at two different pH conditions as observed by circular dichroism is shown in Figure 8. There are several features of these curves that merit special comment. First, in contrast to the epr results, the concentration of guanidine hydrochloride required to unfold each species is highly pH dependent, as would be expected for a denaturation process. Also, a much lower guanidine concentration is required for denaturation at low pH than high pH. Second, the spin-labeled derivatives denature at lower guanidine hydrochloride concentrations than the unlabeled enzyme. Finally, the pentacyclic-labeled molecule is conformationally less stable (as determined by CD) than the monocyclic-labeled enzyme. The displacement of the CD denaturation curve for the unlabeled enzyme at low pH to higher ellipticity values is a result of the spectral changes occurring when α-chymotrypsin is derivatized at the active site. This is shown in Figure 6.

In an attempt to rationalize the apparently contradictory results between the electron spin resonance data and the circular dichroism data, the results obtained from each derivative by both techniques were plotted separately. The data for the monocyclic-labeled derivative are shown in Figure 9. The loss of activity by the unlabeled enzyme in guanidine hydrochloride is shown for reference by the dashed line. It is noteworthy that the esr denaturation curves fall between the circular dichroism curves. That is, at low pH the molecule appears to be more stable when observed by electron spin resonance than when observed by circular dichroism, while at neutral pH the reverse seems to be the case. It should also be noted that the loss of activity follows the electron spin resonance denaturation curves somewhat more closely than it does the circular dichroism curves. The implication of these observations is that the two techniques are actually measuring conformational changes at separate parts of the molecule. This is clearly shown in the comparison of data on the pentacyclic derivative. These results are shown in Figure 10. The pentacyclic spin label on α-chymotrypsin at low pH initially

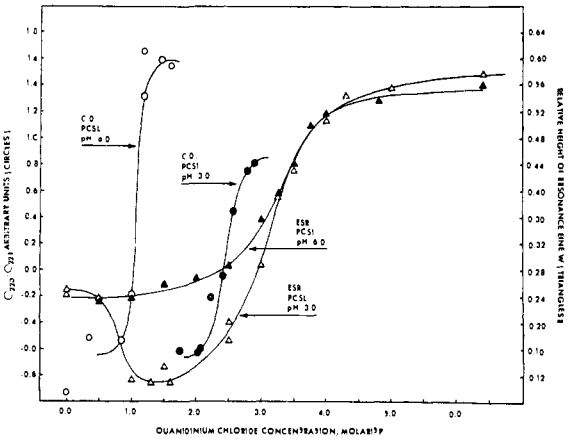

Figure 10. Changes in epr and CD spectra of PCSL-chymotrypsin in guanidine hydrochloride solutions at 27°: ○, CD changes at pH 3.0; ●, CD changes at pH 6.6; Δ, epr changes at pH 3.0; Δ, epr changes at pH 6.6.

decreases in mobility at low guanidine hydrochloride concentrations. Subsequently, the spin probe becomes more mobile as the concentration of guanidine hydrochloride is increased. This observation provides an important clue to the interpretation of the results shown here.

The question of whether the CD spectral changes reflect a general unfolding of the molecule or just loosening in a very limited region could, in principle, be answered by studies of viscosity changes under the same conditions. Unfortunately, the viscosities of solutions of spin-labeled derivatives at low pH in the guanidine hydrochloride concentrations of interest could not be measured due to aggregation of the protein. However, it appears (Figure 11) that the viscosity change levels off well before the change in esr, and then undergoes further changes above 4 *M* guanidine hydrochloride. At the same time, the viscosity continues to change between 2.4 and 2.8 *M* guanidine hydrochloride, well above the concentration required for the CD change. The dotted portion of the viscosity curve in Figure 11 is consistent with the data, but in the absence of critical points must be regarded as conjecture.

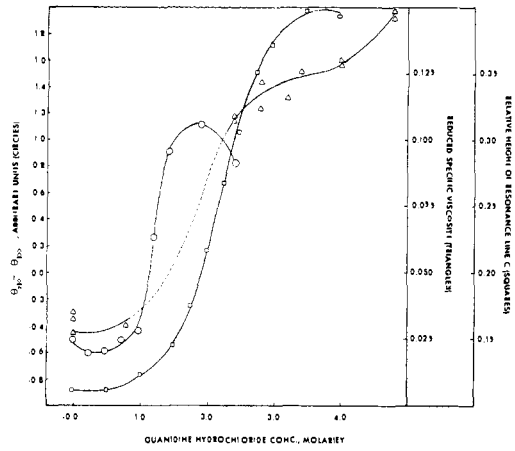

Figure 11. Changes of specific viscosity of MCSL-chymotrypsin in guanidine hydrochloride solutions at pH 3.0, compared with epr and CD changes observed under the same conditions. The dashed portion of the curve includes that region in which aggregation takes place (see text) and, therefore, there are no reliable data to substantiate its shape or exact position.

CT-MCSL is stable for at least 2 months when stored at pH 3 and 5°. At 25° and pH 3 the appearance of a mobile component becomes discernible after about 1 week. At pH 6.6, the rate of appearance of a spectral component indicating high mobility for the radical is significantly greater than at pH 3. In fact, at this higher pH, the spectrum changes rapidly enough to perform time studies. Four such studies are shown in Figure 12. As expected, the process is significantly more rapid at 25° than at 5°. As the change in relative height of the high-field resonance line c begins to level off, only about 7% of the original chymotryptic activity has been regained. In none of the spontaneous reactivation experiments did the recovery of activity exceed 13%. We attribute this low recovery of activity to digestion of unreactivated CT-MCSL by the reactivated chymotrypsin, digestion of the reactivated enzyme by itself,[19] and/or aging.[20] The important feature of these studies is that spontaneous epr spectral changes of CT-MCSL at pH 6.6 are not significant at times less than 2 hr. This gives ample time to carry out a denaturation experiment by CD, epr, or viscosity.

When samples of CT-MCSL and CT-PCSL which had been denatured by 4.0 *M* and 6.5 *M* guanidine hydrochloride, respectively, were dialyzed exhaustively against 0.1 *M* potassium chloride, pH 3.0, the epr spectra of the resulting solutions were identical with those obtained from the same samples before denaturation, thereby establishing the reversibility of the process for both derivatives.

Discussion

It is apparent from these results that electron spin resonance spectroscopy is reflecting changes in a different part of the molecule from that observed by circular dichroism or optical rotatory dispersion. Since the spin label is attached to the active site serine, it is reasonable to assume that those changes observed by electron spin resonance accurately reflect changes in the active site region of the enzyme. Furthermore,

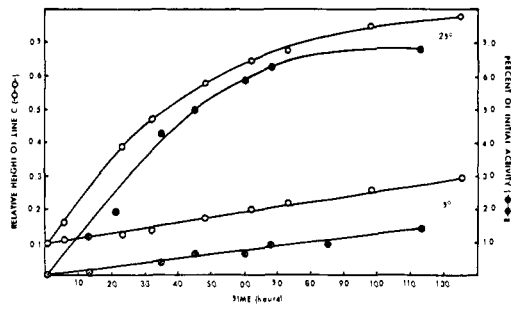

Figure 12. Spontaneous release of spin label by MCSL-chymotrypsin at pH 6.6 at two different temperatures. The upper curves represent the data at 25°, the lower curves the data at 5°.

the size and shape of the monocyclic label approximates that of the *p*-toluenesulfonyl group used by Blow and his associates[21] to tag the active site serine for crystallographic studies and, therefore, probably occupies the same space, and has the same or similar orientation as that group.[15] Therefore, one may safely assume that any structural changes indicated by the monocyclic spin label are very similar to those changes at the active site of the native functioning enzyme, particularly in the presence of a substrate. This is borne out by the fact that activity loss follows so closely the electron spin resonance change (Figure 9). On the other hand, the source of the observed circular dichroism transition is not entirely clear at this time; therefore, the interpretation of circular dichroism changes in terms of conformational changes of particular portions of the protein molecule is necessarily tenuous. It is obvious that circular dichroism does not reflect the same structural changes, *i.e.*, those at the active site, as electron spin resonance does. However, the observation that forming a derivative at the active site serine brings about a structural change reflected by circular dichroism (Figure 6) implies that the structural change must take place very near the active site region. Examination of circular dichroism changes resulting from the activation of chymotrypsinogen to α-chymotrypsin leads one to suspect that the structural changes observed by circular dichroism upon denaturation are in the same region of the molecule as the changes that occur upon activation. Biltonen, *et al.*,[22] have suggested that the ORD changes observed upon activation of the zymogen result from local environmental changes of specific chromophoric groups rather than large conformational changes. This would also be true for CD changes, since the two phenomena are closely related.

The results described in the previous section are most easily rationalized if one accepts the hypothesis that the minimum at 230 nm in the CD spectrum arises from a local chromophore environment. The sensitive chromophore must reside in one of the regions that undergoes structural change upon activation of chymotrypsinogen, or at least the chromophore must be in a position to be influenced by one of these changes. It must also be in a position to be influenced directly or indirectly by small conformational changes brought about by substitution at the active site. We have

(19) A. N. Glazer, *Proc. Nat. Acad. Sci. U. S.*, **64**, 235 (1969).

(20) In this case, aging would involve the release of the spin label from the enzyme by scission of the P–O or O–C bond. The phosphorus moiety remains attached to the active site serine residue.

(21) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature* (*London*), **221**, 337 (1969).

(22) R. Biltonen, R. Lumry, V. Madison, and H. Parker, *Proc. Nat. Acad. Sci. U. S.*, **54**, 1412 (1965).

shown (C. A. Broomfield and J. D. Morrisett, manuscript in preparation) that a number of different substituents at the active site, either on serine-195 or histidine-57, bring about CD and ORD spectral changes similar to those shown in Figure 5. In the terminology of Biltonen, *et al.*,[22] this would be the transition Aa $\rightleftharpoons$ A which they have studied in some detail.[23,24] Therefore, it is not likely that the chromophore is influenced directly, but rather its environment is changed by small conformational changes. The number of chromophores which fit these criteria is quite limited. Based on examination of stereoscopic drawings of the three-dimensional structures of chymotrypsinogen[25] and α-chymotrypsin,[14] it appears that the only chromophores likely to be involved are methionine-192, tyrosine-146, and tryptophan-141. Tryptophans-27 and -29 are also in the N-terminal tail of the B chain, but are several residues removed from the end and would probably not be affected by structural changes upon activation. Tryptophan-215 is near the active site, but could not interact with residues on the B-chain terminus. Biltonen, *et al.*,[22] have shown that oxidation of methionine-192 does not change the ORD spectrum of α-chymotrypsin. We have shown (C. A. Broomfield and J. D. Morrisett, manuscript in preparation) that modification of tryptophan-215 or tyrosine-146 and -171 has no effect on the CD spectrum. This leaves tryptophan-141 as the most likely chromophore to be susceptible to environmental perturbations which produce the observed CD and ORD spectra. This conclusion is consistent with that of Williams, *et al.*,[26] and of Oppenheimer, *et al.*,[27] that although the number of exposed tryptophyl residues remains the same upon reaction with DFP, there is a slight difference in the solvent access to one or two tryptophyls in the modified chymotrypsin compared with the unmodified enzyme. It is also consistent with the conclusion of Hess, *et al.*,[28] based on fluorescence quenching data, that there is a complex formed between histidine-40 and a tryptophan in the native, unmodified enzyme. It would appear, in the stereoscopic drawings of the chymotrypsin structure,[14] that histidine-40 is in such a position relative to tryptophan-141 that such a complex could form between these residues. Assuming this is the case, the experimental observations described in the Results section may be explained as follows. The monocyclic label occupies the same site, with approximately the same orientation, as the *p*-toluenesulfonyl group in the chymotrypsin crystal. The pentacyclic label, however, is too large to fit into the hydrophobic binding site and, therefore, is only partly buried. Reaction of α-chymotrypsin with either of these inhibitors brings about the same changes in the CD spectrum because the spectral change is not brought about by direct interactions between the phosphonyl substituent and the chromophore, but rather by small conformational changes resulting from interruption of the charge relay system between Asp-102, His-57, and Ser-195.[13,14]

When guanidine hydrochloride is added at low pH, the protein begins to unfold, beginning at the ends of the chains. Because tryptophan-141 (or possibly another chromophore) is in the midst of several chain termini, it will be influenced during very early stages of denaturation and, therefore, the unfolding measured by circular dichroism is observed at very low guanidine hydrochloride concentrations. The spin labels, on the other hand, are in a more structurally stable portion of the molecule, and because they form many hydrophobic bonds with the enzyme, they actually help to stabilize that portion of the molecule. In fact, we feel that the pentacyclic label, which probably is initially partially excluded from the specificity site because of its excessive size, is able to plunge all the way into the hydrophobic pocket when the tertiary structure is loosened somewhat by low concentrations of guanidine hydrochloride. By virtue of a large number of hydrophobic contacts formed in this manner, the spin label appears to become almost, if not completely, immobilized with respect to the protein. At the same time it is reasonable to expect that the active center portion of the molecule is stabilized by the pentacyclic label to a much greater extent than it is with the monocyclic label. Thus half of the PCSL-CT is unfolded at about 3.2 *M* guanidine hydrochloride, whereas only about 2.0 *M* is required for the same amount of unfolding in the MCSL-CT. Since the portion of the enzyme interacting with the spin labels is hydrophobic, the lack of pH dependence for breaking up the interaction may be understood.

Examination of the epr spectra of CT-PCSL (Figures 5 and 10) raises the question of why the spectrum at pH 6.6 does not exhibit the same initial decrease of mobility as that at pH 3.0. Those results may be rationalized by the following argument. The transition observed by CD at pH 3.0 reflects a degree of hydrophobic bond disruption sufficient to allow a limited amount of unfolding in the region of the CD-sensitive chromophore. This same degree of disruption is also sufficient to open the active site region to a great enough extent to allow the PCSL to plunge well into the interior of the enzyme, experiencing decreased mobility as a result of *new* hydrophobic or steric interactions. At pH 6.6, however, a higher guanidine hydrochloride concentration is required to bring about the CD-monitored transition because the hydrophobic interactions involved in maintaining the structure in that region are augmented by hydrogen bonds and ionic interactions (*e.g.*, salt bridges) which are formed at this pH, but not at pH 3.0. It is probable that the concentration of denaturant required to bring about the transition observed by CD at pH 6.6 is so high (more than 2.0 *M*, see Figure 10) that the hydrophobic interactions at the active site necessary to decrease the mobility of the spin label are precluded.

The epr spectrum of CT-PCSL at pH 3.0 with no guanidine present is interesting for several reasons. The spectrum very closely resembles that observed by Stryer and Griffith[12] for dansyl nitroxide in 90% glycerol–5% water–5% ethanol at 35°. Using fluorescence polarization, they calculated the rotational correlation time of the radical to be 36 nsec, assuming

(23) R. Biltonen and R. Lumry, *J. Amer. Chem. Soc.*, **91**, 4251 (1969).
(24) R. Biltonen and R. Lumry, *ibid.*, **91**, 4256 (1969).
(25) S. T. Freer, J. Kraut, J. D. Robertus, H. T. Wright, and Ng. H. Xuong, *Biochemistry*, **9**, 1997 (1970).
(26) E. J. Williams, T. T. Herskovits, and M. Laskowski, Jr., *J. Biol. Chem.*, **240**, 3574 (1965).
(27) H. L. Oppenheimer, J. Mercouroff, and G. P. Hess, *Biochem. Biophys. Acta*, **71**, 78 (1963).
(28) G. P. Hess, J. McConn, E. Ku, and G. McConkey, *Phil. Trans. Roy. Soc. London, Ser. B*, **257**, 89 (1970).

that the rotational relaxation time of the nitroxide moiety is identical with that of the fluorescent dansyl group. Significantly, Gerig[29] has reported nmr studies on the binding of tryptophan to α-chymotrypsin and from these studies he concludes that D-tryptophan is bound so tightly that it assumes the same overall rotational characteristics as the enzyme in solution. From the nmr data he calculates a correlation time of 36 nsec, exactly that value obtained by Stryer. The observation that the pentacyclic label, when attached to the enzyme, *can* become more immobilized (Figures 5 and 10) means that, initially it does not quite have the same rotational characteristics as the chymotrypsin molecule. This would suggest that (1) the rotational relaxation times of the two portions of the dansyl nitroxide are not exactly equivalent and/or (2) D-tryptophan does not quite have the same rotational properties as the enzyme to which it is bound.

There is another explanation for these observations that deserves comment. As mentioned previously, there is a tendency for these derivatives to aggregate at low pH, particularly between guanidine hydrochloride concentrations of 0.6 *M* and 2.0 *M*. This concentration range coincides for the most part with the region of decreased mobility of the CT-PCSL. If, as Gerig suggests,[29] the rotational characteristics of the α-chymotrypsin monomer are those reflected by the 36-nsec correlation time, then the decreased mobility could merely reflect the increased size of the protein aggregates. However, there is evidence that this is not the case. Although the length of time required for a given solution to acquire a certain amount of turbidity was not highly reproducible (this time is somewhat dependent upon conditions of mixing), it was found that usually there was no significant increase in turbidity, as measured by scattering of light at 320 nm, in less than 20 min after mixing at the protein concentrations used for epr measurements. Special effort was made to make all measurements as soon as possible after mixing those solutions in which turbidity was expected to develop. There was no evidence that the development of the decreased mobility was time dependent prior to the visible formation of aggregates. And finally, there was no evidence of decreased mobility in those solutions of MCSL-CT that eventually precipitated (though this could be explained by the observation that MCSL has considerable freedom of motion with respect to the protein, anyway, and thus probably would not be affected by aggregation). These observations do not obviate the possibility that particles might form very soon after mixing which are very large compared to monomeric α-chymotrypsin, but yet too small to cause a significant amount of light scattering at 320 nm. This possibility is remote, however, because the shape of the precipitation curves suggests a nucleation–growth mechanism for the process.[30] In this mechanism, growth is very rapid once stable nuclei are formed, and few of the molecules are present in aggregates during the lag phase (*i.e.*, the period during which our measurements were made).

The inhibition of esterases by organophosphorus compounds is sometimes, albeit mistakenly, thought to be irreversible. Typically, measuring recovery of activity has been the means for determining the extent of spontaneous reactivation by dephosphorylation or dephosphonylation. The stability studies shown in Figure 11 clearly show that dephosphonylation of CT-MCSL at pH 6.6 does occur and at a significant rate. We have observed that for both CT-MCSL and CT-PCSL, increasing the pH from 6.6 to 8.0 results in vastly increased rates of spin label release. In fact, we could obtain no meaningful information from studies on either CT-MCSL or CT-PCSL at pH 8.0. Thus, it is questionable whether results obtained from CT-DIP at this or higher pH values are unambiguous.[19] Nevertheless, the stability of CT-MCSL appears to be much greater in the range pH 6–7 than the corresponding acyl derivatives reported by Berliner and McConnell[31] and Kosman, *et al.*[32] It is for this principal reason that the phosphorus spin labels are generally the more useful.

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(30) G. C. Wood, *Biochem. J.*, **75**, 598 (1960).

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