Synchrotron radiation circular dichroism spectroscopy: vacuum ultraviolet irradiation does not damage protein integrity

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Synchrotron radiation circular dichroism (SRCD) spectroscopy is an emerging technique for sensitive determination of protein secondary structures and for monitoring of conformational changes. An important issue for its adoption as a useful technique is whether the high-intensity low-wavelength vacuum ultraviolet radiation in the SRCD chemically damages proteins. In this paper, using horse myoglobin as a test sample, it is shown that extensive irradiation in the SRCD does not produce any change in the chemical nature of the protein as detected by either SDS gel electrophoresis or mass spectrometry. In addition, no changes in the protein secondary structure are detectable from the SRCD spectra after extensive exposure to the SRCD beam.

Keywords: circular dichroism spectroscopy; secondary structure; proteins; vacuum ultraviolet (VUV); myoglobin.

1. Introduction

Developments in synchrotron radiation sources and analytical instrumentation have resulted in the emergence of synchrotron radiation circular dichroism (SRCD) spectroscopy (Sutherland *et al.*, 1980; Snyder & Rowe, 1980) as a potentially important method for the structural analysis of proteins (Wallace, 2000*a*). The intensities of existing SRCD beams in the vacuum ultraviolet (VUV) region (below 190 nm) are more than 10^3 times those of conventional circular dichroism (CD) instruments (Wallace, 2000*a*; Clarke *et al.*, 2000). As a result, data in aqueous solutions can be collected to much lower wavelengths (~160 nm) (Wallace, 2000*b*) than previously possible in laboratory-based CD instruments, resulting in a higher information content in the spectra (Wallace *et al.*, 2001).

In principle, the energy of photons in the VUV wavelength range should only induce electronic transitions and should not be sufficient to produce structural or chemical changes in proteins under study. Previously, CD studies using conventional light sources (Johnson, 1978) had indicated, by comparisons of UV and VUV spectra, that there were no spectral differences detectable following VUV irradiation down to wavelengths of 181 nm. More recently, protein spectra obtained on a conventional CD instrument and with the SRCD down to 160 nm also exhibited identical appearances over their common wavelength range (Wallace, 2000*b*). These similarities suggest there are no overall secondary structural changes induced by the irradiation. However, as a result of the very high intensity beams at the lower wavelengths obtainable in the SRCD, questions have been raised as to the potential for damage to proteins by irradiation in the SRCD. To test whether there are more subtle chemical changes, in the present study we have undertaken a series of experiments to examine the effects of high-intensity VUV radiation in SRCD instruments on the structure and chemistry of a well characterized test protein, horse myoglobin.

2. Materials and methods

2.1. *Materials*

Horse myoglobin from ICN Biochemicals Inc. (98%+ purity) was dissolved in deionized water at a concentration of ~8 mg ml^{-1}.

2.2. *Exposure of samples*

For the mass spectroscopy and gel electrophoresis studies, the samples were placed in a 0.02 cm-pathlength Suprasil (Hellma Ltd) cuvette and the experiment was conducted in two ways:

(i) Spectra were scanned very slowly over the wavelength range 160–300 nm for a period of 1 h at 283 K on station 3.1, which is part of the BBSRC Centre for Protein and Membrane Structure and Dynamics (CPMSD) located at the SRS, Daresbury Laboratory, Warrington, UK.

(ii) The sample sat in the beam whilst being irradiated continuously at a wavelength of 160 nm for a period of 1 h at 301 K on station UV1 located at ASTRID in Aarhus, Denmark.

2.3. *Analyses of samples*

SDS polyacrylamide gel electrophoresis: 1 µl of either a 0.16 or 0.032 mg ml^{-1} solution (1/50 and 1/250 dilutions, respectively) of each of the exposed and unexposed myoglobin samples was loaded on a 8–25% gel and run in a Laemmli buffer on a PHAST gel system (Amersham Pharmacia Biotech) at 250 V, 10.0 mA, 288 K and stained with Coomassie blue. Molecular weight standards were obtained from a low-molecular-weight calibration kit (Pharmacia).

Mass spectroscopy: mass spectra were collected on a Micromass platform single-quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, UK). Samples (10 µl) were injected under standard conditions (50% acetonitrile/0.25% formic acid) and the delivery solvent (50% acetonitrile) was pumped at 10 µl min^{-1}. Twelve 10 s scans in positive ion mode were accumulated for each sample over the m/z range 750–1150. The source temperature was set

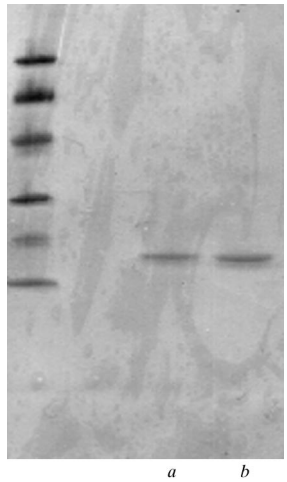

Figure 1
SDS gel of unexposed (lane *a*) and exposed (lane *b*) myoglobin. The left-hand lane contains standards of the following molecular weights: 97000; 66000; 45000; 30000; 20000; 14000.

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at 323 K. Spectra were processed using the Masslynx software supplied by Micromass (version 3.4).

2.4. *SRCD spectroscopy*

SRCD spectra were obtained at station 3.1 using a 0.001 cm-pathlength cell and scanned continuously over the wavelength range 157–300 nm in 0.2 nm intervals at 283 K over a period of 4 h.

3. Results

At both SRCD stations the beam intensities at 160 nm were approximately 5×10^{11} photons s^{-1} (Wallace, 2000*a*; Clarke *et al.*, 2000; D. Clarke, personal communication; J. Kenney, personal communication). Essentially identical results were obtained following both types of exposures.

SDS gel electrophoresis: horse myoglobin migrates as a band of molecular weight ~17000 (Fig. 1, lane *a*). Following irradiation, no new bands of either higher or lower molecular weight were seen (Fig. 1, lane *b*), even on grossly overloaded gels (not shown), nor was the apparent intensity of the myoglobin band decreased. This suggests that no detectable crosslinking or cleavage reactions occurred upon irradiation. However, as this is not a terribly sensitive technique for monitoring minor components, mass spectroscopic studies were also undertaken in order to detect very minor components.

Mass spectroscopy: the calculated molecular weight of horse myoglobin is 16951 and the main peak measured had a molecular weight of 16947 (Fig. 2*a*). The commercially available protein was reported to be of >98% purity. A very small amount of a second component (molecular weight = 16987) was also detected by mass spectroscopy. Identical distributions of components were obtained prior to and following irradiation (Fig. 2*a*). Furthermore, no new molecular species (either of higher or lower molecular weight) were detected following irradiation (Figs. 2*b* and 2*c*). These results suggest that chemical modification did not occur upon exposure in the beam. No evidence was obtained for any oxidation, degradation or adduct formation as a result of this procedure.

SRCD spectroscopy: spectra were collected over a 4 h period of continuous irradiation. These conditions are more severe than would be experienced under normal data-collection procedures. No differences were detected between the SRCD spectra collected at the beginning and end of the run, indicating that the secondary structure of the protein is unchanged by the irradiation (Fig. 3).

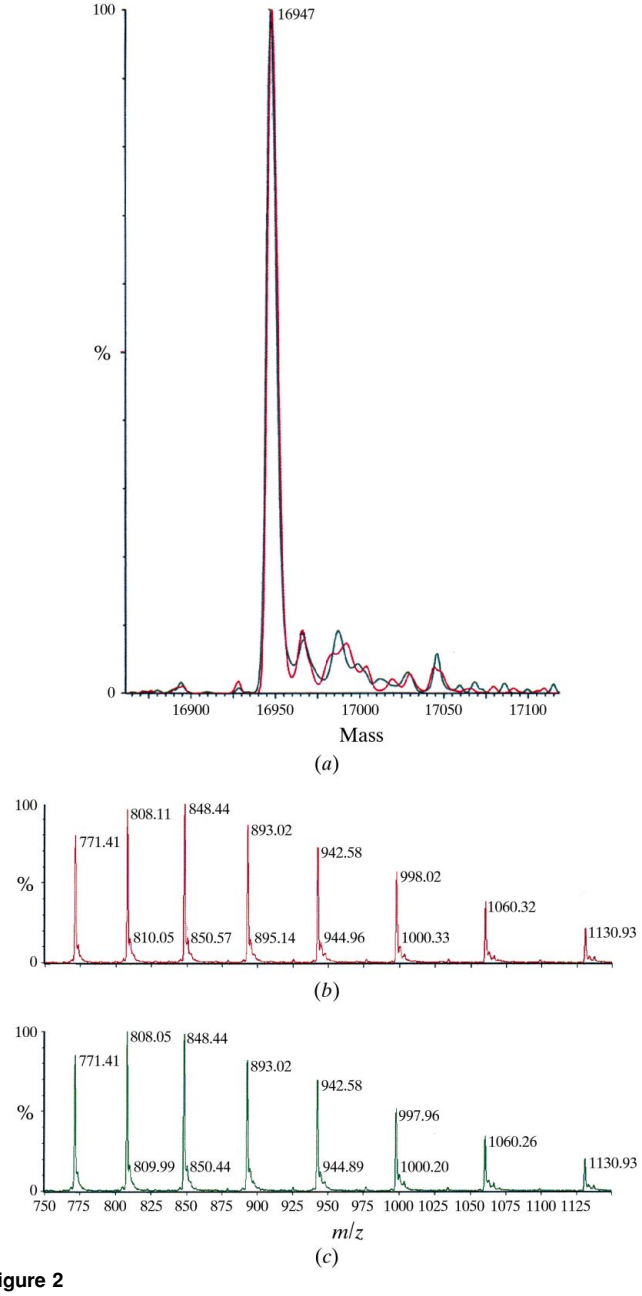

Figure 2
Mass spectroscopy: (*a*) deconvoluted mass spectra of unexposed (green line) and exposed (red line) myoglobin; and mass spectra showing the multiply charged ions obtained from (*b*) unexposed and (*c*) exposed myoglobin.

4. Conclusions

Circular dichroism is an important technique in structural molecular biology. Recent advances in SRCD have suggested it will be a very useful adjunct to conventional CD methods, but for it to be useful on biological samples it must be shown that the method does not cause damage to proteins.

Early studies using CD (Takeda & Moriyama, 1991) and optical rotatory dispersion (Wilson & Foster, 1970) spectroscopies on myoglobin had indicated that very small changes in the spectra were

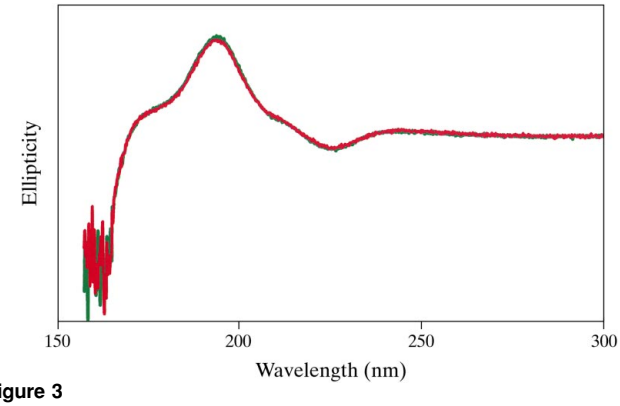

Figure 3
SRCD spectra of horse myoglobin obtained on station 3.1 following continuous irradiation in the beam over a period of 3 h during repetitive scans of the protein in a 0.001 cm-pathlength cell. The red spectrum was taken 4 h after the green spectrum. Note: these are 'raw' spectra, without any smoothing, averaging or baseline subtraction; consequently, the relative peak heights and shapes in these spectra will differ from those found in baseline-corrected spectra.

obtained after irradiation of the samples for up to 3 h at one wavelength in the UV range (~220–230 nm). The results from the present study seem somewhat incompatible with those observations, in that irradiation over the entire range from 160 to 300 nm at much higher intensities produced no such detectable changes. The samples in the previous studies were not characterized in terms of their chemical integrity following the procedures. The present studies, however, have included careful characterizations of the protein by both gel electrophoresis and mass spectrometry to demonstrate the lack of chemical modification to the protein as a result of any of the handling or irradiation procedures.

In summary, irradiation of myoglobin at VUV wavelengths in the SRCD appears to have resulted in no damage, crosslinking or cleavage of the protein, despite the high intensity of the beam. Thus, it should be possible to make SRCD measurements over a considerable period of time without concern of altering the chemistry or structure of the sample.

We thank Neil Freeman of GlaxoSmithKline and Dr Peter Bayley of NIMR for helpful discussions, and the staff and scientists of station 3.1 at Daresbury and station UV1 at ASTRID for use of the facilities. We acknowledge the BBSRC for provision of beam time at the SRS. This work was supported by a grant from the BBSRC to BAW. AJWO was supported by a BBSRC studentship and MH was supported by a project grant from the Wellcome Trust to BAW.

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 Received 5 February 2001 • Accepted 12 March 2001