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A tryptophan synchronous and normal fluorescence study on bacteria inactivation mechanism

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The UV photodissociation kinetics of tryptophan amino acid, Trp, attached to the membrane of bacteria, *Escherichia coli* and *Bacillus subtilis*, have been studied by means of normal and synchronous fluorescence. Our experimental data suggest that the fluorescence intensity of Trp increases during the first minute of irradiation with 250 nm to ~ 280 nm, 7 mW/cm² UV light, and subsequently decreases with continuous irradiation. During this short, less than a minute, period of time, 70% of the 10⁷ cell per milliliter bacteria are inactivated. This increase in fluorescence intensity is not observed when tryptophan is in the free state, namely, not attached to a protein, but dissolved in water or saline solution. This increase in fluorescence is attributed to the additional fluorescence of tryptophan molecules formed by protein unfolding, the breakage of the bond that attaches Trp to the bacterial protein membrane, or possibly caused by the irradiation of 2 types of tryptophan residues that photolyze with different quantum yields.

bacteria inactivation | synchronous fluorescence | photodissociation kinetics

UV irradiation is critically important in many situations where inactivation of bacteria is required to prevent contamination or even infection. This is especially true where environmental decontamination is necessary (1, 2). Yet, despite the fact that UV light has been widely used for many years (3), features of the inactivation process by UV light remain unknown, and in-depth knowledge of the UV light interaction with biological species may lead to a more effective use. In this regard, we report on important phases of the lethal effect of UV light on 2 bacterial species of medical relevance.

Tryptophan (Trp), an aromatic amino acid, is a constituent of peptides/proteins and also a precursor of serotonin and other derivatives such as adenine dinucleotides. Trp is known to be the most intensely fluorescing component of *Escherichia coli* and *Bacillus subtilis* bacteria, and the photooxidation of proteins after absorption of UV radiation is known to proceed by either direct photooxidation that generates excited singlet and triplets states or formation of singlet oxygen, ROS, by energy transfer to ground-state molecular oxygen, process I and process II, respectively (4–7). Although the photostability of Trp-containing proteins has been investigated, the detailed mechanism of this photochemical process is not completely known. To that effect, the data presented in this article provide information that advances knowledge concerning the detail mechanism of the initial bacterial photoinactivation process.

While peptide bonds exhibit a weak absorption band, $n \rightarrow \pi^*$ transition at 210 nm to ~220 nm, $\epsilon = 100 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, the absorption spectrum of Trp, a major chromophoric amino acid, exhibits a strong band at 220 nm and a weaker one at 280 nm with molar extinction coefficients of 22,900 and 3,800, respectively. This suggests that the indole moiety of the Trp molecule is the Trp component that absorbs in the near UV and generates the ROS that reacts efficiently with and photodegrade proteins (8–10). Experimental data indicate that singlet oxygen and possibly other radical species are mostly responsible for the photodissociation of Trp. The dissociation of Trp and tyrosine

(Tyr) induced by UV irradiation was determined by the change in their fluorescence intensity as a function of UV dose by us (11) and others (12–16). Previous experimental data show that there is a bend in the linear decay as a function of UV dose (17), which may be assigned to irradiation of undamaged residues or to the presence of 2 types of Trp residues that are photolyzed with varying UV quantum yields. Our study shows that the quantum yield of Trp photolysis in proteins is several times larger than that in free amino acids, and may vary with protein type. To that effect, our experimental fluorescence data regarding bacteria inactivation with UV light are interpreted using a mechanism which indicates that the initial photodissociation process is the detachment of some Trp molecules from the membrane protein(s) followed by dissociation and dimerization of the DNA thymine bases.

The response of bacteria and other microorganisms to UV radiation may be influenced by several factors including 1) the growth medium, 2) the stage of the culture, and 3) the strain of the microorganisms (18, 19). Photoreactivation is another important factor which determines bacterial response to UV radiation. Most, if not all studies, attribute the “death” of a bacterium by UV irradiation to the damage induced by lesions of its nucleic acids which, subsequently, form pyrimidine dimers and other photoproducts (20). The dominant product of the UV-induced DNA lesion is cyclobutane pyrimidine dimers generated by the adjacent thymine bases. The consequence of these photoproducts is the prohibition of the normal DNA cellular replication process. Such prohibition process leads to cell death and immune suppression.

Significance

The inactivation of bacteria by UV light involves sequential dynamic processes that occur from femtoseconds to minutes and span from simple molecules to proteins and organisms. Detailed understanding of the inactivation mechanism is important and the key to efficient inactivation of harmful pathogens whose antibiotic resistance is critically increasing. In this study, we show that, for *Escherichia coli* and *Bacillus subtilis* bacteria, their tryptophan fluorescence intensity increased within the first minute of UV irradiation and subsequently decreased continuously. These data suggest that, shortly after exposure to UV light, tryptophan may be released from the bacterial membrane and protein unfolding which contributes to the increased tryptophan fluorescence intensity. This study provides a further means for monitoring the rate of bacteria inactivation.

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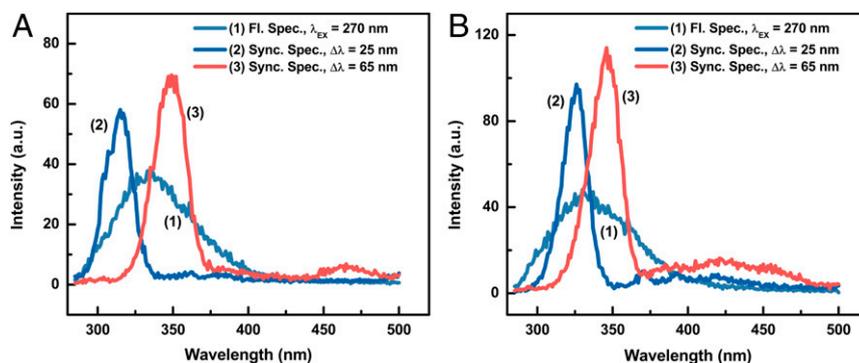


Fig. 4. Fluorescence and synchronous fluorescence spectra of (A) *E. coli* and (B) *B. subtilis* bacteria. The $\Delta\lambda$ used in the synchronous fluorescence measurements are 25 nm and 65 nm for the Tyr and Trp components, respectively.

when they are in the form of spores, which suggests that the DNA is further shielded from UV radiation. In the present study, we are concerned, mostly, with bacteria in their natural state, and, in the case of *B. subtilis* spores, the bacteria were removed from their spore environment before absorption, emission, and UV irradiation measurements were performed.

Sensitivity to UV disinfection can vary for a certain species of a microorganism species with strain type, growth medium, and stage of the culture (19). The *E. coli* bacterial samples in our experiments were irradiated with a nearly collimated beam whose UV power at the sample was measured using a calibrated power meter. And the absorption spectra of bacterial samples were recorded by a Shimadzu UV 160 spectrophotometer.

The average intensity, I_{avg} , in the optical cell that contain the bacteria was calculated by ref. 18,

$$I_{avg} = I_0(1 - e^{-A_e L})/A_e L,$$

where I_0 is the incident light intensity, A_e is the absorbance per centimeter, and L is the path length. As shown previously in Fig. 2, such increase in the fluorescence intensity was unobservable, when Trp was alone, not attached to a protein, and irradiated with UV light. In contrast, similar experiments performed with Human Serum Albumin (HSA) (Fig. 5) shows the same fluorescence intensity changes as a function of UV irradiation dose as the *E. coli* bacteria.

We also observed an initial increase in fluorescence intensity with pure proteins, egg ovalbumin, as a function of UV light irradiation dose. Such initial increase in the fluorescence of pure proteins as a function of UV irradiation has been observed earlier with bovine growth hormone (5) and tentatively attributed to protein(s) unfolding (22, 23), which is a form of UV-induced

damage to the proteins that compromise their functions in the cell and may also induce toxicity. However, as proteins unfold, a number of Trp amino acids become exposed to direct UV radiation, which results in initial fluorescence increase, followed by subsequent intensity decrease as Trp is denatured by the continuous UV radiation. This phenomenon resembles, closely, the initial increase observed for *E. coli* and *B. subtilis* described earlier.

The mechanism for this initial increase in fluorescence may be different in pure proteins and bacteria. The initial increase in fluorescence upon UV radiation represents damage to the proteins which is correlated with the death of bacteria cells. It is known that bacterial inactivation does not take place at very low UV fluencies, most probably because the low rate of inactivation is balanced by the high replication rate of bacteria (24–27). However, as discussed previously, exposure to higher UV fluence, even for very short periods of time, does result in observable bacterial inactivation.

It is known that the inactivation of bacteria irradiated with UV of wavelengths lower than 255 nm, such as 214 nm to ~220 nm, is more effective than irradiation with the 253.7-nm mercury emission line. This may be due to the higher absorption cross-section of the bacteria at the shorter wavelengths and the fact that the higher photon energy of the shorter wavelengths is absorbed by the single C–C and C–H bonds of the bacterial membranes and nucleic acids, which do not absorb at the longer wavelengths. The experimental data also suggest that, when the bacteria were irradiated with 270-nm UV light, the fluorescence spectrum displayed a similar fluorescence intensity behavior. Namely, the bacterial fluorescence intensity decreases, after an initial increase, which scales with bacteria inactivation, and, at longer irradiation time periods, a continuous photodegradation

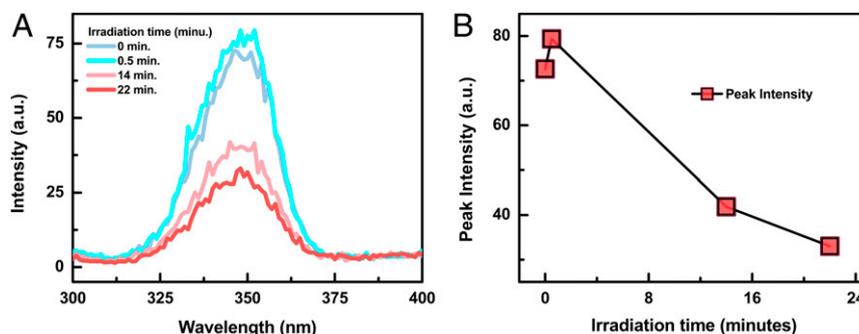


Fig. 5. HSA fluorescence spectra vs. UV irradiation time (A) and the correspondent fluorescence peak intensity vs. UV irradiation time (B). Fluorescence intensity increase was observed in the first minute after UV irradiation. HAS concentration in water solution, 100 $\mu\text{g}/\text{mL}$; UV intensity, 1.3 mW/cm^2 .

of Trp and Tyr is observed which correlates with further bacterial inactivation.

The inactivation rate constant of *E. coli* and *B. subtilis* bacteria in water has been reported by several investigators (17, 28–32) and is summarized in ref. 33. Pathogenic bacterial cells have been found to be more susceptible to UV irradiation than viruses, about which we are not concerned in this study. The inactivation rate constant K (square centimeters per millijoule) for the bacteria irradiated in the present studies varied from 0.065 for *B. subtilis* to 1.37 for *Vibrio cholera* (33). The rate constant for *E. coli* found in our studies varies from 0.63 to 0.52, which is also in very good agreement with the literature value of 0.54 (30, 34). It might be interesting to note that the sensitivity to monochromatic and polychromatic UV radiation in the same narrow wavelength region and same dose was found to be very similar when applied with the same UV flux. These experimental data are not surprising when one considers that the absorption band of the bacteria is broad and of similar optical density in this wavelength range. Our data indicate that *B. subtilis* and especially spores are less sensitive to UV than the *E. coli* bacteria. The *B. subtilis* rate constant was found to be 0.62 in our study, which is similar to the reported literature values (18, 29, 33).

One advantage of UV irradiation over other bacterial inactivation bacterial processes is the fact that the quantum yield of UV inactivation is not affected by temperature, pH, and most non-UV-absorbing impurities. However, several factors may play a role in the UV inactivation rate, including 1) the physiological state of the organism such as particle associations and

repair mechanisms, including DNA double helix restoration after dimer photolysis, and 2) fluence distribution in the sample: absorption, reflection, and scattering.

Conclusion

UV inactivation experiments with *E. coli*, *B. subtilis*, and other bacteria show that, during the first 30 s to 1 min of irradiation (~ 7 mW/cm², 220- to ~ 270 -nm UV light), a number of the Trp amino acids attached to the membrane proteins are exposed to the radiation, which causes an initial increase in the number of fluorescing Trp molecules, and, consequently, the fluorescence intensity increases temporarily. The cause of Trp molecules exposure is attributed to protein unfolding and release of Trp and Tyr amino acid molecules which may fluoresce with varying quantum yields. During this initial irradiation time period, $\sim 40\%$ of the 10^7 to 10^8 bacteria irradiated were found to be inactivated. Further UV irradiation of the bacteria results in the continuous decrease in the fluorescence intensity. The UV light absorbed by the Trp and Tyr amino acid components of the bacterium induces their photodissociation, generating “a hole” in the membrane through which the UV radiation enters the interior of the bacterium where it interacts and dimerizes DNAs, which, in turn, inhibit DNA replication and result in bacterial inactivation.

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