Circular Dichroism Studies of Chymotrypsin and Its Derivatives

CORRELATION OF CHANGES IN DICHROIC BANDS WITH DEACYLATION

(Received for publication, March 27, 1969)

MARGUERITE VOLINI* AND PETER TOBIAS†

From the Institute for Biomedical Research, American Medical Association, Education and Research Foundation, Chicago, Illinois 60610

SUMMARY

The kinetics of the deacylation of trimethylacetyl chymotrypsin were followed by direct observation of changes in the circular dichroism spectrum of the enzymic protein itself. These changes result either from an alteration of the protein conformation or from direct perturbation of aromatic or cystinyl residues in the active site of the enzyme.

The mechanisms by which enzymes effect bond formation and cleavage have been revealed in remarkable detail by kinetic analyses of substrate and product turnover. In addition to substrate- and product-based observations, a complete description of the mechanism must also include direct observations of the enzyme protein during reaction to determine the relevance of structural changes to the catalysis. In careful studies, Wooton and Hess (1) and Himoe, Brandt, and Hess (2) have been able to monitor the breakdown (deacylation) of the covalent enzymic intermediate, monoacetyl chymotrypsin, by measurement of changes in absorption spectra which they attribute to perturbations of an enzymic tryptophyl residue. The magnitude of the changes, however, is small, amounting to about 1% of the total absorbance.

Circular dichroism spectra, since they arise from the preferential absorption by a chromophore of right or left circularly polarized light, have the inherent possibility of displaying large differences under perturbing conditions in which changes in the corresponding absorption spectra are small. This possibility is readily deduced from a comparison of the formulations for the dipole strength of an absorption band and, its counterpart, the rotational strength of a circular dichroism band (see "Discussion"). For the derivative, trimethylacetyl chymotrypsin, this possibility is realized and the present studies take advantage of this feature.

The deacylation of trimethylacetyl chymotrypsin was observed by measurement of changes in circular dichroism spectra. The changes in the derivatized enzyme were of the order of 20 to 30% when compared to unsubstituted chymotrypsin. They result from perturbations of tyrosyl, tryptophyl, or cystinyl (or all three) residues of the enzyme protein since the intermediate contains no substrate-derived chromophore in the wave length region. To implement the interpretation of changes noted on deacylation, time course studies on the CD spectrum of α-chymotrypsin were carried out as a function of pH and the spectrum of the stable intermediate, diisopropylphosphoryl chymotrypsin was also examined. The DIP-intermediate, like trimethylacetyl chymotrypsin contains no substrate-derived chromophore that absorbs in the region of interest. Unlike the trimethylacetyl derivative, its deacylation rate is negligible when water serves as the attacking nucleophile. Differences in the CD spectra for both derivatives compared to α-chymotrypsin resulted from perturbations of the protein functions and were identical in the region from 325 to 215 μm. Hence, the DIP-derivative provided a fixed state with which to compare ellipticity changes noted with the labile trimethylacetyl chymotrypsin.

EXPERIMENTAL PROCEDURE

Enzyme Preparations—Crystalline α-chymotrypsin and DIP-chymotrypsin were obtained from Worthington. Trimethylacetyl chymotrypsin was prepared from α-chymotrypsin and reccrystallized p-nitrotrimethylacetate. Stock solutions of the enzyme and its derivatives were made in 0.1 M KCl adjusted to pH 3.55 with HCl and kept at 0°C. Samples of the stock solutions were diluted in Tris-acetate buffers of 0.1 ionic strength at the appropriate pH. All enzyme solutions for the CD studies were adjusted to protein concentrations of 0.9 ± 0.1 mg per ml. Concentrations were determined spectrophotometrically at 282 μm using a molar absorptivity coefficient of 5 × 104.

Spectra—CD curves were measured on the 6001 CD attachment for the Cary model 60 spectropolarimeter. Absorption spectra were recorded with a Cary model 14 spectrophotometer. Since protein concentrations were kept constant, ellipticities and absorbances varied inversely with wave length. Spectra were divided into three overlapping regions for examination. For most studies, the cell path lengths were (a) 0.016 cm in the region from 260 to 195 μm, (b) 0.051 cm from 270 to 215 μm, and (c) 2.00 cm from 325 to 250 μm. These conditions maximized difference ellipticities for α-chymotrypsin and the derivatives at protein concentrations suitable for kinetic work.

* Postdoctoral Fellow supported by the Education and Research Foundation of the American Medical Association. Present address, Department of Biochemistry, University of Chicago, Chicago, Illinois 60610.
† United States Public Health Service Trainee GM-424, Department of Biochemistry, University of Chicago, Chicago, Illinois 60637.

The abbreviations used are: CD, circular dichroism; DIP-, diisopropylphosphoryl-.
To ensure that the effect of autodigestion (see "Results") on the initial spectra was negligible, separate samples of the stock enzyme solutions were diluted in buffer immediately before examination in each of the three wave length regions. Both CD and absorption spectra were recorded for each sample. The samples were kept at room temperature and their CD spectra were re-examined over a period of 30 hours. Typical of the absorbances used in the CD experiments are those for the experiment in Fig. 1. In A, the absorbance at 202 nm was 1; in B, the absorbance at 229 nm was 0.4; in C, at the positive CD maximum (297 nm), the absorbance was 1.5, and 1.9 at the negative CD maximum (260). In regions of high absorption, the CD spectra were shown to be free from artifact by tests run routinely in this laboratory using d 10 camphorsulfonic acid in tandem with an absorber and, also, by examination of the sample spectra in cells of shorter path length. In all cases ellipticities were proportional to path length. Base-lines were recorded for all CD spectra. Molar ellipticities were calculated on a mean residue weight basis.

Enzyme Activity—Aliquots of the same samples used for the CD measurements were removed for enzymic assay. The assay mixture contained 2.85 ml of Tris-acetate buffer, pH 8.0 (0.1 ionic strength), 0.25 mmole of p-nitrophenyl acetate in 0.05 ml of acetonitrile, and a 0.1-ml sample aliquot. Spontaneous rates were determined in the same system using 0.1 ml of buffer without enzyme at the experimental pH. Rates were measured on the Cary model 14 equipped with a 0 to 0.5 optical density slide channel of which contained distilled water. Sedimentation centrifugation was performed at 25° in a double sector cell, one column, 16 X 1.0 cm, of Sephadex G-25 were equilibrated with Tris-acetate buffers at the pH of the enzyme incubation mixture. The centrifugation was performed at 59,780 rpm for 2 to 3 hours in a Spinco model E ultracentrifuge equipped with a photoelectric scanner. The centrifugation was performed at 25° in a double sector cell, one channel of which contained distilled water. Sedimentation coefficients were calculated from at least 6 scans taken at 15-min intervals.

RESULTS

CD Spectra of cr-Chymotrypsin and Its Derivatives

pH Studies—Fasman, Foster, and Beychok (4) have reported the CD spectrum of a-chymotrypsin at pH 6.8 in the wave length region from 325 to 217 nm. In the present studies, spectra were recorded at nine pH values and were extended to 195 nm which allowed characterization of an additional negative band at 202 nm. Fig. 1 shows the CD pattern of a-chymotrypsin at pH 4.8. In the wave length region from 325 to 195 nm, negative maxima are centered at 305, 262, 229, and 202 nm, respectively. Two positive maxima separated by a positive minimum occur at 297, 288, and 292 nm, respectively. The fine structure near 290 nm had not previously been detected. Over the range pH 3.8 to 9.3 in buffers of constant ionic strength, the positions of the extrema were the same as those at pH 4.8, but the ellipticities of the bands at 229 and 202 nm were altered. In Table I, the ellipticities obtained for a-chymotrypsin and the DIP-derivative are listed for pH 3.8 and 9.0.

In contrast to a-chymotrypsin, the magnitudes of the bands for the DIP-derivative were invariant at the extremes of the pH range. Qualitatively, the CD spectrum of the DIP-derivative was the same as that observed with the unsubstituted enzyme but, quantitatively, at alkaline pH the derivative showed increased ellipticities for bands in the region from 300 to 220 nm (Table I). No change in the magnitude of the extremum at 202 nm was observed. At pH 9.0, the 297 - 260 nm difference ellipticity for

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$\theta_{202\text{nm}}$</th>
<th>$\theta_{229\text{nm}}$</th>
<th>$\theta_{202\text{nm}}$</th>
<th>$\theta_{229\text{nm}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-Chymotrypsin...</td>
<td>2.35</td>
<td>2.35</td>
<td>2.35</td>
<td>2.35</td>
</tr>
<tr>
<td>DIP-Chymotrypsin.</td>
<td>2.35</td>
<td>2.35</td>
<td>2.35</td>
<td>2.35</td>
</tr>
</tbody>
</table>

Table I

Ellipticities of dichroic bands in a-chymotrypsin and DIP-chymotrypsin at pH 3.8 and pH 9.0.
Fig. 2. The pH dependence of the rate of ellipticity change in aged samples of \( \alpha \)-chymotrypsin. The slope referred to in the ordinate is proportional to the first order rate constants calculated from individual plots of \( \log [\theta]_T - [\theta]_260 \text{ nm} \) versus \( t \). In accord with the results of Bender et al. (7), it is assumed that the enzyme undergoes a slow, rate-limiting process (conformational change) followed by a fast hydrolytic reaction which accounts for the first order behavior observed at least up to 6 hours. With the reaction sequence formulated as

\[
EH \xrightarrow{K_1} E \xrightarrow{k_{12}} E' \xrightarrow{k_{13}(EH)} E_{\text{hydrolyzed}}
\]

the curve shown here can be described by the relation:

\[
\log [\kappa' \lambda_2] = \log [\kappa_4] - \log \left( 1 + \left( \frac{[H^+]}{[K]} \right) \right)
\]

The intersection of the solid lines indicates the pK of a group important for the rate-limiting process.

Fig. 3. The time dependence of ellipticity changes in trimethylacetyl chymotrypsin (•—•) and DIP-chymotrypsin (×—×) at pH 9.0 in Tris-acetate buffer, 0.1 ionic strength. The concentration was 0.9 mg per ml for both derivatives as determined from the optical density at 282 nm. The derivative represents an increase of 20% over that of \( \alpha \)-chymotrypsin. The ellipticity of the derivative band at 229 nm for \( \alpha \)-chymotrypsin at acidic pH appears to approach that of the DIP-derivative as a limit.

Fig. 4. Decacylation of trimethylacetyl chymotrypsin at pH 9.0 as determined by changes in (a) enzyme activity and (b) ellipticity. In b, •—• refers to \( \log A_{297} = 160 \text{ mlr} \) where

\[
X-X \text{ refers to } \log A_{260} = 129 \text{ mlr}
\]

where \( A = \frac{[\theta]_260 \text{ nm} - [\theta]_229 \text{ nm}}{[\theta]_260 \text{ nm} - [\theta]_229 \text{ nm}} \)

The reaction of interest is: \( E \rightarrow trimethylacetyl_2 \rightarrow E + trimethylacetic \) acid. The appearance of enzyme activity is a measure of decacylation since acylated enzyme is inert. The sample concentration was 0.9 mg per ml in Tris-acetate buffer, pH 9.0, 0.1 ionic strength. Rate constants were calculated from \( k = (2.3) \) (slope). Slope values were obtained from the linear regression of the ordinate values on time.

Time Course Studies—In all experiments, samples were re-examined over a period of 30 hours to determine whether there were spectral changes resulting from reactions other than decacylation. The ellipticities of bands in the unsubstituted enzyme decreased slowly with time. The rate of change appears to depend on a group with a pK close to 9.0 (Fig. 2). Gel filtration experiments on Sephadex G-25 showed that the loss resulted from autodigestion of the enzyme. When incubated samples which exhibited decreased ellipticities were fractionated, a partial separation into intact and fragmented protein was obtained. In those fractions corresponding to intact protein, the molar ellipticities were the same as in the original sample. For DIP-chymotrypsin, the original ellipticity values were invariant with time, indicating that autodigestion of this derivative did not take place.
CD Spectrum of Trimethylacetyl Chymotrypsin

Time Course Studies—As noted previously, trimethylacetyl chymotrypsin displayed a CD spectrum identical with the DIP-derivative in the region from 325 to 215 μm both in position and magnitude of the bands.4 (The wave length region below 215 μm was not examined for trimethylacetyl chymotrypsin.) In contrast to the DIP-derivative, a time-dependent decrease in ellipticities was observed with trimethylacetyl chymotrypsin (Fig. 3). The logarithmic curve shows an initial rapid decrease that can be attributed to deacylation followed by a slow decrease resulting from autodigestion of the regenerated free enzyme. A comparison of rate constants calculated from the initial ellipticity changes measured at 287 - 260 μm and 260 - 229 μm and from changes in enzymic activity as determined on the same sample (Fig. 4) showed that the decrease in ellipticity resulted from deacylation of the intermediate.7

Sedimentation Studies on α- and DIP-chymotrypsin—Sedimentation studies showed that derivatization does not affect the polymerizability of the enzyme at pH 9.0 under the same conditions used in the CD experiments.8 Identical s20,w values of 2.2, corresponding to that expected for monomer, were obtained for both α-chymotrypsin and the DIP-derivative.

Discussion

In the wave length region from 325 to 260 μm, the CD bands of α-chymotrypsin can be assigned to enzymic tyrosyl, tryptophyl, or cystinyl (or all three) residues. A histidine residue may also be a contributor to the band at 229 μm (10). Phenylalanine is unlikely as the spectra do not display its characteristic fine structure near 260 μm. The low helix content of α-chymotrypsin as determined in the crystal (11), and as calculated from optical rotatory dispersion data (4) suggest that the n-π* transition of the peptide chromophore makes only a minor contribution to the band at 229 μm.

In contrast to the absorption spectra, the CD spectra of the derivatized chymotrypsins display relatively large changes when compared to those of the unsubstituted enzyme. The difference in magnitude of the changes can be explained by a consideration of the dipole strength of the absorption band relative to the rotational strength of its corresponding CD band. The dipole strength, D, is proportional to the area under the absorption curve. It can be calculated from the relation

\[ D = 0.92 \times 10^{-6} \int_0^\infty \frac{\varepsilon(\lambda) \lambda}{\sin (\lambda)} d\lambda = |\vec{\mu}_e|^2 \]

where ε is the extinction, λ is the wave length, and \( \vec{\mu}_e \) is the electric transition dipole moment. In turn the rotational strength, R, is proportional to the area under the CD curve. It can be calculated from the relation

\[ R = 0.696 \times 10^{-6} \int_0^\infty \frac{\theta(\lambda) \lambda}{\sin (\lambda)} d\lambda = Im \{|\vec{\mu}_e| \vec{\mu}_e| \cos \beta\} \]

where \( \theta \) is the ellipticity, λ is the wave length, Im means the imaginary part, \( \vec{\mu}_e \) is the electric transition dipole moment, and \( \vec{\mu}_e \) is the magnetic transition dipole moment, and \( \beta \) is the angle between the latter two. It is apparent that even under perturbing conditions where the electric transition moment and, thus, the magnitude of the absorption band is not greatly altered, an increase in the magnetic moment or a change in relative orientation of the electric and magnetic moments can result in a large change in the rotational strength and, as a consequence, in the magnitude of the CD band.

The spectral changes noted on deacylation could arise from (a) a conformational change in the enzyme, (b) vici nal perturbations resulting from the derivative moiety, or (c) a change in the state of aggregation of the enzyme. The latter possibility was eliminated by the sedimentation studies. Furthermore, changes in aggregation caused by concentration effects were eliminated by keeping the protein concentration at 0.9 ± 0.1 mg per ml. The fact that the spectral changes, relative to α-chymotrypsin, were identical for the two derivatives suggests that the enzyme protein undergoes a conformational change on deacylation. This conclusion, based only on the data reported here, must be qualified since contributions of the disisopropylphosphoryl or trimethylacetyl functions as vicinal perturbing groups may not be greatly dissimilar (12). However, evidence consistent with the conclusion that the band at 229 μm results from a conformational change in the protein comes from the work of Himoe et al. (2), Oppenheimer, Labouesse, and Hess (13), and McConn et al. (14). These investigators have demonstrated pH-dependent equilibria between an active and an inactive form of the enzyme. Their data indicate that the active conformation is stabilized by a positively charged amino group with a pK (apparent) of approximately 9.0. In the DIP-derivative as well as in enzyme-substrate complexes formed with specific substrates, the pK of this group is shifted to a higher value with the result that the active form of these compounds is stabilized at alkaline pH values where α-chymotrypsin exists primarily in the inactive form. In the present work, the pH dependence of the ellipticity at 229 μm in α-chymotrypsin, as well as the observation that the value approaches that of the derivatized enzyme as a limit at acidic pH, are in accord with the findings of Himoe et al. (2), Oppenheimer et al. (13), and McConn et al. (14).

The difference ellipticity at 297 - 260 μm does not show a dependence on pH in this range, however, further studies with derivatives containing aromatic moieties, such as cinnamoyl chymotrypsin, should distinguish whether these changes result from actual movement of the protein chromophore or from direct perturbations by the derivative moiety. If the changes are caused by residues impinging on the active site of the enzyme, then reciprocal relations with transitions in, for example, the cinnamoyl moiety should be observed (15, 16). Such coupling of transitions can provide information on the geometry of groups in the active site region (17).

Wooton and Hess (1) and Himoe et al. (2) have suggested the involvement of a tryptophyl residue in the acylation process. It is of interest to consider the magnitude of the ellipticity changes based on a single amino acid residue. For the band at 229 μm, the change in ellipticity on a molar protein basis was 2.3 x 10^4. The change at 297 - 260 μm was 6 x 10^4. These are large values; if assignable to vicinal perturbations of a single enzymic residue, they would indicate that the residue is approached within a few angstroms by the perturbing group on the active site serine.
The pH dependence of the autodigestion process as measured by CD kinetics is also in accord with that observed by Bender et al. (7) for irreversible denaturation as measured by enzyme activity. Furthermore, the rate constant for the deacylation of trimethylacetyl chymotrypsin as determined in the present studies, both from CD kinetics and from enzyme activity, is in agreement, within the range of statistical significance, with that calculated by Bender et al. (8) from \( k_{\text{cat}} \) of the enzymic reaction.

Acknowledgments—The major part of this work was carried out in the laboratory of Dr. Dan W. Urry. We wish to express our thanks to Dr. Urry for his guidance and counsel. We also wish to thank Professor John Westley and Professor F. J. Kezdy for helpful discussions and Dr. Clyde Goodheart for the use of the model E ultracentrifuge. We acknowledge the excellent technical assistance of Mr. Leo Barry.

REFERENCES
