Structure–Function Relationships in Lysozyme by Circular Dichroism[1]

Sir:

In a previous communication,[2] we reported on the finding of a solvent-sensitive aromatic Cotton effect in egg white lysozyme. Since X-ray crystallographic,[3,4] physical,[5] and chemical studies[6] have shown tryptophan residues to be present at the substrate-binding site in this enzyme, it was of interest to determine in what manner these particular residues contributed to the observed Cotton effect. Johnson and Phillips[4] have reported that N-acetyl-D-glucosamine (NAG), a competitive inhibitor of lysozyme, was bound stoichiometrically at the substrate-binding site. Examination of the optical rotatory dispersion of lysozyme in the presence of NAG indicated that the positive aromatic Cotton effect was enhanced by the presence of this compound. However, it was difficult to evaluate precisely the magnitude of this enhancement because of the deep background levorotation originating from the α-helical content of lysozyme and of the need to compensate for the positive rotation of NAG. Since circular dichroism (CD) has neither of these disadvantages, we continued these studies on the Jasco–Durrum CD recording spectrophotometer made available to us through the kind cooperation of Dr. W. F. H. M. Mommaerts.

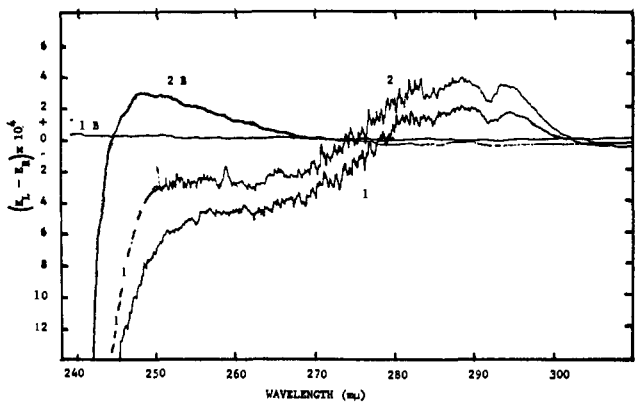

Figure 1. CD spectra of lysozyme (0.9 mg/ml) in the presence and absence of N-acetyl-D-glucosamine. Curve 1, lysozyme in 0.05 *M* phosphate buffer, pH 7.0; curve 2, lysozyme in 0.05 *M* phosphate buffer, pH 7.0, containing 0.25 *M* NAG; curve 1B, 0.05 *M* phosphate buffer, pH 7.0; curve 2B, 0.25 *M* NAG in 0.05 *M* phosphate buffer, pH 7.0. Optical path length: 1 cm.

Crystalline lysozyme, Lot No. 6194204, was obtained from C. F. Boehringer and Soehne, Gmbh. Chromatographically pure sugars and sugar derivatives were obtained from Mann Research Labs, Inc. Freshly prepared solutions were used for all measurements.

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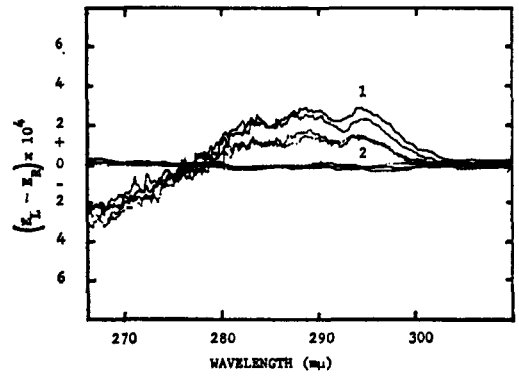

Figure 2. CD spectra of lysozyme (0.67 mg/ml) in 0.1 *M* phosphate buffer, pH 7.0, containing: curve 1, 0.25 *M* NAG, or 0.25 *M* N-acetyl-D-galactosamine; curve 2, 0.25 *M* glucose, or 0.25 *M* sucrose, or lysozyme in absence of added sugar, all superimposed. The base lines for these solutions virtually superimpose in this wavelength region. Optical path length: 1 cm.

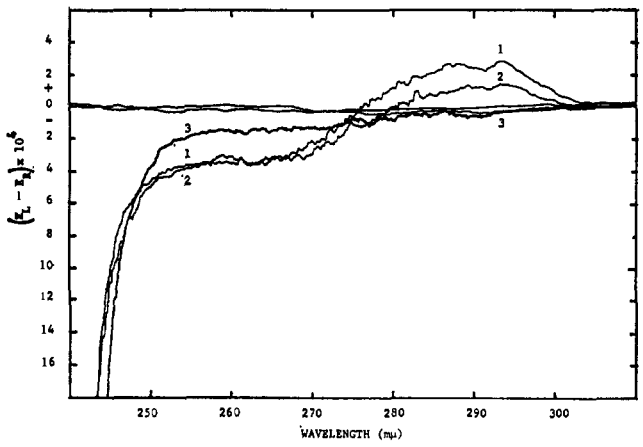

Figure 3. CD spectra of lysozyme (0.67 mg/ml) in: curve 1, 50% aqueous ethylene glycol (v/v); curve 2, water; curve 3, 2% sodium dodecyl sulfate in water, pH 5. Optical path length: 1 cm.

Most measurements were performed with an optical path length of 1 cm. The CD spectra were found to be strictly proportional to protein concentration over the wavelength range examined (240–340 mμ). The data in Figures 1–3 are photographed directly from the recorder charts and are, therefore, presented as $\Delta E = E_L - E_R$, as measured under the conditions given in the figure legends. Conversion to molar ellipticities (θ) may be made by first converting the data to the molar extinction coefficients ϵ_L and ϵ_R, then $\theta \approx 3300(\epsilon_L - \epsilon_R)$.

The near-ultraviolet CD of lysozyme (Figure 1, curve 1) was resolved into three positive bands in the region 280–300 mμ and a negative band centered at about 262 mμ. Presumably, the positive aromatic Cotton effect observed in our earlier study,[2] as well as the broad positive CD band observed by Beychok,[7] represented a summation of the three positive bands. The negative band has been ascribed by Beychok[7] largely to disulfide, on the basis of studies on model compounds. In the presence of NAG, the CD of lysozyme in the aromatic absorption region was approximately double that observed in the absence of the sugar derivative (Figure 1, curve 2). Glucose and

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sucrose, at the same molarity, did not produce this enhancement (Figure 2, curve 2). It is of interest to note that N-acetyl-D-galactosamine produced an enhancement similar to that seen with NAG (Figure 2, curve 1). These findings indicate that the exposed aromatic residues in the substrate-binding site of lysozyme contribute to the optical activity observed and that *their orientation is probably affected by substances binding at this site.*

Study of the effect of sodium dodecyl sulfate and of ethylene glycol on the CD of lysozyme (Figure 3) confirmed and extended our earlier findings. Ethylene glycol increased the magnitude of the CD bands in the region 280–300 mμ, but had no influence on the negative band centered near 262 mμ. The CD of lysozyme in 50% ethylene glycol resembles closely that obtained in the presence of NAG. Presumably, in aqueous ethylene glycol solution, the orientation of the optically active aromatic chromophores is similar to that obtained in the presence of NAG. Perhaps in the case of both NAG and ethylene glycol the enhancement of optical activity arises as a consequence of displacement of water from the substrate-binding crevice,[4] permitting some reorientation of the aromatic residues. Sodium dodecyl sulfate (Figure 3, curve 3) completely eliminated the aromatic CD bands. This result is in total accord with our earlier optical rotatory dispersion findings.[2] In addition, however, sodium dodecyl sulfate greatly diminished the magnitude of the negative 262-mμ band. A similar observation was reported by Beychok[7] for the effect of urea on the CD of insulin. From our earlier optical rotatory dispersion[2] and these CD data, the detergent appears to disorganize profoundly all those regions of lysozyme not in α-helical segments.

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The Mechanism of Direct *cis–trans* Photoisomerization of the Stilbenes

Sir:

Hitherto available data on the *cis–trans* photoisomerization of the stilbenes can be accommodated by either of two mechanisms: (1) the Lewis mechanism,[1] which assumes that $S^1 \rightarrow S^0$ radiationless conversion produces a freely rotating ground state; (2) the Förster mechanism,[2-7] in which the stilbene isomers lose their identity upon $S^1 \rightarrow T^1$ crossing to interconvertible or common triplet states.

Preference for the triplet-state mechanism is based on the Kassel–Rice theory, which predicts that energy available upon internal conversion to the ground state is rapidly distributed among various vibrational modes. The Lewis mechanism is, therefore, not expected to compete with loss of vibrational excitation to solvent.[8]

Two findings are not easily compatible with the triplet mechanism: (1) the frequency factor (10^{12} sec^{-1}) for the main path leading to isomerization of *trans*-stilbene is larger than would be expected for a spin-forbidden process;[3,6] (2) the azulene effect on the sensitized isomerization is accounted for by eq 1.[7]

$$t^{T1} + Az^{S0} \longrightarrow t^{S0} + Az^{T1} \quad (1)$$

However, azulene's effect on the direct photoisomerization cannot be quantitatively accounted for by eq 1.[7] The discrepancy is shown in Figure 1, where the dependence of the $([trans]/[cis])_S$ ratio at the photostationary state on azulene concentration is compared to the straight line expected for the triplet mechanism.

We present data which show that the discrepancy in Figure 1 is best interpreted as evidence against the involvement of triplet states in the direct photoisomerization of the stilbenes. We consider the following kinetics for the Lewis mechanism

$$c^{S0} \xrightarrow[3130\ A]{h\nu} c^{S1} \quad (2)$$

$$t^{S0} \xrightarrow[3130\ A]{h\nu} t^{S1} \quad (3)$$

$$t^{S1} \longrightarrow t^{S0} + h\nu \quad (4)$$

$$t^{S1} \longrightarrow at^{S0} + (1 - a)c^{S0} \quad (5)$$

$$c^{S1} \longrightarrow \beta t^{S0} + (1 - \beta)c^{S0} \quad (6)$$

$$t^{S1} + Az^{S0} \longrightarrow t^{S0} + Az^{S1} \quad (7)$$

Energy transfer from c^{S1} to azulene is neglected because the lifetime of c^{S1} is expected to be shorter than that of t^{S1}.[1,4,9] The self-quenching step (8) is neglected

$$t^{S1} + t^{S0} \longrightarrow 2t^{S0} + \text{energy} \quad (8)$$

for low concentrations of *trans*-stilbene.[7] The mechanism yields stationary-state relationship 9 where

$$\left[\frac{[t^{S0}]}{[c^{S0}]}\right]_S = \left[\frac{\epsilon_c}{\epsilon_t}\right]\left[\frac{\beta}{1-\alpha}\right]\left[1 + \frac{k_4}{k_5} + \frac{k_7[Az]}{k_5}\right] \quad (9)$$

ϵ_c and ϵ_t are extinction coefficients of *cis*- and *trans*-stilbene at the exciting wavelength. The initial slope to intercept ratio of curve 1 in Figure 1 gives $k_7/(k_4 + k_5) \leq 40\ M^{-1}$.

trans-Stilbene fluorescence can be induced by β-ray radiation.[10,11] A scintillation counting method was developed to evaluate singlet excitation transfer from *trans*-stilbene to azulene (eq 7). The scintillation system consisted of benzene-^{14}C as solvent and β-ray source, *trans*-stilbene as fluor, and azulene as

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