Circular Dichroism Measurement of a Protein in Dried Thin Films

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(Received November 26, 2001; CL-011185)

Solid-state CD of bovine serum albumin in dry thin films exhibited different spectra depending on the sample preparation method and the rotation positioning of a film. It was revealed that this is due to the macroscopic anisotropies of the samples and not due to the protein conformation transition during the film formation, as suggested in literature.

Solid-state spectroscopy provides valuable information on solid-state structure and supuramolecular properties which are not obtainable from the solution phase. This applies not only to organic or inorganic compounds but also to biological substances. Circular Dichroism (CD) spectroscopy of proteins in dry thin films may provide useful information on their unique conformation or an aggregate form in the condensed phase. The information may be particularly relevant to some neurodegenerative disorders such as Alzheimer and prion diseases in which the production of abnormal aggregates of β-amyloid peptide or prion protein seems to constitute an important step.[1,2] However, circular dichroism (CD) spectra in the solid phase are necessarily accompanied by artifacts which originate from the macroscopic anisotropies of a sample such as linear birefringence (LB) and linear dichroism (LD), and the interaction between the macroscopic anisotropies and the non-ideal characteristics of polarization-modulation instruments.[3–7] Thus, measurement of solid-state chiroptical properties using a commercially available CD spectrophotometer has to be carried out with extreme care.

An interesting paper[8] appeared last year which reported CD spectra of six proteins in both solution and in dry films. Their CD spectra in the solid and solution states were different for one class of proteins (e.g. bovine serum albumin (BSA) and α-synuclein), whereas similar for the other class of proteins (e.g. insulin, lysozyme and luciferase). Based on these findings, it was claimed[8] that some proteins undergo structural transformation from native structures in solution into β-sheet predominant structures in the solid state. In their work, no macroscopic anisotropies of a solid sample were considered. Thus, we have independently studied CD of BSA using our specially constructed Universal Chiroptical Spectrophotometer (UCS:J-800KCM).[9] This instrument houses two lock-in amplifiers for the simultaneous detection of 50 and 100 kHz signals, an analyzer which can be inserted or taken out easily from the light path, and a computer controlled sample rotation system.[9] It also contains a good quality detector (PM) and a photoelastic modulator (PEM).[9] As a consequence, this instrument is capable of measuring all the chiroptical properties of CD, LD, LB, and CB (circular birefringence = Optical Rotatory Dispersion), in solution and solid states.[9,10]

All the CD measurements were performed on the UCS: J-800KCM. The spectra were recorded over a wavelength range of 260–200 nm with 'Low' sensitivity at 100 nm/min, time constant of 1 s and bandwidth of 1 nm. Data were further processed for noise reduction when necessary. BSA was purchased from Sigma and used without further purification. The concentration of the solution sample was about 0.2 mg/ml in a buffer of 6.5 mM Tris and 16.5 mM HCl (pH 7.4). For the solid-state CD measurements, about 150 μl of the BSA solution (ca. 5 mg/ml) was cast onto a 2-cm diameter cylindrical quartz glass for evaporation. In one case, evaporation was carried out overnight (24 h) at room temperature, and in another case over 10 min using a dryer, until a dry thin film formed on the surface of the glass. Rotation measurements were carried out by rotating the sample 360° in the plane perpendicular to the light beam at 230 (LB) or 210 nm (CD) with a rotation speed of 2.5°/s.

For UCS:J-800KCM possessing good quality optical and electrical elements, the 50 kHz signals detected by the lock-in amplifier with and without an analyzer can be given as follows[9]:
without an analyzer

$$50\,\mathrm{kHz(CD)} = G_1(P_x{}^2 + P_y{}^2)[\mathrm{CD} + 1/2(\mathrm{LD'LB} - \mathrm{LDLB'})] + G_1(P_x{}^2 - P_y{}^2)\sin 2a(-\mathrm{LB}\cos 2\theta + \mathrm{LB'}\sin 2\theta) \quad (1)$$

with an analyzer

$$50\,\mathrm{kHz(LB)} = G_3\{\mathrm{CD} + 1/2(\mathrm{LD'LB} - \mathrm{LDLB'}) - \mathrm{LB}\cos 2\theta + \mathrm{LB'}\sin 2\theta\} \quad (2)$$

Here, LD' and LB' are 45-degree linear dichroism and 45-degree linear birefringence, respectively. P_x^2 and P_y^2 are the transmittance of the photomultiplier along the x and y directions and "a" is the azimuth angle of its optical axis with respect to the x axis. θ is the rotation angle of the sample. G_1, G_3 are the apparatus constants with or without an analyzer, respectively.

Experimental results for the quickly formed (10 min) BSA cast film are presented in Figure 1. Figures 1a and 1b show a LB and a PM voltage (HT) rotation spectra, respectively, which were measured by rotating the cast BSA film 360° in the (x-y) plane at 230 nm with an analyzer. The LB value changed on the rotation non-concomitantly with HT. Thus, not only the uneven thickness of the film but also anisotropy of the specimen must be the origin of the change. It is clear from Figure 1, eq (1) and eq (2) that there is a substantial contribution of LB and LB' as well as that of coupling of LB with LD to the CD signals. The CD signal measured at the wavelength of an absorption maximum (210 nm) without an analyzer changed on rotating the sample (Figure 1c).

From the CD rotation spectrum, we can locate the CD_{max} and CD_{min} positions. The wavelength scans carried out at these positions are quite different from each other: CD_{max} spectrum exhibits a negative peak at 223 nm, while CD_{min} spectrum shows a broad negative band (Figure 1d). Thus, the CD spectra differed substantially depending on the sample rotational positioning, θ. A solution CD of the same BSA sample in relative ellipticities is included in the Figure for comparison. It is clearly different from

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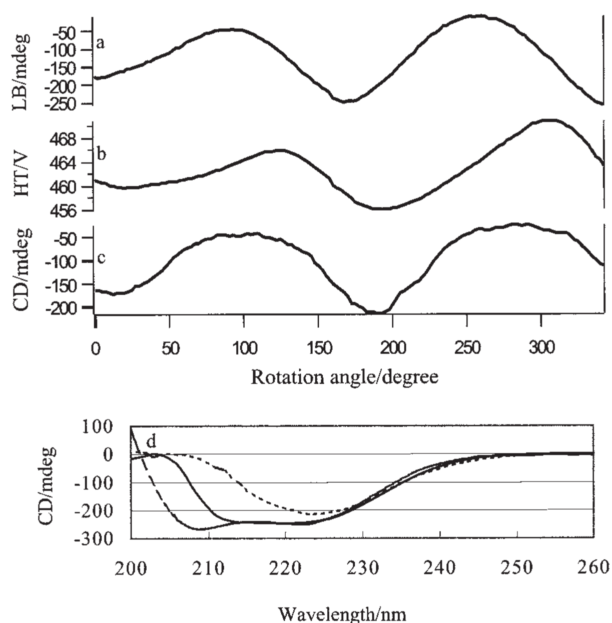

Figure 1. LB (a), HT (b) and CD (c) rotation spectra at 230 (LB, HT) and 210 nm (CD) of a BSA film (5 mg/ml) formed by quick evaporation. d: CD spectrum of the film at the CD_{max} (dotted line) and CD_{min} (solid line) positions. The solution CD spectrum (broken line) is shown for comparison.

these two solid-state CD spectra, presenting a double negative broad spectrum with a peak at 209 nm and a shoulder at 222 nm.

The solid state CD at the CD_{max} position is incidentally similar to what was reported in the literature.[8] The authors concluded that CD in the solution is a typical of proteins with a high α-helical content, whereas that in the solid state with a strong negative peak at 224 nm and a small shoulder at around 208 nm reflects the structure containing β-sheets and aggregate into an amorphous form. Our results unambiguously deny their statement. The spectral differences must originate from the substantial artifacts, mainly LB and its coupling with LD.

To confirm this, we have carried out an experiment on a BSA cast film which was formed by slow evaporation (24 h) to obtain a relatively strain-free film of even thickness. Strangely, this slow casting procedure is similar to what was reported in the reference.[8] The LB and the LD (data not shown) signals were smaller than those of the quickly dried film and PM voltage was almost constant throughout the rotation to assure the even thickness of the sample. Although the LB signal changed on the sample rotation, the CD signal hardly changed (Figure 2a,c), as our instrument was specially designed to minimize moderate and rotation-dependent artifacts. Thus, we could obtain nearly identical wavelength-scan spectra at any rotation position. As is shown in eq (1), there are artifact terms which are independent of the sample rotation (θ). The contribution from these terms is negligible in this case, as the LD signals were small. Thus, the observed CD must be close to the true CD. Indeed, the solution CD spectrum is similar to the solid-state spectrum, as shown in Figure 2d.

Different CD spectra were obtained for the cast films made from a same stock solution depending on the evaporation speed, and, in one case, also at different rotation positions even for the

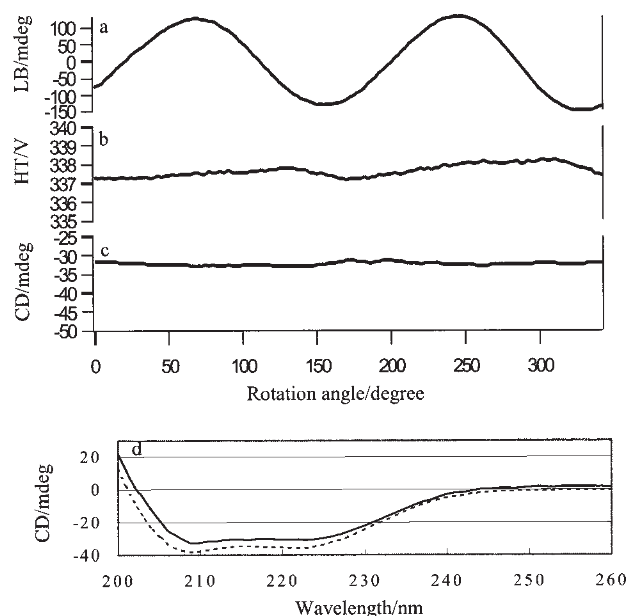

Figure 2. LB (a), HT (b) and CD (c) rotation spectra at 230 (LB, HT) and 210 nm (CD) of a BSA film (5 mg/ml) formed by slow evaporation. d: CD spectra of the film (solid line) with the solution CD (broken line) for comparison.

same film. It depends whether the LB and LD signals affect the CD signals. We have previously devised a method for removing artifact signals to obtain true CD based on the Mueller Matrix analysis.[9] This procedure, however, cannot be applied to inhomogeneous samples like cast films with lot of strain.

Based on these results, we can conclude that the structural change of BSA does not occur in the process of film formation. The difference in CD is simply due to artifacts arising from macroscopic anisotropies. For the CD measurement of samples with possible macroscopic anisotropies such as films, gels, micells, and liquid crystals, it is necessary to measure the anisotropies using specially designed spectrophotometer such as our UCS:J-800KCM. Only with this consideration, an important information on protein and peptide characteristics in the condensed phase will be elucidated.

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