Photosensitized splitting of *cis-syn* 1,3-dimethyluracil dimer by tryptophan and its peptides*

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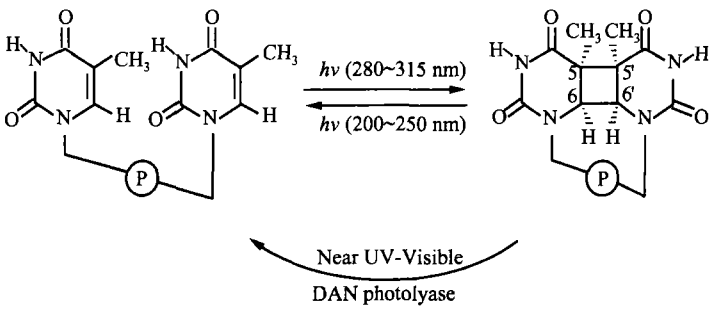
Abstract Photosensitized splitting of *cis-syn* 1,3-dimethyluracil dimer by tryptophan (Trp) and its peptides (Trp-Tyr and Trp-Phe) is studied by fluorescence quenching experiments and irradiation experiments. It reveals that 1,3-dimethyluracil dimer is mainly split by e_{aq}^- resulting from two-photon ionization of Trp and its peptides under irradiation with light ($\lambda > 290$ nm). Moreover, the excited singlet state of Trp and its peptides can transfer an electron to the dimer, which splits and gives two pyrimidines. In addition, because of its lower oxidative potential, the excited triplet state of Trp-Tyr can also split the dimer via transfering an electron to the dimer.

Keywords: tryptophan, cyclobutane dimethyluracil dimer, DNA photoreactivation.

Ultraviolet (UV) light is harmful to the biological function of DNA. The major lesion in DNA by this irradiation is the formation of cyclobutane pyrimidine dimer (CPD) which results from [2+2] photocycloaddition between 5, 6 C=C double bonds of two adjacent pyrimidine bases in the same strand of the DNA double helix (Scheme 1). The CPD would induce cell death by blocking replication and transcription and result in mutation and even skin cancer. Although the reverse photochemical reaction is symmetry-allowed by orbital symmetry consideration, the CPD does not absorb UV-B light (280~315 nm) significantly as it does not possess the conjugate π system as the original pyrimidine, and thus the CPD accumulates in DNA. Cells protect themselves against these effects by eliminating these photoproducts from their genome either by excision or by photoreactivation under near UV and visible light, and the latter is catalyzed by photolyase. In recent years due to concerns about the depletion of atmosphere ozone, which is expected to increase the flux of UV irradiation at the earth surface over the next decade, DNA repair, especially DNA photoreactivation has received heightened attention of chemists and biologists[1,2].



Scheme 1. Formation of thymine dimer between two adjacent thymine bases in the same strand of DNA double helix irradiated by UV-B light (280~315 nm). Reverse reaction is photoreactivation catalyzed by photolyase under UV-Vis light (300~500 nm).

In photoreactivation, DNA photolyase can specially recognize the damaged DNA and bind to it at the site where the CPD is located. Under the irradiation of near UV and visible light the second cofactor of DNA photolyase as a light-harvesting antenna absorbs a photon and, by dipole-dipole interaction transfers the excitation energy to the catalytic cofactor $FADH_2$ ($FADH^-$ is the active form) which, in turn,

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transfers an electron to the CPD in DNA, the CPD splits and back electron transfer restores the dipyrimidine and the functional form of $FADH_2$ ready for a new cycle of catalysis[3]. In addition to energy and electron transfer during the process of repairing, two other photoinduced electron transfer processes have been observed[4]. First, during purification, the catalytic cofactor $FADH^-$ is easily oxidized to the catalytically inert neutral radical form, $FADH^{\cdot}$. Under the irradiation of white light this form can be reactivated by electron transfer from Trp to the excited quartet state of $FADH^{\cdot}$. Second, in the presence of near UV light, the CPD can be split by the direct electron transfer from a Trp in apoenzyme with high efficiency ($\Phi = 0.56$[5]). Both of these electron transfers which play an important role in DNA photoreactivation[6] concern Trp residues of polypeptide. The former was called photoactivation whose intraprotein electron transfer has been observed directly[7,8]. In contrast, relatively little knowledge is obtained for the latter. The present work is aimed at investigating the splitting mechanism of the CPD by protein enzyme based on the photosensitized splitting of *cis-syn* 1, 3-dimethyluracil dimer by Trp and its peptides (Trp-Tyr and Trp-Phe).

1 Material and methods

1.1 Material

Trp, Trp-Tyr and Trp-Phe were purchased from Sigma Chemical Co. and used without further purification. Uracil was obtained from Shanghai Chemical Co. (China) and used as received. Other reagents were commercially available and were used after recrystallized or redistilled twice.

1.2 Equipment

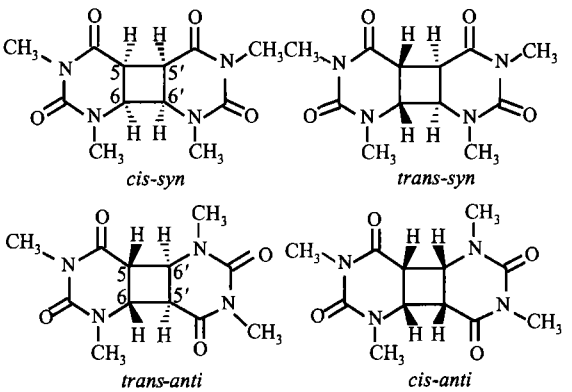
UV-Vis spectra were measured on a Lambda Bio20 UV/VIS spectrometer using a 10 mm path length quartz cell. Fluoresence emission spectra were measured on a 970R fluoresence spectrometer (Shanghai Analysis Instrument Co.). Cyclic voltammography was performed by a BAS CV-27 Voltammograph using a Hg/Hg_2Cl_2 electrode (SCE) as a reference with tatraethylammonium perchlorate as supporting electrolyte.

1.3 Preparation of 1, 3-dimethyluracil (DMU)

Methyl sulfate was dipped into the alkaline aqueous solution containing uracil at 0 ℃, then heated to boiling, extracted with chloroform. After removing the solvent by rotary evaporation, the product was purified through recrystallizing in ethanol.

1.4 Preparation of 1, 3-dimethyluracil dimer (DMUD)

The solution containing DMU and acetone was placed in a Pyrex reactor (>290 nm), bubbled with high purity nitrogen (99.99%), and irradiated with a 300 W high pressure Hg lamp until DMU was depleted. Then the solvent was removed by rotary evaporation, and the residue was isolated by elution using 6:1 EtOAc/petroleum ether with a silica gel column, which produced four DMT dimers (Scheme 2), *cis-syn*, *cis-anti*, *trans-syn* and *trans-anti*, whose percentages were 48%, 26%, 16% and 10% respectively.



Scheme 2. Structures of the isomeric 1, 3-dimethyluracil dimers.

1.5 Fluorescence quenching experiment and irradiation experiment

All samples were prepared in triply distilled water and buffered with phosphate buffer (Na_2HPO_4-KH_2PO_4) at pH 7.0, and deaerated by high purity nitrogen (99.99%), oxygen (99.99%) or nitrous oxide (99.99%), bubbling for 15 min before experiment. All experiments were performed at room temperature.

As the molar extinction of DMU at 266 nm ($\varepsilon = 11500\ dm^3 \cdot mol^{-1} \cdot cm^{-1}$) is much stronger than that of DMUD ($\varepsilon = 750\ dm^3 \cdot mol^{-1} \cdot cm^{-1}$) (Fig. 1), the absorbance at 266 nm will increase when DMUD splits to give DMU. Hence, the splitting extent of DMUD can be evaluated by the absorbance change at 266 nm, and the absorbance change can be transformed to a concentration of DMU in solution. For example, if $4 \times 10^{-5}\ mol \cdot dm^{-3}$ DMUD totally splits

to give 8×10^{-5} mol·dm^{-3} DMU, the change of absorbance at 266 nm will be $\Delta A_{266}=11500\times0.08\times10^{-3}\times1-750\times0.04\times10^{-3}\times1=0.89$. According to this, the splitting extent of DMUD can be calculated. In Fig. 2 the splitting percentage of DMUD is 42% after irradiating for 2 h.

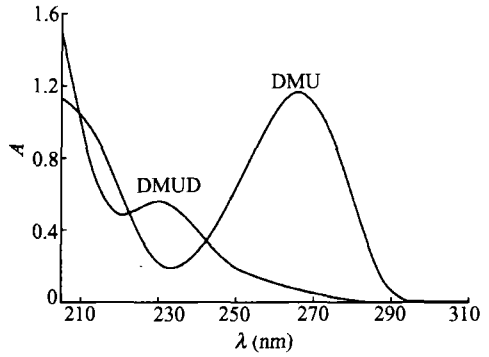


Fig. 1. UV absorption spectra of 1×10^{-4} mol·dm^{-3} dimethyluracil (DMU) and dimethyluracil dimer (DMUD) in water.

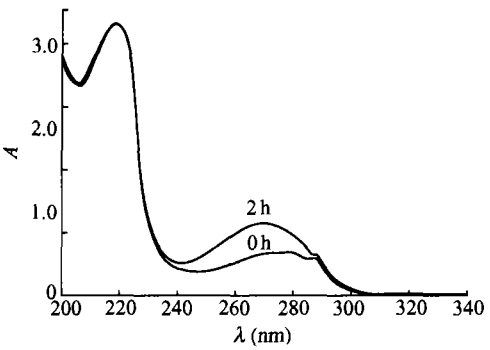


Fig. 2. UV absorption spectra of 4×10^{-5} mol·dm^{-3} DMUD in the presence of 1×10^{-4} mol·dm^{-3} Trp deaerated aqueous solution (pH 7.0) before and after irradiation for 2 h.

2 Results and discussion

2.1 Fluorescence quenching experiment

Upon the excitation of 295 nm fluorescence quenching of the sensitizers, 1×10^{-5} mol·dm^{-3} Trp, Trp-Tyr and Trp-Phe, by DMUD at different concentration, no absorption at wavelength above 290 nm was detected. The K_{sv} values were obtained from the slopes of Stern-Volmer plots (Fig. 3) and listed in Table 1. The quenching effect of Trp and its peptides by DMUD shows an order of Trp-Phe > Trp > Trp-Tyr. Furthermore, the result showed that compared to nitrogen, there is only a little decrease (1%) in fluorescence intensity as saturated by oxygen and no difference in the presence of air. The fluorescence quenching extent can also be obtained from Ref. [9]. In aqueous solution the value of quenching rate constant k_q of Trp by oxygen is 5.9×10^9 mol^{-1}·dm^3·s^{-1} and that of fluorescence lifetime τ_s is 2.5 ns. And at 25 ℃ the values of oxygen concentration in water at 1 atm and 0.21 atm partial pressure are 1.27×10^{-3} mol·dm^{-3} and 2.7×10^{-4} mol·dm^{-3} respectively. According to Stern-Volmer equation in the presence of oxygen, $(F_0-F)/F=k_q\tau_0[Q]=0.019$, while in the presence of air, $(F_0-F)/F=0.004$. This result is in agreement with that of experiment.

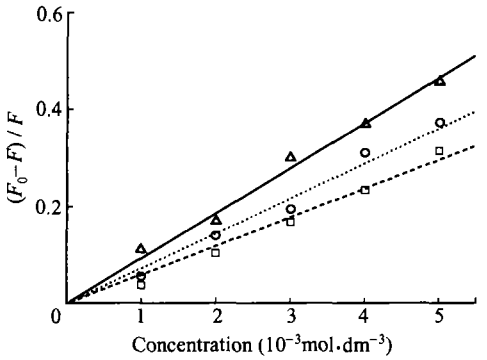


Fig. 3. Stern-Volmer analysis for fluorescence of Trp(○), Trp-Tyr(□) and Trp-Phe(△) in water (pH 7.0) quenching by DMUD.

UV absorption spectrum indicated that the singlet excited energy of DMUD (~420 kJ·mol^{-1}) was more than that of Trp (399 kJ·mol^{-1}). On the basis of energetic ground, energy transfer (singlet, triplet) mechanisms can be excluded in the splitting reactions. Therefore, DMUD quenched the fluorescence of Trp through electron transfer mechanism and it was split at the same time[10]. The reaction can be presented by

$$\begin{aligned}&{}^1\mathrm{Trp}^* + \mathrm{DMUD} \longrightarrow {}^1(\mathrm{Trp}\text{—}\mathrm{DMUD})^*\\ &\longrightarrow (\mathrm{Trp}^{\cdot+}\text{—}{}^{-}\cdot\mathrm{DMUD})\\ &\longrightarrow (\mathrm{Trp}^{\cdot+}\text{—}{}^{-}\cdot\mathrm{DMU}-\mathrm{DMU})\\ &\longrightarrow \mathrm{Trp}+\mathrm{DMU}+\mathrm{DMU}\end{aligned}$$

2.2 Irradiation experiment

The solution containing 2×10^{-4} mol·dm^{-3} DMUD and 5×10^{-4} mol·dm^{-3} Trp, Trp-Tyr or Trp-Phe (pH 7.0) was placed in the Pyrex tube (≥290 nm), each was bubbled with nitrogen, oxygen and nitrous oxide respectively, then irradiated with a 300 W high pressure Hg lamp. The absorbance at wavelength 266 nm was measured at regular intervals.

Different gases exert different effects on the photoreactivation. Nitrogen is an inert gas that will not react with the reactive intermediates of the photoreactivation. At 0.21 atm pressure the concentration of oxygen in the aqueous solution is 2.7×10^{-4} mol·dm^{-3} [1)] which will quench the excited triplet effectively and give reactive singlet oxygen. Moreover, it can react with hydrated electron (e_{aq}^-) at high constant rate ($k=1.9\times10^{10}$ dm^3·mol^{-1}·s^{-1} [11]). Nitrous oxide, an electron scavenger, can change an e_{aq}^- to a hydroxyl radical ·OH. In the nitrous oxide saturated solution (2.5×10^{-2} mol·dm^{-3}), more than 98% of hydrated electrons are converted into ·OH by nitrous oxide ($k(e_{aq}^- + N_2O)=9.1\times10^9$ dm^3·mol^{-1}·s^{-1}). In the presence of t-BuOH, ·OH reacts with t-BuOH and then converted into the nonreactive radical ·$CH_2(CH_3)_2COH$. The reaction can be presented by

$$e_{aq}^- + N_2O + H_2O \longrightarrow N_2 + {}^{\cdot}OH + OH^- \quad (1)$$

$$^{\cdot}OH + \text{t-BuOH} \longrightarrow H_2O + {}^{\cdot}CH_2C(OH)(CH_3)_2 \quad (2)$$

Under the irradiation at wavelength above 290 nm no photoreaction occurs for Trp and its peptides. In experiment, after irradiation for 2 h the UV spectrum of the solution containing Trp which was placed in the Pyrex tube and saturated with nitrogen gave no difference, while Trp-Tyr and Trp-Phe only had a little change.

2.2.1 Photosensitized splitting of DMUD by Trp and Trp-Phe Fig. 4 is the change of splitting percentage of the solution containing DMUD and Trp with irradiating time in three systems: nitrogen, air and nitrous oxide. It indicates that the splitting percentage of the system saturated with nitrogen is much higher than that of the other two systems. While the splitting percentage of the system saturated with air is similar to that of nitrous oxide. This result is in agreement with that of the solution containing DMUD and Trp-Phe (Fig. 5). Based on the above result we conclude that DMUD could be split by e_{aq}^- efficiently. In Eqs. (3) and (4) DMU-DMU$^{\cdot-}$ is a DMU molecule and a DMU radical anion linked with C6-C′6 bond. The splitting reaction of DMUD and e_{aq}^- is a very fast process whose rate constant is diffusion controlled ($\sim10^{10}$ dm^3·mol^{-1}·s^{-1})[12].

$$e_{aq}^- + \text{DMUD} \longrightarrow \text{DMUD}^{\cdot-} \longrightarrow \text{DMU} - \text{DMU}^{\cdot-} \quad (3)$$

$$\text{DMU} - \text{DMU}^{\cdot-} + \text{DMUD} \longrightarrow 2\text{DMU} + \text{DMU} - \text{DMU}^{\cdot-} \quad (4)$$

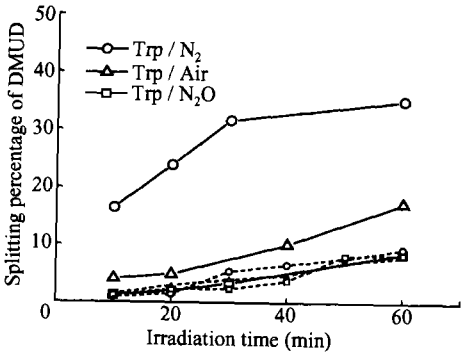


Fig. 4. The change of splitting percentage of 2×10^{-4} mol·dm^{-3} DMUD in water (pH 7.0) in the presence of 5×10^{-4} mol·dm^{-3} Trp with irradiation time.

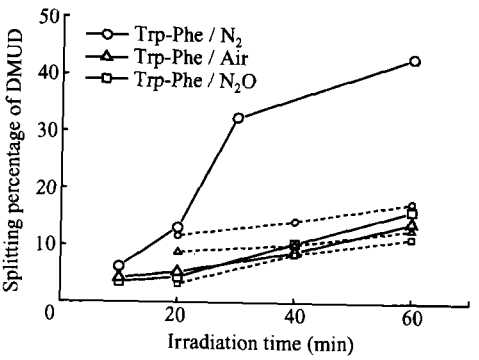


Fig. 5. The change of splitting percentage of 2×10^{-4} mol·dm^{-3} DMUD in water (pH 7.0) in the presence of 5×10^{-4} mol·dm^{-3} Trp-Phe with irradiation time.

However, because the energy of a photon at wavelengh above 290 nm ($\leqslant$4.3 eV) is not high enough to make Trp ($E_{ion}^S=4.5$ eV[13]) and its peptide ionize, there is only two-photon ionization left. According to the fluorescence lifetime of Trp (2.5 ns), it is impossible to lead to two-photo ionization through excited singlet pathway. Moreover, if singlet pathway exists, the splitting percentage of nitrous oxide saturated system will be higher than that of air in which oxygen will quench ^{1}Trp*. But the result of experiment showed that the splitting percentage is higher than or near to the air's. So e_{aq}^- is the resulting product of two-photon ionization through excited triplet pathway. In air saturated system oxygen can quench ^{3}Trp* efficiently as soon as it is created. Therefore, in air and nitrous oxide saturated systems it is excited singlet state of Trp and its peptide (S) that transfer an electron to DMUD and

1) All data in this section were obtained at 25 ℃.

make it split.

$$S \xrightarrow{h\nu(>290\ nm)} {}^1S^* \xrightarrow{ISC} {}^3S^* \quad (5)$$

$${}^3S^* \xrightarrow{h\nu(>290\ nm)} S + e_{aq}^- \quad (6)$$

$${}^3S^* + O_2 \longrightarrow S + {}^1O_2 \quad (7)$$

Under the irradiation of a low intensity light the same experiment was performed. It indicated that the splitting percentages of three systems were similar to each other (Figs. 4 and 5). This result further confirmed that e_{aq}^- results from two-photon ionization through excited triplet pathway and only excited singlet state of Trp and its peptide could split DMUD by transfering an electron to DMUD because two-photon ionization hardly occurs under a low intensity of light.

2.2.2 Photosensitized splitting of DMUD by Trp-Tyr The splitting percentage of DMUD by Trp-Tyr saturated with nitrous oxide was between that of nitrogen and air saturated systems (Fig. 6). This result is different from that of Trp and Trp-Phe.

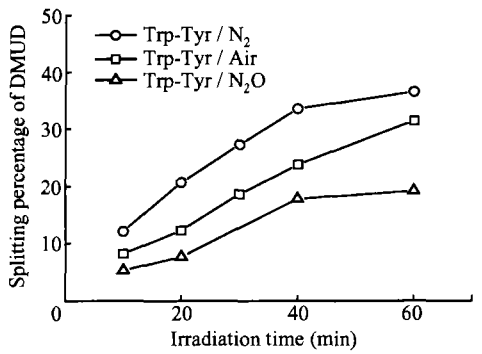


Fig. 6. The change of splitting percentage of 2×10^{-4} mol·dm^{-3} DMUD in water (pH 7.0) in the presence of 5×10^{-4} mol·dm^{-3} Trp-Tyr with irradiation time.

Moreover, the free energy changes (ΔG_S, ΔG_T) of these supposed electron transfer reactions from excited singlet and triplet of sensitizers Trp, Trp-Phe and Trp-Tyr to DMUD were calculated according to Rehm-Weller equation (Eq. 8). The reductive potential for DMUD (E_{red}) is -2.3 V (*vs.* SCE) and the lowest energy level of excited singlet and triplet Trp are 399 kJ·mol^{-1}, 298 kJ·mol^{-1} respectively. Calculated ΔG values are listed in Table 1. It shows that both the excited singlet state of Trp and its peptides can transfer an electron to the dimer and make it split. In addition, only the triplet state of Trp residue (^{3}Trp-*) of Trp-Tyr can split the dimer via transfering an electron to DMUD. Based on the above result, we conclude that in the system saturated with nitrous oxide and due to the photosensitized splitting by ^{3}Trp*-Tyr, the splitting percentage is between that of nitrogen and air.

$$\Delta G = 96.5(E_{ox} - E_{red}) - \Delta E_{0,0} \quad (8)$$

Table 1. Calculated free energy change and the rate constant for the electron transfer reaction from excited Trp, Trp-Phe and Trp-Tyr to DMUD

Compounds	K_{SV} (M^{-1})	E_{ox} [a] (V)	ΔG_S [b] (kJ·mol^{-1})	ΔG_T [b] (kJ·mol^{-1})
Trp	72	0.78	−102	−0
Trp-Phe	93	0.82	−98	+3
Trp-Tyr	59	0.67	−112	−11

a) From Ref. [14].

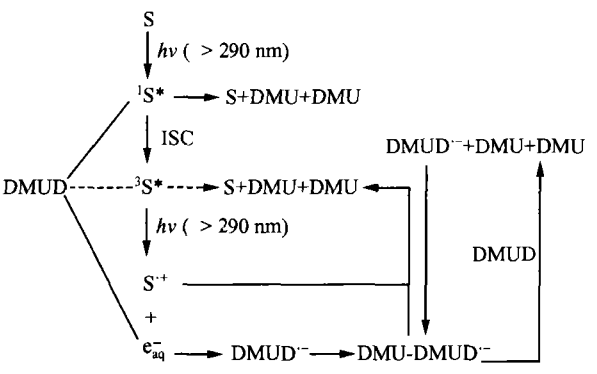
b) The excited singlet and triplet energy of Trp ($\Delta E_{0,0}$) were used in all ΔG_S and ΔG_T calculation.

3 Conclusion

From above results and discussion we could conclude the photosensitized splitting pathway of DMUD by Trp and its peptides (Scheme 3) as the following:

(i) Under the irradiation of a high intensity light DMUD is mainly split by e_{aq}^- resulting from two-photon ionization of Trp and its peptides. Besides this, the excited singlet state of Trp and its peptides can transfer an electron to the dimer and make it split. Under the irradiation of a low intensity light only the latter exists.

(ii) Owing to the lowest oxidative potential the excited triplet state of Trp-Tyr can also split DMUD via transfering an electron to the dimer.



Scheme 3. Reaction pathway of photosensitized splitting of DMUD by Trp, Trp-Phe and Trp-Tyr. — only for Trp-Tyr.

In DNA photolyase the residue Trp^{277} is proximate to the proposed substrate binding site, which makes the distance become shorter between donor and acceptor[4], the excited singlet state of Trp^{277} splits

the dimer by electron transfer with a high efficiency under the irradiation of UV light. In contrast, another DNA repair mechanism, excision repair existing in humans is light-independent. Endonuclease V which functions in the removal of the UV-induced pyrimidine dimer photoproducts has a binding site involving a Trp-Tyr-Lys-Tyr-Tyr[15] sequence. So we infer that endonuclease V would possess light-repair function for the pyrimidine dimer under UV light, besides its excision repair function.

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