ON THE PHOSPHORESCENCE OF COLLAGEN[+]

M.W. Grimes[*], D.R. Graber, and A. Haug

MSU/AEC Plant Research Laboratory
East Lansing, Michigan 48823

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Summary. Phosphorescent collagen emits blue light from two independent triplet states, which are of a Π-Π^*-type. At 77°K, collagen in solution and solid collagen are characterized by lifetimes of 0.9 ± 0.1 sec. and 4.5 ± 0.5 sec. The short-lived component, which is tenatively attributed to the tyrosine chromophore, is about ten times more intense than the long-lived component, which has been assigned to phenylalanine. At room temperature, phosphorescent solid collagen has lifetimes of 0.5 ± 0.1 sec. and 2.3 ± 0.2 sec., respectively. At 77°K, solid collagen shows a very weak $\Delta m = 2$ transition in the electron paramagnetic resonance spectrum with a zero-field-splitting value of $D^* = 0.1294\ cm^{-1}$. This transition probably occurs in phenylalanine.

Introduction. Collagen consists of three separate protein chains which form a triple helical structure stabilized by interchain hydrogen bonds.[1] Soluble collagen in acid solution is a stiff, rod-shaped macromolecule which is 290 nm long, has a diameter of 1.5 nm, and has a molecular weight of about 350,000. Dry collagen is only 1.0 nm thick.[2] Vertebrate collagen contains small amounts of tyrosine and phenylalanine, but contains no trypotophan residues.[3] Because of this low aromatic amino acid content, collagen doesn't exhibit the absorption peak around 280 nm, which characterizes most proteins.

Upon irradiation with ultraviolet light of 254 nm, collagen in solution undergoes a photopolymerization. Irradiated collagen exhibits a blue fluorescence which results from photochemically modified residues of

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tyrosine and phenylalanine.[4] The phosphorescence spectrum of collagen in the solid state has a broad peak around 390 nm. It was concluded that this phosphorescence emission results from the excitation (365 nm) of the carbonyl group and does not require the excitation of the aromatic ring system.[5]

It is the purpose of this paper to examine the phosphorescent triplet state of the collagen macromolecule, in solution as well as in the solid state. The lifetime of this excited state is of the order of seconds, and therefore the triplet state may play an important role in the formation of photoproducts when collagen is irradiated with ultraviolet light.

Materials and Methods. Denatured and non-denatured collagen from tendon was purchased from Sigma Chemical Corp. and from Calbiochem. Corp., respectively. Cow tendon collagen was also obtained as a gift from the Food Science Laboratory of Michigan State University, East Lansing, Michigan. Collagen in solution was obtained from solid collagen by extraction with 0.45 M NaCl.

Phosphorescence spectra and lifetimes of collagen were measured with an instrument described recently.[6] This apparatus employs a 0.5 m Jarrell-Ash emission monochromator and a multichannel analyzer operated in the signal averaging mode. The sample was placed in a quartz tube and filtered or non-filtered light (Corning filters, Bausch and Lomb monochromator) from a 1 KW mercury-xenon lamp was focused on it. To obtain a rigid glass collagen in 0.45 M NaCl was mixed with ethylene glycol (3:2). Solid collagen was used directly.

The electron paramagnetic resonance (EPR) experiments were carried out with a Varian 4502-15 X-band spectrometer equipped with a liquid nitrogen accessory.

Results. The phosphorescence spectrum of collagen, measured at the temperature of boiling liquid nitrogen, is presented in Fig. 1. This emission spectrum was obtained by irradiation of collagen with either non-filtered light or using a Corning 7740 cut-off filter which transmits light above 280 nm.

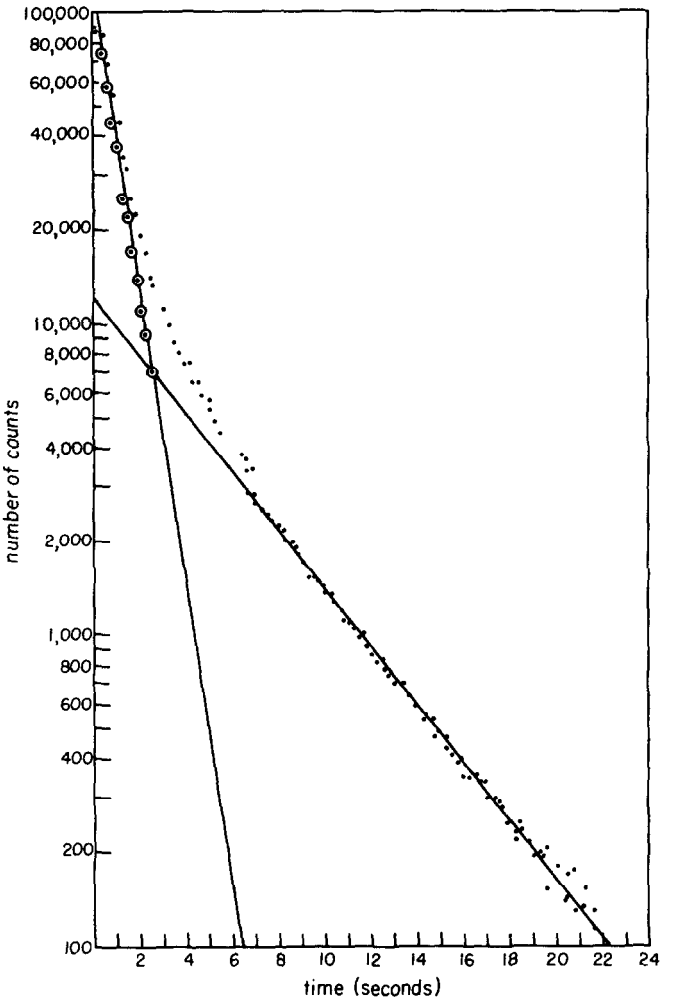

Fig. 1 Phosphorescence spectrum (uncorrected) of collagen, (a) solid collagen at 77°K, (b) collagen in 0.45 M NaCl/glycol (3:2) at 77°K, (c) solid collagen at room temperature.

At 77°K, the phosphorescence spectrum of collagen in solution exhibits pronounced vibrational levels at 396 nm, 435 nm, and 460 nm (Fig. 1). The emission of solid collagen at 77°K is red-shifted when compared to that of dissolved collagen and has vibrational bands at 415 nm and 440 nm. The phosphorescence spectrum of solid collagen at room temperature is further shifted towards longer wavelengths and the broad unstructured peak is centered around 470 nm. The intensity of the phosphorescence emission at 300°K is

of the order of 0.1 times that at 77°K.

The decay curves of photoexcited phosphorescent collagen can be time-resolved into two independent exponential decay curves, which result from two separate triplet states (Fig. 2). At 77°K, the long-lived component of the decay curve of solid collagen or collagen in salt solution at pH 4.5 is characterized by a life-time of 4.5 ± 0.5 sec. Under these conditions the short-lived component is characterized by a lifetime of 0.9 ± 0.1 sec. The emission of blue light by solid phosphorescent collagen at room temperature has lifetimes of 2.3 ± 0.2 sec. and 0.5 ± 0.1 sec., respectively. At 333°K it was not possible to detect phosphorescence. For both 77°K and room temperature, the fast component is about ten times more intense than the long-lived component. Within error of measurement, the lifetimes showed no difference whether pumped or non-pumped samples were used.

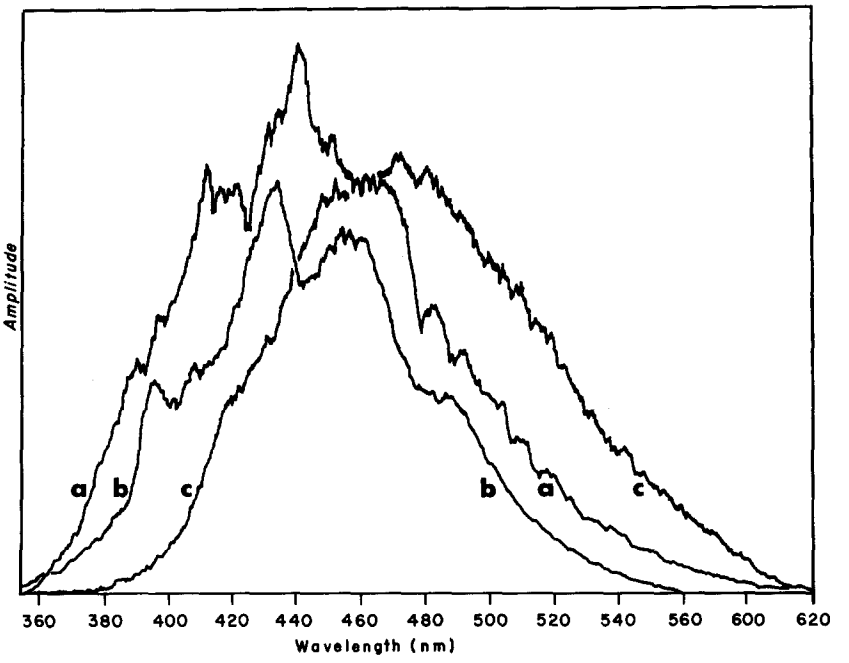

Fig. 2 Phosphorescence decay of solid collagen at 77°K. This graph shows that the decay curve can be represented by two superimposed exponential decay curves.

At 77°K, solid collagen shows a very weak $\Delta m = 2$ transition in the EPR spectrum. For a frequency of 9239 MHz, $H_{min} = 1443$ gauss, from which a zero-field-splitting parameter of $D^* = 0.1294\ cm^{-1}$ is obtained.[7] Experiments to determine the lifetime of this weak transition were not successful.

For purposes of comparison phenylalanine in a water/glycol matrix (3:2) was also investigated. It has a lifetime of 8.4 ± 0.6 sec., $H_{min} = 1435$ gauss and $D^* = 0.1318\ cm^{-1}$. For a frequency of 9239 MHz, tyrosine has a $\Delta m = 2$ transition at 1405 gauss from which a zero-field-splitting parameter of $D^* = 0.141\ cm^{-1}$ is obtained.[8]

Discussion. The data demonstrate that photoexcited collagen emits blue light from two triplet states. Because the lifetimes are of the order of one second and more these triplet states are of the Π-Π^*-type.

Since vertebrate collagen does not contain any tryptophan[3], it is reasonable to attribute the short-lived component of the decay curve to tyrosine and the long-lived component to phenylalanine. This assignment is supported by the following facts.

The excitation light used populates both tyrosine and phenylalanine.[9] The absorption spectrum of tyrosine in aqueous solution shows a maximum at 275 nm; its phosphorescence spectrum at low temperature has a broad emission peak in the range from 370 to 410 nm, and a shoulder around 415 nm. The absorption spectrum of phenylalanine in aqueous solution is distinctly structured with vibrational bands in the 230 to 280 nm region.[9],[10] The phosphorescence spectrum at 77°K shows vibrational levels at 425, 449, and 493 nm. At 77°K collagen in solution and solid collagen has vibrational bands which may result from the bound aromatic amino acids.

The decay time of the phosphorescent tyrosine molecule, at 77°K, has been measured as 2.1 ± 0.1 sec. or 2.7 ± 0.2 sec.[11], respectively. Ultraviolet light irradiated dry tyrosine shows a lifetime of 1.8 ± 0.3 sec.[11] For phenylalanine, values of 5.5 ± 0.5 sec.[12] and 7.3 sec. have been reported.[10]

We found a lifetime of 8.4 ± 0.6 sec. At 77°K in ultraviolet light irradiated dry trypsin, tryptophan, shows a lifetime of 5.0 ± 0.4 sec. as compared to a lifetime of 6.6 ± 0.2 sec. for free tryptophan in solution.[11] A similar shortening of the lifetime is observed in the case of collagen.

The phosphorescence spectrum of solid collagen at room temperature is red-shifted and the lifetimes are about half as long as those measured at 77°K. Compared to collagen in solution, the phosphorescence peak of solid collagen at 77°K is also shifted to longer wavelenghts. It is reasonable to assume that such a phosphorescent behavior reflects conformational changes of the protein structure in the vicinity of the phosphorescent aromatic amino acids. At higher temperatures additional deactivation processes become operative on the radiative levels which leads to a shortening of the lifetimes. It should be recalled that wet collagen macromolecules are 1.5 nm thick while the dry molecules are only 1 nm thick.[2]

Similar temperature dependent changes of protein structure have been observed in the tyrosine fluorescence of ribonuclease.[13] The blue fluorescence of ultraviolet light irradiated collagen, arising from photoproducts of tyrosine and phenylalanine, is somewhat quenched in the helical conformation of the macromolecule. The quenching is removed when the macromolecule undergoes a thermal transition to a random coil.[4] Altering the conformation of bovine serum albumin by urea or heat causes the phosphorescence emission to disappear.[14]

The lack of oxygen quenching of the triplet states of collagen may indicate that the local environment of the protein protects the bound aromatic chromophores from being perturbed by the oxygen. Absence of oxygen quenching has been found in flash experiments of the triplet state of eosin bound to albumin.[15]

It is highly probable that the $\Delta m = 2$ transition in the EPR spectrum of phosphorescent collagen results from photoexcited phenylalanine.

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