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The Inactivation Effects of UV Light on Bacteriophage f2

Chi-Kyung Kim and Quae Chae
Dept. of Biólogy and Dept. of Chemistry Chungbuk National
University, Chongju Korea
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박테리오파지 f2에 대한 紫外光線의 殺菌効果

김치경 · 채 쾌
충북대학교 생물학과 ·화학과

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The effects of ultraviolet light on bacteriophage f2 were investigated to determine the inactivation kinetics and its mechanism. The 260nm light showed a little higher inactivation rate than the one of 300 nm. In this work, our main concern was whether structural and/or conformational changes in the protein capsid could occur by UV irradiation. The inactivation for the first 20 minutes irradiation was rapid with a loss of about 4 logs and followed by a slower rate during the next 40 minutes with no survival noted in the samples irradiated for 90 minutes or longer. The structural change of the protein capsid was examined by optical spectroscopic techniques and electron microscopy. The absorption spectra of the UV irradiated phages showed no detectable differences in terms of the spectral shape and intensity from the control phage. However, the fluorescence emission spectroscopic data, i.e. 1) fluorescence quenching of tryptophan residues upon irradiation of 300 nm light, 2) enhancement of fluorescence emission of ANS (8-anilino-1-naphthalene sulfonate) bound to the intact phages compared to the one in the UV-treated phages, and 3) decrease of energy transfer efficiency from tryptophan to ANS in the UV-treated samples, presented remarkable differences between the intact and UV-treated phages. Such a structural alteration was also observed by electron microscopy. The UV-treated phages appeared to be broken and empty capsids. Therefore, the inactivation of the bacteriophage f2 by UV irradiation is thought to be attributed to the structural change in the protein capsid as well as damage in the viral RNA by UV irradiation.

Increasing population and expanding urbanization force increasing use and reuse of waters for domestic and industrial purposes. Therefore, water supplies and wastewater effluents must be disinfected for the public health. Disinfection by post-chlorination in water and wastewater treatment has been sufficient against enteric bacterial pathogens. But enteric viruses have been demonstrated to be much more resistant to chlorine than bacteria[1] and extremely high dosages of chlorine must be applied to achieve adequate disinfection of the viruses in waters[2]. The absence of bacteria from the treated water is not infallible proof of safety against waterborne diseases and viruses may still exist in water which is to be reused[3,4,5,6].

When chlorine for disinfection is added to waters containing organic compounds, various kinds of chlorinated organic by-products are produced[7,8]. These chlorinated organic materials may have adverse effects on health of humans as well as on the aquatic ecosystem[9,10]. The serious health concerns over the problems produced by chlorination of waters have prompted a search for alternatives.

Ultraviolet (UV) light irradiation, one of the alternatives, has advantage of no by-product production. Inactivation kinetics with UV light has been studied actively in many laboratory using a variety of microorganisms, including viruses[11,12,13]. Hill *et al.*[11] reported that UV light can be highly effective and provide a reliable safety factor in treating estuarine water.

The inactivation mechanism of UV light has been partially studied with viruses[12, 13, 14]. When poliovirus was exposed to UV light[12, 14], the virions were rapidly converted to empty capsids, with degradation of the viral RNA and a change in the antigenic specificity of the capsid. The

antigenic change is probably triggered by the release of the viral RNA[12] and is related to the release of the smallest of the structural proteins of poliovirus, VP4[15]. Miller *et al.*[13] reported that UV irradiation affects mengovirus in quite a different manner, especially in that no empty capsids are formed. Upon degradation of the virus, the viral RNA is released intact, associated with about 30% of the capsid protein in the form of 80S ribonucleoprotein (RNP) particles. Dissociation of the virions, however, is preceded by a progressively increasing instability of the virus capsid which correlated with the loss of its hemagglutination (HA) activity and ultrastructural changes in the capsid proteins. Also, some of the proteins or parts thereof become covalently linked to the viral RNA.

The bacteriophage f2 has been thought to be an adequate model to study the inactivation mechanism of UV irradiation, because it consists of a single stranded RNA and two structural proteins having known amino acid sequences[16] and because it is inactivated by chlorine and other disinfectants in a mannedr similar to the enteric viruses[17]. The RNA-containing virus has been used by many researchers[17,18,19,20,1] for the study of inactivation kinetics and inactivation mechanisms using various disinfecting agents. UV light has not been used in an inactivation study with the f2 bacteriophage.

The objective of this study was to determine the effects of UV light on inactivation of the phage f2 and particularly to observe alterations to its protein capsid using spectroscopic and electron microscopic methods.

Materials and Methods

Preparation, purification, and titration of bacteriophage f2

Bacteriophage f2 (ATCC #15766-B1) was supplied by Dr. O. J. Sproul at The Chio State University and propagated by using *Escherichia coli* K-13 Hfr (ATCC #15766) grown in tryptone yeast extract (TYE) broth medium and the ammonium sulfate method described by Loeb and Zinder[21], with minor modifications. These modifications were to prepare one liter of the host bacterial culture and to prepare the lysate by omitting filtration through a filter candle. Further purification of bacteriophage f2 was carried out by cesium chloride density gradient centrifugation (conc. of CsCl = 0.65 g/ml, 125,000 xg for 20 hrs). A Sorvall OTD 75B ultracentrifuge equipped with a T-875 rotor (Du Pont Co.) was used for the centrifugation. The phage band was collected from the middle of the tube and dialyzed twice at 4°C for 24 hours against one liter of 0.01M phosphate buffer (pH 7.2) containing 8.0g of sodium chloride per liter. The dialysate was diluted with sterile double distilled water to about 1.3 x 10^9 plaque forming units (PFU) per mililiter and stored at -30°C until used. All of the phage samples used in this study were titrated using *E. coli* K-13 Hfr as the host, according to the overlay method of Adams[22].

UV irradiation and the spectroscopical method

The suspension of purified bacteriophage f2 was diluted with sterile double distilled water to 10^7 PFU/ml. Three mililiters of the diluted sample was placed in a quartz cell and irradiated in a Rayonet photoreactor (The Southern New England UV Co., Model no. RPR-208). This photoreactor can be mounted with UV lamps which can provide different wavelength ranges of UV light (260, 280 and 300 nm regions, respectively). The sample for the spectroscopic analysis was irradiated with the 300 nm lamps (approximate lamp intensity was 60 watts with 4 lamps) at a distance of 16 cm from the light source as a functions of time. Absorption spectra of each sample were monitored by a Cary-17 spectrophotometer. Fluorescence emission and excitation spectra were measured by an Aminco-Bowmann spectro-fluorometer equipped with the IP-20 photo-multiplier tube. ANS (8-anilino-1-naphthalene sulfonate) purchased from Sigma Chemical Co. was used after recrystalization three times from dilute aqueous $MgCl_2$[23]. Concentration of ANS in the phage solution was 1 mM.

Electron microscopy

One drop of control and UV-irradiated phage samples was put on a Formvar and carbon-coated 300 mesh copper grids and dried for 30 minutes[18]. Excess water was drained with filter paper and then negatively stained with 1% phosphotungstic acid, pH 6.5, for 3 minutes. After the excess stain was drained off and air dried, the grids were examined with a Hitachi H-500 electron microscope at an accelerating voltage of 75 kV.

Results and Discussion

The inactivation kinetics of the bacteriophage f2 irradiated at different wavelengths of UV light are shown Fig. 1. The 260 nm light showed a higher inactivation rate than those of 280 and 300 nm, but no significant difference was observed between 280 and 300 nm. This result suggests that the RNA molecules of the phage are more vulnerable

to 260 nm UV light than the capsid protein. Miller and Plagemann[13] reported that the RNA of poliovirus was accessible to RNAse when the virus had been irradiated by UV light for 20 minutes and that the larger portion of the viruses were progressively broken by longer exposure. Breindle[15] and Katagiri *et al.*[12] also reported that the RNA of poliovirus type 1 was released by UV irradiation.

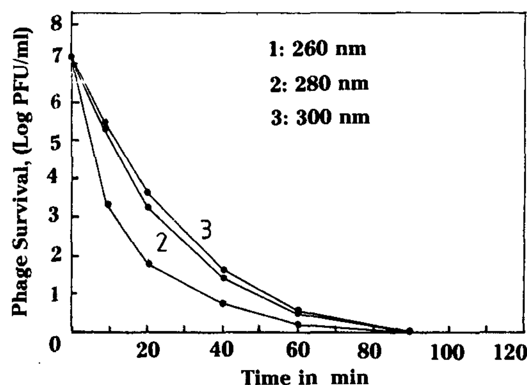

Fig. 1. Inactivation kinetics of bacteriophage f2 by UV irradiation at 260, 280, and 300 nm wavelengths, respectively

This study was primarily focussed on UV effects on the capsid proteins of the phage, so we chose 300 nm UV light as the source in the following experiments expecting that the protein capsid would be more affected by 300 nm UV light than 260 nm. The UV irradiation kinetics of the phage f2 at 300 nm wavelength are shown in Table 1. The inactivation rates during two-hour irradiations from three experiments were very similar each other. The inactivation during the first 20 minutes of irradiation was rapid with a loss of about 4 logs of virus. During the next 40 minutes, the phages were gradually inactivated at a slower rate in each case. In the phage samples irradiated for 90 minutes or longer no viable phage was found.

Such a two-step inactivation was also reported by Hill *et al.*[11]. They explained that the two-step kinetics could be attributed to virus aggregation. The virus sample used in this study did not show any aggregation by electron microscopy. Our results had a pattern similar to those observed by Kim *et al.*[18] and Katzenelson *et al.*[24] with the f2 phage. Katzenelson *et al.*[24] explained the two-step kinetics as a threshold effect in virus inactivation. Galasso and Sharp[25] pointed out that rotating or stirring the visus suspending fluid during UV exposure would increase the inactivation rate. In this study, the samples were hung in the irradiation box and the rotating effect of the sample was

Table 1. Inactivation kinetics of bacteriophage f2 by UV light of 300 nm wavelength

Irradiation time (min)	F2 phage survival (PFU/ml) in three experiments		
	1	2	3
control	1.2×10^7	1.5×10^7	7.6×10^6
5	3.4×10^6	---[a]	---
10	1.3×10^6	2.2×10^5	3.6×10^4
15	6.0×10^4	---	---
20	3.8×10^4	6.4×10^3	2.8×10^3
30	5.2×10^3	1.8×10^3	1.4×10^3
40	---	8.2×10^2	---
50	---	2.1×10^1	3.2×10^1
60	1.2×10^1	6.8×10^0	5.3×10^0
90	---	0	0
120	0	0	0

a, not determined.

not examined.

The phage particles inactivated by 300 nm UV irradiation must have had both their RNA and protein capsids changed. Structural changes in the capsid by UV irradiation has been reported by several workers in some types of viruses[12,14]. Altered proteins in the capsid were demonstrated by Miller *et al.*[13] using hemagglutination and equilibrium centrifugation techniques. Since the structural and/or conformational change of the proteins can be detected with fluorescence techniques, these techniques were used to examine structural changes in the irradiated protein capsid of the phage f2. The fluorescence parameters such as quantum yield (Φ_F), energy transfer, spectral shape, life time (τ_F), and rotational relaxation time are quite sensitive to the microenvironmental change of the fluorophores[25,27,28].

In this work, we focussed on the structural and/or conformational alterations of the protein capsid, which were attributed to the UV light, rather than alterations of RNA buried in the protein coat. Fig. 2 shows the absorption spectra of the phages (conc.: 10^7 PFU/ml) as a function of irradiation time. The irradiation by 300 nm was employed for better separation of RNA and protein absorption of UV light. However, no detectable differences in spectral shape and intensities were observed between each sample. The results imply two possibilities: 1) no dramatic structural change of a protein moiety, or 2) a slight microenvironmental change which can not be detected by the spec-

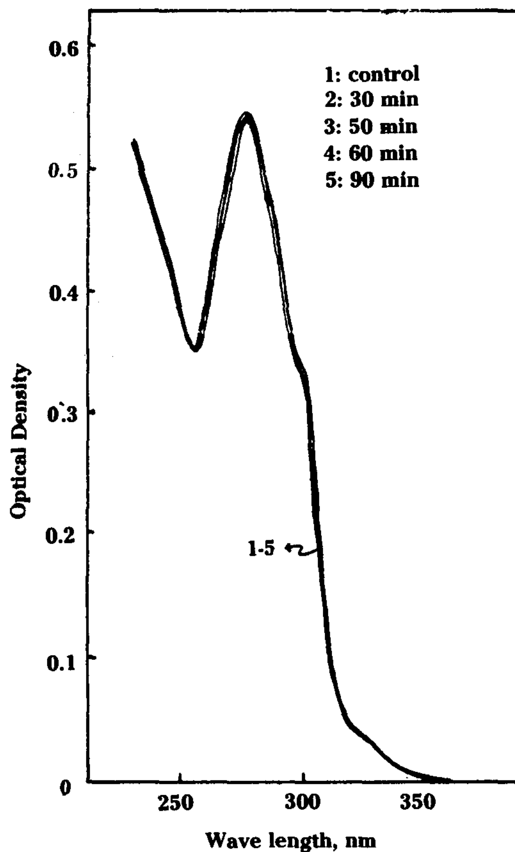

Fig. 2. Absorption spectra of bacteriophage f2 in distilled water as a function of irradiation time of UV light.

The concentration of the phage is 10^7/ml and the irradiation time intervals are inserted in the figure.

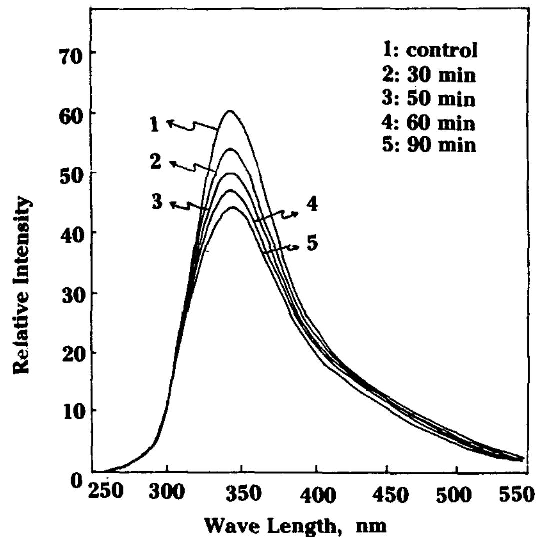

Fig. 3. Fluorescence emission spectra of bacteriophage f2 in distilled water (room temperature) as a funofion of irradiation time of UV light.

The sample (10^7/ml) was diluted 3 times with distilled water and excitation wavelength was 280 nm.

trophotometer.

In Fig. 3, a slight, but systematic decrease of the fluorescence intensities of tryptophan residue (λem: 350nm) as a function of irradiation time is seen. According to amino acid analysis, the phage f2 contains two tryptophan residues in its protein capsomere[16]. It is a well known fact that tryptophan in the hydrophobic environment exhibits higher fluorescence intensity than in the polar environment. Based on the above phenomenon, it is very likely that the hydrophobic environment of tryptophan residue gets become to be exposed by the UV light effect. At the moment the detailed molecular change of the protein capsid is unknown, but it is almost certain that there must be a kind of conformational change of the protein. This result qualitatively agrees with the electron micrographs.

In order to ascertain the above results, we carried out ANS (hydrophobic fluorescence porbe) experiments[29]. Fig. 4 shows the fluorescence emission spectra of ANS bound to the hydrophobic site of the protein capsid treated with and without UV light (λ max = 300 nm). The dramatic enhancement of fluorescence intensity of ANS in the control phage (intact phage f2 in water) is observed in contrast to the ANS only in the aqueous environment. However, the slight increase of fluorescence intensity of ANS was shown in the case of phage treated with UV before addition of ANS. This result also suggests that the environment of ANS is different between the UV treated sample and the untreated one.

Energy transfer behavior of tryptophan to the ANS was also checked (Fig. 5). The strong fluorescence of the sample 1 (ANS + intact phage) was observed at the 515 nm region by exciting at 280 nm. In contrast, the sample 2 (ANS + UV treated phage) shows a strong fluorescence at the 350 nm region (tryptophan's emission band) and a weaker fluorescence at the 515 nm region. An emission intensity of tryptophan is shown in sample 1. This means a partial energy transfer from tryptophan to ANS in sample 1. However, it is apparent that more energy transfer has occurred in sample 1 than in sample 2. The absorbance of

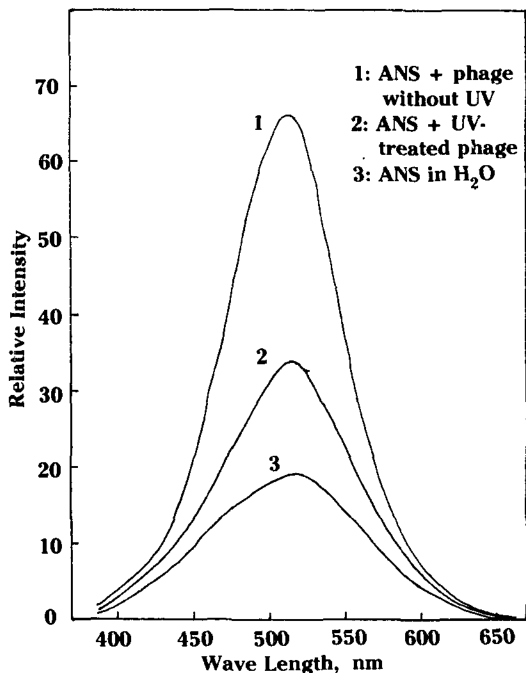

Fig. 4. Fluorescence enhancement of ANS (1 mM) upon binding to the bacteriophage f2.

ANS bound to the phage without UV Irradiation: 1, ANS bound to the phage with UV irradiation for 90 minutes: 2, ANS in aqueous environments: 3.

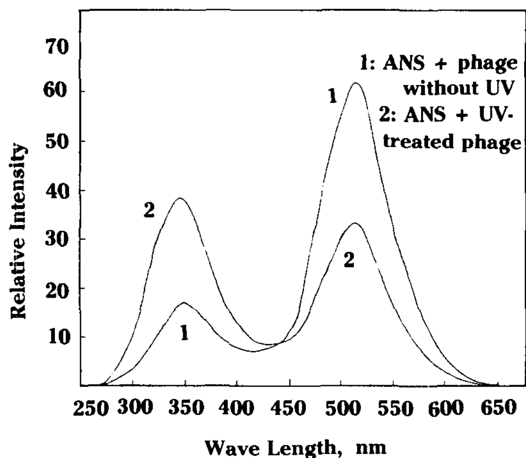

Fig. 5. Fluorescence emission spectra of ANS bound to the intact bacteriophage f2 and to the UV treated one (90 min.) upon exciting at 280 nm light.

The first sample shows a dramatic enhancements of ANS fluorescence and decrease of intensity of 280 nm band. However, the opposite behavior in the second sample has been shown.

ANS at the region of 280 nm (λmax of tryptophan) confuses the interpretation of the results. The different fluorescence intensities at 515 nm with the same concentration of ANS (1 mM) in both sample rules out the confusion however. Considering the important molecular factors required for the energy transfer from energy donor to the acceptor, i.e. distance and orientation between those molecules, the energy transfer data consistently support the structural and/or conformational change of the viral capsid protein by UV irradiation.

In order to prove the change of the protein capsid in the UV-treated phages examined by fluorescence techniques and to correlate the change with the inactivation rates of the phage, the control and UV-treated phages were observed with an electron microscope. The control phage (Fig. 6A) had the integrity of the icosahedral structure. Almost all phages were full with RNA and were structurally intact. In the phage samples which had been irradiated by 300 nm UV light for 40 minutes (Fig. 6B), some phages were broken and others were structurally intact. The intact phage particles are shown to be empty. Formation of empty capsids by UV irradiation was reported by Katagiri *et al.*[12] and Roizman *et al.*[14] in poliovirus, but was not observed in mengovirus[13]. The empty capsids were also observed by Kim *et al.*[18], when the same f2 phage was ozonated. From the fact that the f2 phage is very similar to poliovirus

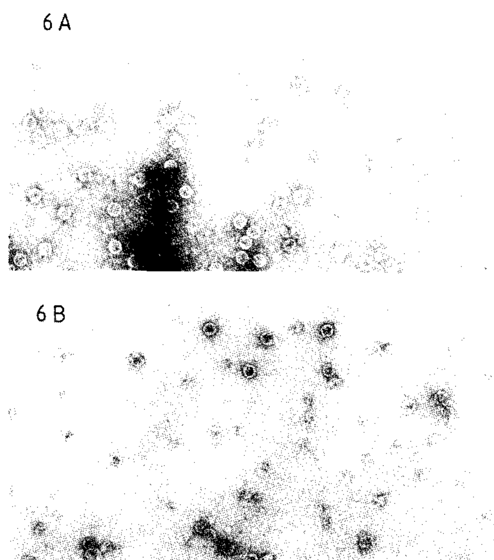

Fig. 6. Electron Micrographs of Bacteriophage f2.

control phages Control Phages (A) are structurally intact, but the phages (B) irradiated by UV light at 300 nm wavelength for 40 minutes show many borken and empty capsids.

in chemical and physical condition and in a manner of its response to many disinfectants, we assume that the protein capsid was structurally altered when the phages were UV irradiated, and the RNA was released from the capsids.

Therefore, the inactivation of the phage by UV irradiation is thought to be attributed to damage to both the RNA and the protein capsid. The higher inactivation rates during the first 20 minutes irradiation and at 260 nm UV light suggest that the first order inactivation may be due to the effect on RNA and the second order inactivation due to the effect on capsid protein. In other words, it seems that the UV effect on RNA is a one-hit effect and the effect on capsid protein is progressive and accumulative. At this time we have found only an approximate and slight conformational change of the capsid protein by UV treatment of the phage. To show the detailed molecular behavior due to the UV effect, more work must be done. In particular, the relationahips between the survival rate of the phage and the protein's structural change must be investigated.

요 약

Bacteriophage f2에 대한 紫外光線의 殺菌効果와 외투막 蛋白質의 構造에 미치는 影響을 Rayonet photoreactor PPR-208을 使用하여 300nm의 光線으로 研究하였다. 처음 20분간의 照射 에서는 약 4 log의 phage가 減少되고 그후 완만한 殺菌効果를 보이다가 90분 以上의 照射에서는 生存 바이러스가 発見되지 않았다. Tryptophan residue의 fluorescenve quenching, 紫外線으로 照身한 phage에 附着시킨 ANS (8-anilino-1-naphthalene sulfonate)의 fluorescence emission의 減少, tryptophan에서 ANS로의 energy transfer 의 減少 등 spectroscopic technique에 의한 結果와 紫外線 照射에 의하여 蛋白質 외투막이 破壞되는 電子顕微鏡 観察의 結果에 의하여 紫外光線은 phage f2의 외투막 蛋白質의 構造에 変化를 일으킨다는 것이 밝혀졌다.

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