ALTERATIONS IN PROTEINS AND POLYAMINO ACIDS CAUSED BY ULTRAVIOLET IRRADIATION IN A SPECTROPOLARIMETER[1]

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Summary: The Xenon lamp in a Cary 60 spectropolarimeter caused a photochemical reaction in proteins and polyamino acids which was traced to cleavage in the polypeptide chains. Viscosity studies on helical and coiled polyamino acids indicated that the cleavage occurred in both forms, but ORD changes were obtained only with helical and not with coiled and β conformations. Proteins of high helix content exhibited a significant change in ORD which was enhanced in the presence of denaturants. Quantum yields based on ORD change were greater in the $n \rightarrow \Pi^*$ than in the $\Pi \rightarrow \Pi^*$ transition region.

While it is well known that ultraviolet light can cause changes in proteins (1), the possibility of damage coincident to measurements in spectrophotometers and spectropolarimeters appears not to have been considered. In studies of the denaturation of bovine plasma albumin (BPA), conducted in a Cary 60 spectropolarimeter, we were surprised to observe significant changes in ORD accompanying prolonged exposure to light of wavelength below 240 mμ. In an attempt to clarify the nature and generality of this effect, other proteins and two polyamino acids, polyglutamic acid (PGA) and polylysine (PL) have been studied.

Experimental and Materials: ORD experiments were conducted in a Cary 60 spectropolarimeter employing stoppered cells of two types: (1) a water jacketed cell with 20 mm light path and 8 mm diameter sample compartment, (2) a 20 mm path length cell with 22 mm diameter which was kept at constant temperature by means of a metal thermostatting block.

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Viscosities were measured at 25.00 ± 0.01°C in a Cannon Ubbelohde Dilution Viscometer which had a flow time greater than four minutes for water.

Solutions were irradiated either in the spectropolarimeter or in a Bausch and Lomb Grating Monochromator equipped with a 200 watt Mercury-Xenon lamp and ultraviolet grating. The irradiations in the Cary 60 were carried out in the cells described above. Except where indicated otherwise, the sample was irradiated at the desired wavelength for a period of time, removed with a syringe, stirred, replaced in the cell, and the ORD curve recorded from 330 to 220 mμ. Irradiations in the monochromator were done with magnetic stirring in stoppered cells of 5.0 cm or 2.0 cm path length and 22 mm diameter. Since deaeration did not appear to affect the rate of change in $\alpha]_{233}$, solutions were not deaerated.

The lamp intensity in the Cary 60 was determined using potassium ferrioxalate (2). Quantum yields for this actinometer below 250 mμ were determined by comparison with uranyl oxalate (3) in the Bausch and Lomb monochromator. The values obtained were 1.19 at 230 mμ, 1.24 at 220 mμ, and 1.29 at 210 mμ. Since there is some error in the use of uranyl oxalate quantum yields in this wavelength region, a constant value of 1.24 was assumed for the quantum yield of potassium ferrioxalate over this region. Uranyl oxalate proved to be much too insensitive for measuring the low intensity light from the Cary 60.

Spectrophotometric grade guanidine hydrochloride (G.HCl) was purchased from Heico, Inc. Sperm whale myoglobin and ultrapure urea were purchased from Mann Research Laboratories. Polyamino acids, purchased from Pilot Chemical Company, were PGA, lot G-80 and PL hydrobromide, lot L-59.

BPA, Armour Fraction V, lot D27309, was charcoal defatted (4), passed through a Sephadex G-150 column to obtain monomeric protein, sulfhydryl blocked with iodoacetamide, dialysed, lyophilized, and stored at 2-3°C in a desiccator over calcium sulfate.

All other chemicals were of the highest purity commercially available. Solutions were prepared from glass redistilled water.

Results and Discussion: In studies of the kinetics of BPA denaturation, by continuously monitoring the rotation at 233 mμ, we observed an unusual decrease in the trough depth which did not occur on storing the protein at somewhat longer wavelengths. The rate of change was fastest for BPA in urea or G.HCl near the threshold of denaturation, or at elevated temperature near the on-set of thermal denaturation. Similar effects were observed with myoglobin. Results for these proteins are shown in Figure 1. These results were obtained by constant exposure and recording at 233 mμ with no stirring and cannot be interpreted quantitatively because of diffusion of the protein into and out of the light beam.

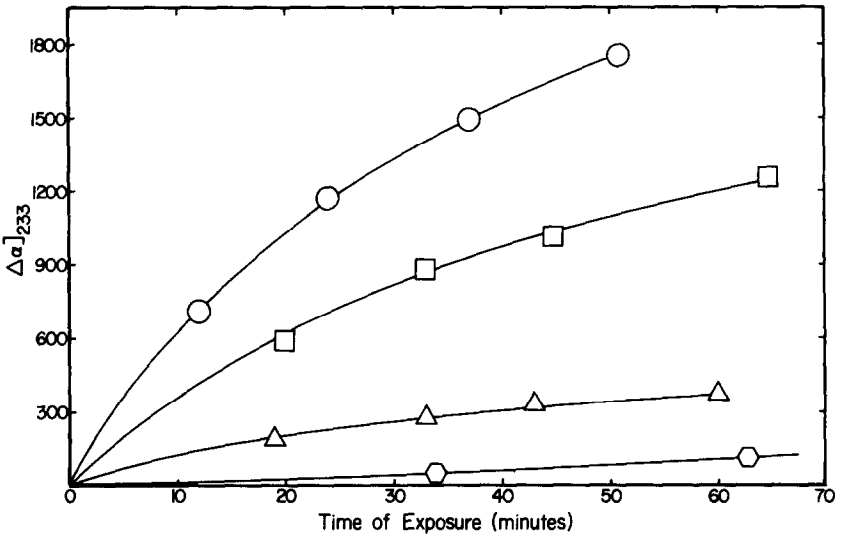

Figure 1. $\Delta\alpha]_{233}$ ($\alpha]_{233}$ of the exposed protein minus the same quantity for the unexposed protein) vs. time of exposure in minutes:
□ myoglobin in 1.5 M G.HCl, ⬡ myoglobin in 0.1 M NaCl,
○ BPA in 1.5 M G.HCl, △ BPA in 0.1 M NaCl.

Photochemical studies of proteins have generally been confined to wavelengths greater than 250 mμ and the observed effects attributed to absorption by either aromatic residues or cystine (1). Myoglobin has no cystine and the following experiments suggest that aromatic residues are not solely responsible for the photochemical changes found with BPA and myoglobin. PGA in the 100% helical form (pH 4.90, 0.05 M NaCl) (5) gave a change in $\alpha]_{233}$ that was

similar to that observed for the proteins on the threshold of denaturation, but which could only be caused by light absorbed by the peptide bonds. Figure 2 gives $\Delta\alpha]_{233}$ for solutions of helical PGA exposed for two hours and BPA in 1.5 M G.HCl exposed for 60 minutes at the wavelengths indicated. Both BPA and PGA exhibit the maximum change at 225 mμ. No doubt the reactivity of BPA at wavelengths above 250 mμ reflects absorption by the groups mentioned above, while the more rapid decline of the BPA curve at low wavelengths probably results from the strong absorption of G.HCl in this region. These samples were stirred before recording the ORD as described in the Methods Section and can, therefore, be compared directly to actinometry data on lamp intensity.

The peak at 225 mμ in Figure 2 occurs in the wavelength region generally assigned to the $n\rightarrow\Pi^*$ transition in helical polyamino acids (6). To determine

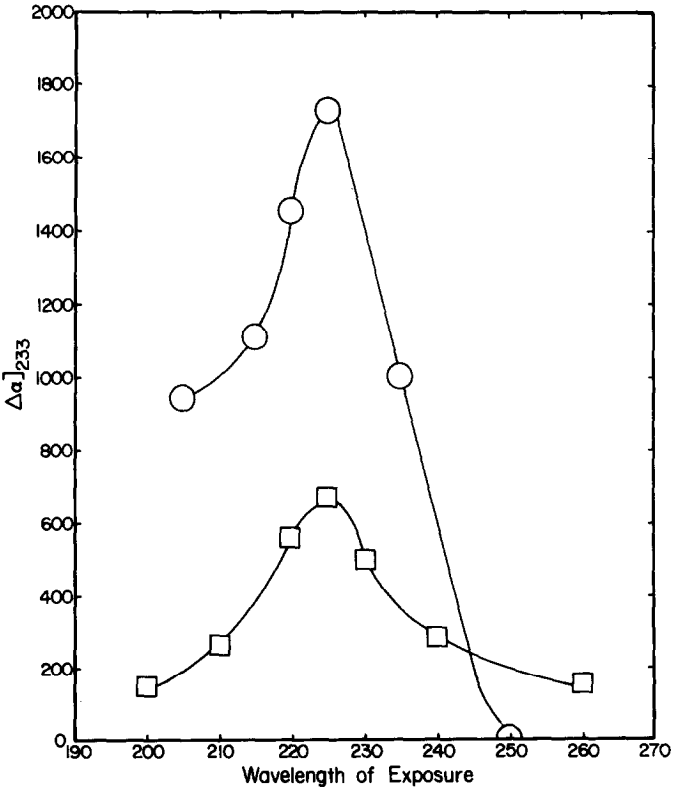

Figure 2. $\Delta\alpha]_{233}$ vs. wavelength of exposure for PGA (2 hours exposure), ○, and BPA in 1.5 M G.HCl (one hour exposure), □.

whether this absorption band favors reaction more than others, it is necessary to calculate quantum yields which are generally expressed in terms of moles reacted (1). In this case we have no way of determining moles of product formed, but an ORD quantum yield, Φ', has been calculated for PGA using $\Delta\alpha]_{233}$ as a measure of reaction on exposure at each wavelength, that is

$$\Phi' = \frac{\Delta\alpha]_{233}}{(1-10^{-A})\ I\ t} \qquad (1)$$

Here A is the absorbance of the irradiated PGA solution, I is the intensity of the light in quanta/second striking the cell, and t is the time of exposure in seconds. The quantity $(1-10^{-A})$ I t gives the quanta absorbed by PGA at each wavelength. The fraction of light absorbed by PGA, $(1-10^{-A})$, is increasing from 235 to 200 mμ, but the intensity of the light emitted by the Cary 60 monochromator is rapidly decreasing over this wavelength region. Correcting the change in specific rotation from Figure 2 for these factors gives Φ' which is plotted in Figure 3. Assuming the same reaction(s) to be responsible for

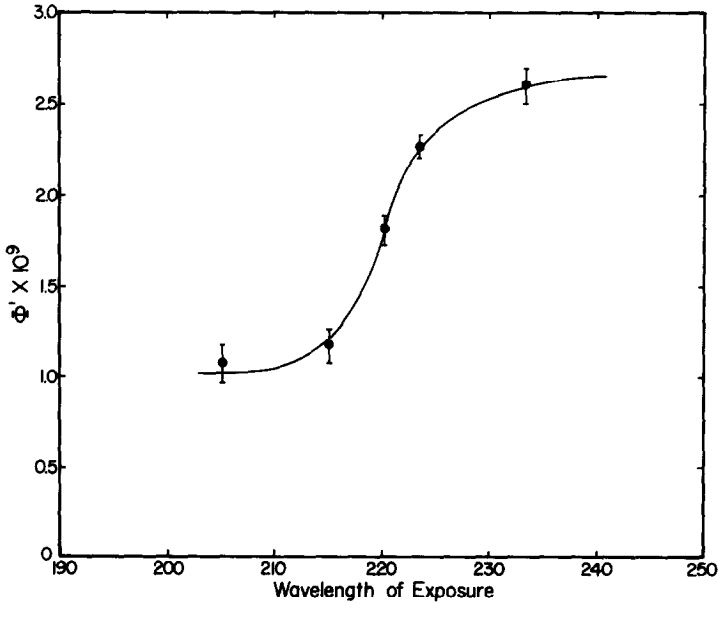

Figure 3. The ORD quantum yield (see text for explanation) for PGA as a function of wavelength of exposure.

the rotation change over the wavelengths 240 to 200 mμ, these results clearly indicate excitation of the $n \rightarrow \Pi^*$ mode to be more effective than $\Pi \rightarrow \Pi^*$ excitation. Experiments are in progress to ascertain the nature of the reaction or reactions over this entire wavelength range.

Polylysine in the α-helical form (7) gave results quite similar to those for α-helical PGA. The polyamino acid was then converted to the β form by the method of Davidson and Fasman and irradiated with constant recording at 233 mμ. No detectable change in ORD was found on irradiation for one hour at this wavelength or at several other wavelengths in the 200 to 240 mμ region. Since the secondary structure of the polyamino acids proved to be quite important for the ORD change, PGA was converted to the coiled form (pH 7.30, 0.05M NaCl) (5) and irradiated at several wavelengths between 205 and 230 mμ. No change in the ORD curve of coiled PGA was found in several hours.

It is possible that the β form is reacting on exposure to light, but does not show a detectable ORD change due to its highly cooperative structure. Also the coil might react but not give a detectable ORD change. To resolve this question, the intrinsic viscosity of PGA was measured before and after exposure. The intrinsic viscosity of helical PGA decreased from 108 to 71 cc/g and of coiled PGA from 212 to 175 cc/g upon exposure to ultraviolet light of 225 mμ for three hours in the Bausch and Lomb monochromator.

These results indicate that ultraviolet light is capable of promoting cleavage of the polypeptide chain. The cleavage seems to occur regardless of the conformation of the peptide, but only in the helical conformation is a significant alteration of optical rotation observed. This explains our observation of substantial changes in $\alpha]_{233}$ of the highly helical proteins, BPA and myoglobin, and the very small effects observed in two proteins of low helix content, β lactoglobulin and cytrochrome C. The results on PL in the β conformation also suggest an explanation for the much more dramatic effects seen in BPA and myoglobin in the presence of denaturants. The native protein can doubtless withstand some bond cleavage without significant alterations in

conformation, whereas in the presence of denaturants even slight damage results in loss of folding and a consequent reduction in the magnitude of the 233 mμ trough. This possibility is under further investigation.

Investigators employing ORD or spectrophotometric equipment for studies of peptides or proteins should take precautions, especially where slow scans through the peptide absorption bands are involved. Degradation effects can obviously be minimized by using cells of large volume with respect to the light beam, and by storing samples with the slit closed or at a wavelength above 300 mμ when doing experiments involving repeated measurements on the same sample. Stirring between ORD recordings would also help to minimize errors due to degradation. With highly helical proteins and especially under denaturing conditions it might be advisable to change the solution frequently.

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