Circular Dichroism Studies of Diethyl Pyrocarbonate-modified Histidine in Hen Egg White Lysozyme*

Chingwen Li, Dexter S. Moore, and Robert C. Rosenberg†

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From the Department of Chemistry, Howard University, Washington D.C. 20059

The single histidine residue (His-15) in hen egg white lysozyme (EC 3.2.1.17) was chemically modified by diethyl pyrocarbonate (DEPC) to form exclusively the mono-N-carbethoxylimidazole adduct (second order rate constant of 252 ± 16 m-1 min-1). Irreversible bis-carbethoxylation of the His-15 imidazole ring by DEPC was observed when lysozyme was pretreated with 2-mercaptoethanol (2-ME), 2-ME plus 8 M urea, or 2-ME plus 1% (w/v) sodium dodecyl sulfate (SDS). Circular dichroism difference spectra were measured for the mono-N-carbethoxylimidazole derivatives of lysozyme, N-acetyl-L-histidine, angiotensin-II, and O-carbethoxy-N-acetyl-L-tyrosine. The circular dichroism difference spectrum for mono-N-carbethoxy lysozyme had one main band (∆[θ]244 nm = +17,000 degree-cm2-dmol-1) in the 240–260 nm region. Denaturing mono-N-carbethoxy lysozyme with 2-ME and 8 M urea (55 °C) or 1% SDS (100 °C) essentially abolished its circular dichroism difference spectrum in the 240–260 nm region without any decarbethoxylation. In this same region the circular dichroism difference spectra of DEPC-modified N-acetyl-L-histidine and DEPC-modified angiotensin-II had two much weaker bands (∆[θ]233 nm = +1000 degree-cm2-dmol-1 and ∆[θ]222 nm = -600 degree-cm2-dmol-1 for N-acetyl-L-histidine). This study reports the first characterization of circular dichroism associated with mono-N-carbethoxylimidazole in an enzyme (lysozyme), a peptide (angiotensin-II), and a model compound (N-acetyl-L-histidine).

Diethyl pyrocarbonate (DEPC) is a group-specific reagent for the modification of histidine in the neutral pH range and has been extensively used to study the catalytic and structural role of histidine in enzymes (Miles, 1977; Nakanishi et al., 1984; Lambiris and Leadlay, 1981; Favorova et al., 1978; Cheng and Nowak, 1989; Sams and Matthews, 1983; Melchior and Fahrney, 1970; Loosemore and Pratt, 1976; Osterman-Golkar et al., 1974; Jawali and Bhagwat, 1987; Burstein et al., 1974; Wallis and Holbrook, 1973; Gomi and Fujioka, 1983; Morris and McKinley-McKee, 1972; Andersen and Ebner, 1979; Carrillo and Vallejos, 1983; Hegiy et al., 1974; Kita et al., 1982; Wijmans and Muller, 1982; Abdulwajid and Wu, 1986; Daron and Aull, 1982; Greenfield, 1974; Iglesias and Andrea, 1983; Bateman and Hersh, 1987; George and Borders, 1979; Vincent et al., 1975; Bhattacharyya et al., 1992; Zhang et al., 1992; and Pelton and Ganzhorn, 1992). In all of these studies, the reaction of DEPC with histidine was followed spectrophotometrically in the UV.

When DEPC is used in large excess, irreversible bis-carbethoxylation and subsequent cleavage of the imidazole ring (Fig. 1A) can occur (Miles, 1977). Except for some simple model systems (Loosemore and Pratt, 1976; Roosomont, 1975), few studies have focussed on the irreversible bis-carbethoxylated histidine and its significance in protein systems. Not only is the formation of irreversibly bis-carbethoxylated histidine related to quantitatively determining the amount of mono-N-carbethoxylimidazole formed, but, as is shown in this report, it can also provide structural information about the environment of the histidine residue under investigation. In this work, UV spectroscopic data are used to estimate the relative amounts of mono- and irreversibly bis-carbethoxylated histidine in human angiotensin-II and hen egg white lysozyme, both of which contain a single histidine residue. In order to understand the conditions under which irreversible bis-carbethoxylation occurs, techniques of protein denaturation were used to make the His-15 in lysozyme more accessible to DEPC.

Although circular dichroism (CD) has been widely used to study protein secondary structural changes, including those following DEPC modification (Nakanishi et al., 1984; Cheng and Nowak, 1989; Favorova et al., 1978), CD measurements associated with carbethoxylhistidine in proteins have not been reported. Most of the CD studies of DEPC-treated proteins demonstrated that modification with DEPC did not significantly alter the protein conformation, as judged by the content of α-helix, β-sheet, and random coil structures (Nakanishi et al., 1984; Cheng and Nowak, 1989; Favorova et al., 1978). Since the reaction of histidine with DEPC causes a substantial UV spectral change, and since a histidine in a protein is located in a chiral environment, it would be interesting to determine if there is any significant CD activity associated with mono-N-carbethoxyl histidine in proteins.

The single histidine residue (His-15) in hen egg white lysozyme (EC 3.2.1.17) occurs at the carboxyl end of helix 5–15 and is located on the surface of the enzyme well away from the active site (Banyard et al., 1974). A hydrogen bond is formed between the N1 of the His-15 imidazole ring and the hydroxyl group of Thr-89 (Phillips, 1974; see Fig. 1 for the imidazole ring numbering system used in this paper). Reaction of His-15 and the 6 lysines (at positions 1, 13, 33, 96, 97, and 116, all at the surface and away from the active site) in lysozyme with iodoacetic acid did not inhibit the glycosidic activity of the enzyme (Kravchenko et al., 1964). A role for His-15 in the esterase activity of lysozyme has been suggested.

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† To whom correspondence should be addressed. Dept. of Chemistry, Howard University, Washington, D.C. 20059.

The abbreviations used are: DEPC, diethyl pyrocarbonate; 2-ME, 2-mercaptoethanol.

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(Piszkiewicz and Bruice, 1968). Thus hen egg white lysozyme is a suitable simple system to further characterize the effects of the chemical modification of histidine by DEPC. Here we report difference ultraviolet absorption and difference ultraviolet CD studies on the reactions of DEPC with hen egg white lysozyme, N°-acetyl-L-histidine, and human angiotensin-II, an octapeptide containing a single histidine.

EXPERIMENTAL PROCEDURES

Materials—N°-Acetyl-L-histidine monohydrate, hydroxylamine hydrochloride, and (1R)-(−)-10-camphorsulfonic acid were purchased from Aldrich. DEPC, hen egg white lysozyme, N°-acetyl-L-tyrosine, 2-ME, human angiotensin-II, o-nitrophenyl acetate, and Micrococcus lysodeikticus suspension were from Sigma. Urea was from J. T. Baker Chemical Co., and SDS was from Bio-Rad.

Carbethoxylation by DEPC—UV absorbance spectra were measured on a Varian 2390 Spectrophotometer using a Varian DS-15 data station or on a Perkin-Elmer Cetus Instruments Lambda Array 3840 Spectrophotometer interfaced with a Perkin-Elmer Cetus Instruments 7300 Professional Computer System. The formation of mono-N-carbethoxyhistidine (Fig. 1A) was followed by the increase in absorbance occurring at 242 nm (εmax = 3200 M⁻¹ cm⁻¹ (Miles, 1977)). CD spectra for native and DEPC-modified samples were obtained on a JASCO J-41A Spectropolarimeter. (1R)-(−)-10-Camphorsulfonic acid was used as a standard for calibration of the CD measurements. Lysozyme concentration was determined spectrophotometrically (εmax = 3.9 × 10⁴ M⁻¹ cm⁻¹ at 281 nm (Yamasaki et al., 1968)). An aliquot of absolute ethanol or DEPC in absolute ethanol was added to the enzyme or other sample solutions, and the reaction was allowed to proceed until no further change was observed in UV or CD measurements. Hydroxylamine hydrochloride (0.35 M final concentration) was used to hydrolyze mono-N-carbethoxyhistidine. Although the decarbethoxylation of angiotensin-II was relatively fast, the decarbethoxylation for lysozyme was calculated from a nonlinear least squares fit of the UV data using the Math feature of the SigmaPlot (Statsoft, Inc.) and by the recovery of the original absorbance spectrum following the reaction of the DEPC-modified lysozyme with hydroxylamine (Fig. 2B). This behavior is characteristic of a mono-N-carbethoxylated histidine imidazole ring (Miles, 1977; Jawaili and Bhagwat, 1987; Favorova et al., 1978; Burstein et al., 1974). Comparable molar increases in absorbance

RESULTS AND DISCUSSION

Mono-N-carbethoxylation of Lysozyme and Angiotensin-II with DEPC—The reversible reaction of the histidine residue in hen egg white lysozyme with DEPC was identified by observation of an absorbance increase at 242 nm (Fig. 2A) and by the recovery of the original absorbance spectrum following the reaction of the DEPC-modified lysozyme with hydroxylamine (Fig. 2B). This behavior is characteristic of a mono-N-carbethoxylated histidine imidazole ring (Miles, 1977; Jawaili and Bhagwat, 1987; Favorova et al., 1978; Burstein et al., 1974). Comparable molar increases in absorbance
of lysozyme at each DEPC concentration were calculated using a fitting procedure that takes into account the rate of DEPC hydrolysis (Pelton and Ganzhorn, 1992). The deviation of the fitted lines from the experimental data at both 150- and 200-fold molar excess of DEPC over lysozyme (Fig. 3) could reflect the formation of a small amount (5%) of irreversibly bis-carbethoxylated product. A value of 0.126 ± 0.051 min⁻¹ was obtained for the pseudo first order rate constant for DEPC hydrolysis by taking a weighted average of the DEPC hydrolysis constants obtained from the fitting procedure at each initial DEPC concentration. As shown in Fig. 3 (inset), kobs shows a linear dependence on DEPC concentration. A second order rate constant of k = 252 ± 16 M⁻¹ min⁻¹ for the reaction of lysozyme with DEPC was calculated from the slope of the fitted line. The intercept on the kobs axis in the plot of kobs versus DEPC concentration was not statistically significant at the 95% confidence level. Spectrophotometric studies at 242 nm indicated that the reaction between DEPC human angiotensin-II or lysozyme was completed in less than 40 min. Thus His-15 in hen egg white lysozyme and the single histidine in angiotensin-II appear readily accessible to DEPC.

Irreversible Bis-carbethoxylation—To determine whether irreversible bis-carbethoxylation occurred, angiotensin-II or lysozyme was reacted with DEPC at increasing equivalents of DEPC to histidine (Fig. 4). The curves labeled with A (open triangles) for angiotensin-II and lysozyme (Fig. 4, a and b, respectively) show the observed ΔA₂₄₂nm data obtained one hour after the indicated equivalents of DEPC were added to angiotensin-II or lysozyme. The histidine in both angiotensin-II (Fig. 4a) or lysozyme (Fig. 4b) was not fully modified at low DEPC to histidine ratios (lower than 8 and 100 equivalents of added DEPC for angiotensin-II and lysozyme, respectively), as indicated by a smaller difference absorbance (ΔA₂₄₂nm) increase than would be expected if all the histidine residues had reacted to form mono-N-carbethoxyimidazole. At low equivalents of DEPC to histidine, rapid DEPC hydrolysis and other possible side reactions, such as reaction with the free amino group of the peptide or with the buffer, consumed much of the reagent, so there would be incomplete reaction between DEPC and the histidine residue. N-Carbethoxylation was pushed to a greater extent by increasing the equivalents of DEPC. At high equivalents of DEPC to histidine, significant irreversible bis-carbethoxylation was observed for angiotensin-II but not for lysozyme. As the number of equivalents of DEPC was increased, much larger ΔA₂₄₂nm values were observed for angiotensin-II (curve A in Fig. 4a). A shift of the difference absorption maximum from 242 nm to shorter wavelengths was also observed for angiotensin-II (data not shown) but not for lysozyme. Since the absorbance increase accompanying irreversible bis-carbethoxylation of a histidine residue is not reversible by treatment with NH₂OH, the carbethoxylated angiotensin-II and lysozyme formed at each value of added equivalents of DEPC were treated with NH₂OH to determine the fraction of irreversible bis-carbethoxylation. The curves labeled B (solid triangles) in Fig. 4, a and b, show the difference absorbance at 242 nm determined 4 h after the addition of NH₂OH to the samples represented by curve A in each figure. Curve C in Fig. 4, a and b (curve A minus curve B), reflects the amount of mono-N-carbethoxyhistidine present in each sample as a function of the initial added equivalents of DEPC. At a DEPC to histidine ratio of 200:1, 95% of the total DEPC-modified lysozyme was mono-N-carbethoxylated, whereas only 53% of the total DEPC-modified angiotensin-II was monocarbethoxylated. Optimal DEPC to imidazole ring ratios can be determined in order to

![Figure 2](image1.png)

**Figure 2.** A, difference UV spectra (ΔA) of carbethoxylated lysozyme (0.033 mM, 200 equivalents of DEPC); B, 0.35 mM NH₂OH-treated mono-N-carbethoxy His-15 derivative of lysozyme (0.033 mM, 200 equivalents of DEPC) versus native lysozyme, at 25 °C in 0.1 M potassium phosphate, pH 6.0, buffer. Here and for all other reported difference absorbance data, ΔA = absorbance for DEPC-treated species minus absorbance for control species.

![Figure 3](image2.png)

**Figure 3.** Time dependence of the reaction of lysozyme (0.033 mM) with 25 (C), 50 (O), 100 (V), 150 (¥), and 200 (C) equivalents of DEPC, in 0.1 M potassium phosphate, pH 6.0, buffer at 25 °C. Inset, dependence of the pseudo first order rate constant on initial DEPC concentration.
minimize the amounts of unreacted and irreversibly bis-carbethoxylated histidine. So under the conditions of Fig. 4, DEPC to imidazole ratios of 8:1 and 200:1 maximize the amounts of mono-N-carbethoxylated imidazole in angiotensin-II and lysozyme, respectively.

**Carbethoxylation of Lysozyme Treated with Denaturants**—No more than 5% of His-15 in native lysozyme appears to be susceptible to irreversible bis-carbethoxylation when DEPC is in 200-fold molar excess (Fig. 4b). This lack of reactivity at the second imidazole nitrogen of the His-15 with DEPC could be explained by the presence of a hydroxyl bond between the hydroxyl of Thr-89 and the N1 of the His-15 imidazole ring (Phillips, 1974). This hydrogen bond could prevent free rotation of the His-15 imidazole ring in native lysozyme as well as the exposure of the N1 imidazole ring nitrogen atom for reaction with DEPC. This explanation is supported by a study of spin-labeled His-15 in lysozyme which showed that the spin label is immobilized with respect to the protein (Schmidt and Kuntz, 1984; Wien et al., 1972). Furthermore, we have found that even after treatment of native lysozyme with 8 M urea at 25 °C, no significant irreversible bis-carbethoxylation of His-15 was observed, even up to a 200-fold molar excess of DEPC (Table I). We conclude that 8 M urea does not denature lysozyme sufficiently in the region of the His-15 residue to disrupt the hydrogen bond involving the imidazole ring N1 nitrogen atom of His-15.

Additional support for this conclusion was provided by experiments involving the reaction of DEPC with lysozyme pretreated with the disulfide bond reducing agent 2-ME. Reaction of reduced lysozyme (pretreated with 2-ME) with 200 equivalents of DEPC resulted in significant irreversible bis-carbethoxylation of His-15, analogous to the reaction of native angiotensin-II with high equivalents of DEPC. Moreover, when lysozyme was reacted with DEPC in the presence of 2-ME plus 1% SDS or 2-ME plus 8 M urea, the result was similar to that with 2-ME alone. The UV data show only a moderate increase in irreversible bis-carbethoxylation over that observed, when lysozyme was modified with DEPC in the presence of 1% SDS alone (Table I).

![Graph](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Monocarbethoxylation histidine*</th>
<th>Bis-carbethoxylation histidine*</th>
<th>Relative activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>95</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Lysozyme + urea (8 M)</td>
<td>95</td>
<td>5</td>
<td>125</td>
</tr>
<tr>
<td>Lysozyme + 2-ME (200 eq)</td>
<td>65</td>
<td>35</td>
<td>95</td>
</tr>
<tr>
<td>Lysozyme + 1% SDS</td>
<td>85</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Lysozyme + 2-ME (200 eq) + 1% SDS</td>
<td>60</td>
<td>40</td>
<td>0</td>
</tr>
</tbody>
</table>

*Unmodified species are considered not present when DEPC is used in large excess (200 equivalents).

![Graph](image)
CD Studies of DEPC-modified Lysozyme

for both His-15 and Thr-89 with consequent disruption of the hydrogen bond between them. Thus, reduction of the disulfide bonds of lysozyme should lead to a greater accessibility of both N1 and N3 of His-15 for reaction with DEPC.

Enzymatic Activities of DEPC-modified Lysozyme—No significant change was found in the glycosidic hydrolysis activity of lysozyme when the carboxethylation was carried out at high (200) equivalents of DEPC to lysozyme (Table I). Thus carboxethylation of the His-15 imidazole ring does not appear to significantly alter the glycosidic catalytic site of lysozyme. The relative activities of lysozyme treated with denaturing reagents are listed in Table I. Although an activity increase was observed for lysozyme treated with 8 mM urea, the enzyme treated with 1% (w/v) SDS lost its activity completely, and the treatment with 2-ME alone did not significantly change the activity. An esterase activity facilitated by His-15 has also been reported for hen egg white lysozyme (Piszkiewicz and Bruce, 1968). We have also observed this weak esterase activity using o-nitrophenyl acetate as substrate (second order rate constant of 3.1 M⁻¹ min⁻¹ at pH 6, compared with the reported value (Piszkiewicz and Bruce, 1968) of 2.75 M⁻¹ min⁻¹). However, we observed no change in this esterase activity for mono-N-carboxethoxylated His-15 lysozyme, which suggests that His-15 is not involved. This observation confirms and extends the results of Jolles and Jolles (1983) who found that duck lysozyme, with Leu in place of His at position 15 (Hermann et al., 1971), also shows this weak esterase activity.

ΔCD of Mono-N-carboxethoxylated Lysozyme and Model Systems—CD measurements are particularly sensitive to the overall geometries of molecules lacking a point or plane of symmetry and thus should provide a sensitive probe of the overall conformational changes that might occur when optically active molecules are subjected to perturbants. Since the imidazole rings in lysozyme, angiotensin-II, and Nα-acetyl-L-histidine are in such asymmetric molecules, we used CD to examine the structural perturbations caused by histidine ring N-carboxethylation. As in the case of the difference absorbance spectra, we concentrate on the spectral region that has been shown to be indicative of histidine ring N-carboxethylation, i.e., the region near 242 nm. The ΔCD spectra (ΔCD = CD spectrum of DEPC-modified species minus the CD spectrum of unmodified species) for lysozyme, Nα-acetyl-L-histidine, angiotensin-II, and Nα-acetyl-L-tyrosine are shown in Fig. 5. In obtaining these ΔCD spectra, we used ratios of DEPC to imidazole ring (for the treated species) that only produce mono-N-carboxethoxylated imidazole rings (i.e., 200:1, 2:1, and 8:1 for lysozyme, Nα-acetyl-L-histidine, angiotensin-II, respectively). In the 230–270 nm spectral region, the ΔCD for monocarboxethoxylated lysozyme exibits a large positive band with a maximum near 244 nm (Δ[θ]244 nm = +17,000 degree-cm²-dmol⁻¹). In this same spectral region, the Nα-acetyl-L-histidine ΔCD spectrum exhibits two much weaker bands: a positive band with the maximum near 233 nm (Δ[θ]233 nm = +1000 degree-cm²-dmol⁻¹) and a negative band between 265 and 242 nm with a minimum near 252 nm (Δ[θ]252 nm = −260 degree-cm²-dmol⁻¹). Two weak bands were also observed in the ΔCD spectrum of angiotensin-II, Δ[θ]244 nm = +700 degree-cm²-dmol⁻¹ and Δ[θ]252 nm = −400 degree-cm²-dmol⁻¹. Thus, the location of the 244 nm maximum in the lysozyme ΔCD spectrum seems to correlate with the 242 nm maximum in the ΔCD of denatured lysozyme. Thus, it is clear from these ΔCD spectra (Fig. 5) that mono-N-carboxethylation of His-15 in lysozyme results in a substantially more intense ΔCD spectrum than is observed for mono-N-carboxethylation of the imidazole ring in Nα-acetyl-L-histidine or angiotensin-II. This substantial difference in spectral magnitudes suggests that the imidazole ring in lysozyme is much more perturbed by its asymmetric environment than the imidazole ring in Nα-acetyl-L-histidine or angiotensin-II. However, since the imidazole ring of His-15 in lysozyme has only limited motionl freedom (Schmidt and Kuntz, 1984; Wien et al., 1972) and assuming that mono-N-carboxethylation does not disrupt the His-15-Thr-89 hydrogen bond, the orientation of the ring relative to its molecular environment should be approximately the same in both the mono-N-carboxethoxylated and uncarboxethoxylated lysozyme. This limited motional freedom would mean that the ΔCD that we have obtained for lysozyme is a measure of the asymmetry induced, in the molecular environment of the ring, by N-carboxethylation of this one particular ring orientation. In other words, with the assumption that the hydrogen bond to the N1 nitrogen is not broken, the ΔCD spectrum of lysozyme (Fig. 5) reflects the extent of imidazole ring N-carboxethylation occurring at the N3 atom of essentially one rotational conformer of the imidazole ring in lysozyme. On the other hand, the imidazole ring in Nα-acetyl-L-histidine as well as the substituents on the ring in both the mono-N-carboxethoxylated and uncarboxethoxylated species will have a considerable degree of motional freedom. Therefore, for both the mono-N-carboxethoxylated and the uncarboxethoxylated species, there may be large cancellations of CD bands as a result of the overlap of positive and negative CD contributions by the many different rotational conformers (Williams and Moore, 1983). Thus, for the 233 nm ΔCD band of Nα-acetyl-L-histidine, positive ΔCD contributions from the many conformers predominate, whereas negative ΔCD contributions predominate in the band at 247 nm. The ΔCD spectrum for angiotensin-II is similar to that obtained for Nα-acetyl-L-histidine, as substantial motional freedom of the mono-N-carboxethoxylated imidazole ring is also expected in angiotensin-II in solution. The ΔCD from treatment (at pH 8) of Nα-acetyl-L-tyrosine with DEPC was negligible in the 240–280 nm region (Δ[θ] < 600 degree-cm²-dmol⁻¹ at 256 and 277 nm (Fig. 5).
oxylated His-15 in lysozyme versus the model systems is that His-15 is in a strongly chiral environment mainly due to its location at the C-terminal end of the 5-15 α-helix. When mono-N-carbethoxyethylated lysozyme was treated with 2-ME plus urea and the CD spectrum was measured at 25 °C, the overall α-helix content was reduced, as indicated by the observed decrease in molar ellipticity at 222 nm (O’Neil and DeGrado, 1990); however, the ΔCD at 244 nm remained unchanged (Table II). But when the sample was heated to 55 °C, the relatively large difference CD spectrum due to mono-N-carbethoxyimidazole is substantially reduced. These treatments do not, however, cause any decrease in the observed absorbance at 242 nm. This treatment with 2-ME plus urea at 55 °C does cause a substantial decrease in the observed [θ]222 nm (Table II), which indicates a dramatic loss in total α-helical secondary structure (O’Neil and DeGrado, 1990). Although we cannot directly correlate the decrease in total α-helical content to specific unfolding of the 5-15 helix, we believe such direct correlation is likely. Due to the relatively high background CD in the 240–260 nm region from other groups in lysozyme, we were unable to detect the expected weak CD spectrum associated with mono-N-carbethoxyimidazole in denatured lysozyme, analogous to mono-N-carbethoxyethylated Nα-acetyl-L-histidine or mono-N-carbethoxyethylated angiotensin-II (see Fig. 5, spectra B and C), under these denaturing conditions. In order to further investigate whether or not the adjacent α-helix is correlated to CD band enhancement observed for the DEPC-modified lysozyme, DEPC-modified lysozyme was treated with SDS or SDS plus 200 equivalents of 2-ME, as described under “Experimental Procedures.” Whereas the CD spectrum of lysozyme treated with SDS or SDS plus 2-ME does not show any decrease of α-helix content (at 222 nm), the difference CD band in the 240 nm region associated with the N-carbethoxylation is essentially eliminated (Table II). Actually the overall α-helix content was increased by the SDS treatment, as judged by the molar ellipticity at 222 nm. The latter result has been reported in previous studies of SDS-treated lysozyme and a number of other proteins (Mattice et al., 1976; Takeda and Moriyama, 1989). When lysozyme was modified with DEPC in the presence of SDS (1%) alone, the UV data did not show significant irreversible bis-carbethoxylation (Table I). However, under such conditions, no difference CD band in the 240 nm region could be detected (Table II). It appears that under these experimental conditions, SDS does not destroy the α-helices in lysozyme, but it does eliminate the strong environmental perturbation which is responsible for the CD spectral enhancement. The catalytic activity of lysozyme is also destroyed by treatment with 1%, w/v, SDS. Although these observations can not rule out the possibility that the loss of the 5-15 helix is more than compensated for by formation of additional α-helical region(s) elsewhere in lysozyme, we believe that the simplest explanation for our results is that the source of the optical asymmetry at His-15 in lysozyme is not due to the 5-15 helix.

We have identified a CD band associated with mono-N-carbethoxyethylated histidine in lysozyme, angiotensin-II, and Nα-acetyl-L-histidine. The molar ellipticity of this CD transition associated with the monocarbethoxyhistidine is substantially enhanced in lysozyme relative to angiotensin-II and Nα-acetyl-L-histidine. The enhancement may reflect the presence in lysozyme of one particular rotational conformation of the mono-N-carbethoxyethylated imidazole ring relative to the asymmetric environment. As the data in Table II indicate the absolute magnitude of the molar ellipticity of the transition associated with mono-N-carbethoxyhistidine in lysozyme is between 50 and 100 times weaker than the absolute magnitude of the molar ellipticity associated with protein secondary structures. Thus, DEPC modification of histidine in proteins combined with careful CD studies could be used to detect the presence of a histidine residue in proteins in which the imidazole ring is rigidly held with respect to its protein environment. The data indicate that histidine ring immobilization is not induced by the presence of the carboxethoxy moiety.

Such studies could also be used to determine whether histidine side chain immobility observed in the crystalline state of a protein is maintained when the protein is dissolved in aqueous solution. Furthermore, changes in the molar ellipticity associated with a carboxethoxyhistidine residue in a protein could also be used to monitor the extent of denaturation or to follow the kinetics of denaturation of the local region surrounding the carboxethoxyhistidine residue.

**REFERENCES**


**TABLE II**

CD of monocarbethoxyethylated His-15 lysozyme after chemical treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[θ]222 nm (degree cm2 dmol–1) × 104</th>
<th>Δ[θ]222 nm (degree cm2 dmol–1) × 104</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (no treatment)</td>
<td>-1.4</td>
<td>17,000</td>
</tr>
<tr>
<td>Lysozyme + 8 M urea + 200 eq of 2-ME</td>
<td>-0.9</td>
<td>15,000</td>
</tr>
<tr>
<td>Lysozyme + 5 M urea + 200 eq of 2-ME treated with heating</td>
<td>-0.2</td>
<td>&lt;4000</td>
</tr>
<tr>
<td>Lysozyme in 1% SDS + 200 eq of 2-ME</td>
<td>-1.7</td>
<td>&lt;4000</td>
</tr>
<tr>
<td>Lysozyme in 1% SDS</td>
<td>-1.6</td>
<td>&lt;4000</td>
</tr>
</tbody>
</table>

* [Lysozyme] = 0.001 mM, at 25 °C.
* [Lysozyme] = 0.033 mM, at 25 °C.
* The sample was heated to 55 °C, then cooled to 25 °C, and measurement was done at 25 °C immediately after cooling.
* Sample was placed in boiling water bath for 5 min and then cooled to 25 °C, and CD spectrum was obtained at 25 °C.
CD Studies of DEPC-modified Lysozyme

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Wien, R. W., Morrisett, J. D. & McConnell, H. M. (1972) Biochemistry 11, 3707-3716