Monomerization of thymine dimers photosensitized by aromatic amino acids

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Aromatic amino acids D,L-tryptophan and D,L-phenylalanine photosensitize monomerization of the *cis-syn* isomer of thymine dimer of the cyclobutane type in aqueous solutions irradiated with light outside the region of the absorption bands of the isolated compounds (from 340 to 380 nm). The yields of the photosensitized monomerization increase many times in a frozen matrix. The yields decrease with increasing ionic strength of the solution and in the presence of KNO_3 as an electron acceptor. From the results it follows that the reaction proceeds through an excited molecular complex ionically dissociated by charge transfer between the dimer and the amino acid. The dimer monomerization involves a radical reaction. The concentration of the complexes in the solution raises on its freezing.

Циклические аминокислоты D,L-триптофан и D,L-фенилаланин фотосенсибилизируют мономеризацию *цис-син* изомеров тиминовых димеров циклобутилового типа в водных растворах при возбуждении светом вне полос поглощения изолированных соединений (340—380 нм). В результате замораживания растворов выход реакции мономеризации увеличивается во много раз. Выход фотосенсибилизированной мономеризации понижается при увеличении ионной силы раствора и в присутствии акцептора электронов KNO_3. Из результатов следует, что реакция протекает через возбужденное состояние молекулярного комплекса с переносом заряда между димером и аминокислотой, который диссоциирует на ионы после возбуждения. Димер мономеризуется радикальной реакцией. Концентрация комплексов в растворе повышается при его замораживании.

Irradiation of nucleic acids with light of the wavelength between 220—280 nm leads to the formation of photoproducts of which the pyrimidine dimers of the

cyclobutane type are the most important from a biological point of view [1]. The dimers are responsible for 50—80 % of lethal and mutagenic lesions of cells [2, 3] and are also known to exhibit carcinogenic effects [4, 5]. The enzyme DNA—cyclobutadipyrimidine—lyase photosensitizes monomerization of the pyrimidine dimers in DNA both *in vivo* and *in vitro*, thus eliminating the harmful effect of the dimers [6]. Because of the great biological role of this reaction, a considerable attention is devoted to studies of its mechanism. Difficulties associated with isolation of the enzyme in a pure state represent the main reasons why the monomerization of the dimers is investigated in enzyme-free model systems.

As models for the photoenzymic monomerization various photosensitizers were tested. The best reaction yields were obtained with quinone derivatives [7—10] or transition metal ions [11]. With these photosensitizers the dimer monomerization was envisaged as a reaction of a radical mechanism, the first step of which involves the formation of a cationradical of the dimer [10, 11]. In the systems with quinones, however, only the cationradical of thymine was observed, therefore it is assumed that this radical was formed in the reaction course. In connection with the modelling the enzyme action more interesting are the data about indole-sensitized monomerization [10, 12]. Indole is a part of the tryptophan molecule. It has been shown that tripeptides containing a tryptophyl moiety are bound to the thymine dimers in DNA and photosensitize monomerization of thymine dimers Thy[ ]Thy *c,s* up to 60 % [13]. The extremely low quantum yields (0.007) of the tryptophan sensitized monomerization of thymine dimers [10], in comparison to the enzyme-catalyzed reaction, points to a diffusion-controlled process.

Hélène and coworkers [12, 27], based on the data of reflexion UV VIS spectroscopy and fluorescence spectroscopy, and *Balgavý* [14], based on e.p.r. spectroscopy measurements, have proposed a model for the monomerization in frozen aqueous solutions. According to this model the dimer forms with tryptophan a charge-transfer complex which undergoes ionic dissociation after excitation, and the subsequent monomerization proceeds as a radical reaction starting with an anionradical of the dimer. The goal of the present work was to verify the model in photochemical experiments.

Experimental

Thymine, D,L-tryptophan, D,L-phenylalanine, KNO_3, and KCl (Lachema, Brno) were of anal. grade. All compounds were recrystallized before use from redistilled water. Thymine dimer was prepared by a modification of the procedure given in [15]. A frozen 3—5 mm layer of saturated solution of thymine in distilled water was irradiated for 3—4 h from a 5 cm distance by a low-pressure mercury vapour lamp made of a quartz glass (TUV 15 W, Philips, Eindhoven, Netherlands) emitting light of the wavelength of 253.7 nm. After thawing the

irradiated solution, thymine dimer was isolated from the mixture with monomer by severalfold crystallization from distilled water. The identity of the product of the photochemical reaction was confirmed spectroscopically. The spectrum of the isolated product, in contrast to that of the monomer (Fig. 1), did not contain the band with a maximum at

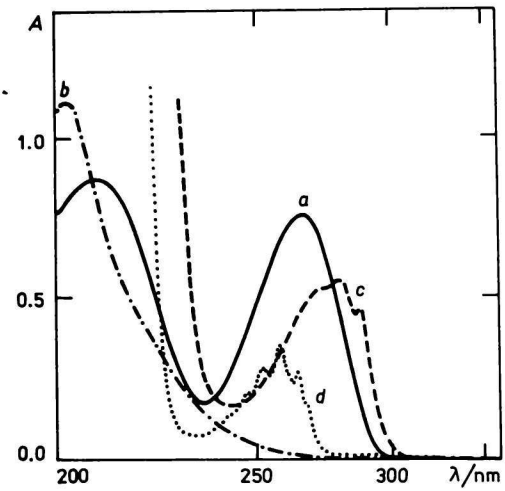

Fig. 1. Electronic absorption spectra of thymine (*a*), *cis-syn* isomer of thymine dimer of the cyclobutane type (*b*), D,L-tryptophan (*c*), and D,L-phenylalanine (*d*).
Concentration of compounds 10^{-3} mol dm^{-3}, optical length 0.1 cm (*a*, *b*, *c*) and 2 cm (*d*), solvent — redistilled water.

256 nm, which is characteristic of the $C_5=C_6$ double bond of pyrimidines. A comparison of the infrared spectrum of the product (Fig. 2) with literature data [16] pointed out that the prepared dimer is the *cis-syn* isomer (abbreviated as Thy[ ]Thy *c,s*) which occurs almost exclusively in DNA *in vivo* and *in vitro* [1].

^{14}C-Labelled thymine dimer was prepared as described [17]. A solution of 2-^{14}C-thymine (Institute for Research, Production, and Utilization of Radioisotopes, Czechoslovakia) in water (1 mg cm^{-3}) of a radioactivity 1.85×10^4 s^{-1} cm^{-3} was frozen in a 1 mm layer and irradiated as above. Thymine was separated from the dimer by descending chromatography on Whatman paper in n-butanol—glacial acetic acid—water (80:12:30, V/V). The band corresponding to the dimer (R_f 0.24—0.29) was eluted with water and the eluate was evaporated to dryness.

A stock solution containing unlabelled and labelled thymine dimer at a concentration *ca.* 10^{-3} mol dm^{-3} and radioactivity 10^6 cpm in 1 cm^3 was prepared. The dimer concentration was determined spectrophotometrically. The reaction mixtures were prepared by mixing the dimer solution with appropriately diluted stock solutions of aromatic amino acids.

Photochemical reactions were monitored in dimer solutions present in cylinder-shaped glass cuvettes of a 4 mm inner diameter as a function of the time of irradiation with a high-pressure 500 W mercury vapour lamp HBO (Narva, Berlin, GDR). The wavelength

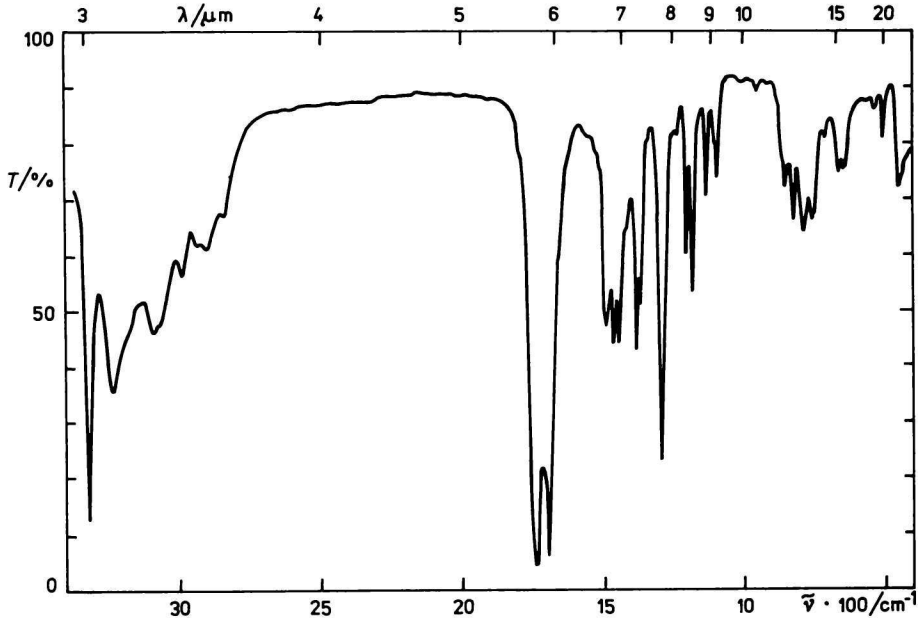

Fig. 2. Infrared spectrum of *cis-syn* isomer of thymine dimer of the cyclobutane type. Recorded in KBr matrix at a concentration 0.9 mg/800 mg KBr.

interval of the light (340—380 nm) was selected by means of glass filters (Schott u. Gen., Jena, GDR). During irradiation the samples were kept in an alcohol bath in a glass cylinder of a 5 cm diameter. Five samples were irradiated simultaneously: thymine dimer alone (1.2×10^{-3} mol dm^{-3}), dimer with tryptophan (both at 4.0×10^{-4} mol dm^{-3}), dimer with tryptophan (both at 6.0×10^{-4} mol dm^{-3}) and KNO_3 (10^{-2} mol dm^{-3}), dimer with tryptophan (both at 6.0×10^{-4} mol dm^{-3}) and KCl (10^{-2} mol dm^{-3}), and dimer with phenylalanine (both at 6.0×10^{-4} mol dm^{-3}). The sample volume was 1 cm^3. Three experiments were carried out at 10 °C and three at −10 °C. The samples were irradiated for 4 h. Preliminary experiments with a frozen matrix containing equimolar concentration of thymine dimer and tryptophan showed that thymine concentration in the sample did not substantially change after irradiation longer than 21/2 h. Cooling of the bath was done by pumping alcohol through a metal tube immersed in an alcohol—solid CO_2 mixture using a thermostat. The temperature of the bath in the glass cylinder was measured with a thermometer and was kept in the range ±2 K. To assure the same irradiation of all samples, they rotated together with the glass cylinder in the light field.

The ratio of thymine concentration to total concentration of thymine dimer and thymine was determined radiochromatographically. During the irradiation, aliquots (0.1 cm^3) were taken directly or after thawing and were applied to Whatman No. 1 paper strips 5 cm wide and 50 cm long and chromatographed as described above. The chromatograms were cut to

0.5 cm wide pieces and their radioactivity was counted in a scintillation fluid containing 7.5 g of diphenyloxazole and 0.1 g of 1,4-bis(2,5-phenyloxazolyl)benzene in 1500 cm^3 of toluene using a Packard Tri-Carb scintillation spectrometer (U.S.A.). In accordance with literature [17, 18], R_f values 0.24—0.29 corresponded to Thy[ ]Thy *c,s* region and R_f values 0.60—0.66 to the region of thymine. The ratio of thymine concentration to concentration of thymine plus thymine dimer was calculated from the ratio of radioactivities in the corresponding regions on paper chromatograms.

Spectra in the u.v. region were measured with a Specord spectrophotometer (Zeiss, Jena) and spectra in the i.r. region with a Perkin—Elmer 377 spectrophotometer (U.S.A.) using the KBr technique.

Results and discussion

The results of the photochemical experiments carried out varied in absolute values of the ratio of thymine to the total concentration of pyrimidines in solution, but the relative ratios for the tested samples were preserved. Data from a typical experiment in which the irradiation was done with light of the 340—380 nm region (thymine dimer and aromatic amino acids do not absorb here; Fig. 1) are shown in Table 1. Within the scope of experimental deviations, the light of the given interval does not induce monomerization of thymine dimers in aqueous solutions frozen or not frozen. Aromatic amino acids D,L-tryptophan and D,L-phenylalanine photosensitized the monomerization under both conditions. The reaction yields were considerably higher in frozen matrix. In both cases D,L-tryptophan appears as a more effective photosensitizer than D,L-phenylalanine. KNO_3 and KCl inhibit the reaction. Inhibitory effects of KNO_3 are more pronounced in frozen matrix in comparison to those of KCl.

The observed effects could be envisaged as follows: The reaction components do not absorb light in the region from 340 to 380 nm, so that the photosensitized monomerization must be due to the formation of complexes with a new absorption

Table 1

Ratio of radioactivity in thymine to the total radioactivity of the analyzed irradiated sample (sum of radioactivity in thymine and thymine dimer)

Sample	+10 °C	−10 °C
Thy[ ]Thy	0.006	0.002
Thy[ ]Thy + Trp	0.039	0.463
Thy[ ]Thy + Phe	0.022	0.384
Thy[ ]Thy + Trp + KNO_3	0.012	0.109
Thy[ ]Thy + Trp + KCl	0.028	0.221

Thy[ ]Thy — thymine dimer; Trp — D,L-tryptophan; Phe — D,L-phenylalanine.

band in the given spectral region. The equilibrium constant of the formation of complexes of thymine dimer with tryptophan or phenylalanine is known to be low [19]. Consequently, low will be the concentration of the complex in the solution and low will be the yields of monomerization. Higher reaction yields with tryptophan than with phenylalanine may be related to higher values of the equilibrium constants of the tryptophan-containing complex [19]. Other possible interpretation is that the irradiation at 340—380 nm leads to the formation of a neutral radical of the aromatic amino acid and to the formation of $H^{\bullet}$ radical [20]. Both radicals can interact with the dimer and induce its monomerization. The latter alternative is considered to be a less probable.

The effect of KCl can be explained as a result of reduced stability of the complexes due to changes in ionic strength of the solution. It is known that increased ionic strength lowers the equilibrium constant of complex formation between pyrimidines and indole [21] and between the enzyme and substrate in the case of a photolyase [6]. Higher inhibitory effect of KNO_3 at the same ionic strength than that produced by KCl is conditioned by the electron-withdrawing properties of KNO_3. Analogous KNO_3 effects were observed during the interaction with excited indole derivatives [20, 22] and in the case of photosensitized destruction of pyrimidine bases in aqueous solution of 3-indolylacetic acid [23].

Considerably higher yields of thymine dimer monomerization were obtained in frozen aqueous solutions. Freezing of the solutions of tryptophan and nucleic acid bases results in a substantial enhancement of the concentration of their intermolecular complexes. This situation is reflected in the red shift in the reflexion spectra, in the appearance of a new fluorescence band characteristic of such a complex, in lower intensity of the tryptophan fluorescence [24] and in the appearance of a new band in the excitation spectra in the region over 300 nm [25]. Evidence has also been presented for reduction of the fluorescence of 5--hydroxytryptophan with thymine dimer in frozen aqueous solutions at 77 K [12] and of the fluorescence of indole and tryptophan in aqueous solutions (room temperature) containing *N*,*N*′-dimethylthymine dimer [10]. There is also a report concerning reduced quantum yields of the fluorescence of the tripeptide Lys-Trp--Lys on interaction with UV-irradiated DNA [13]. The increase of the monomerization yields in our frozen matrix experiments may be ascribed to a rise in the concentration of the complexes between the dimer and aromatic amino acids. The effect of KCl points to the fact that the overall complex concentration in frozen solutions is related to complex stability in liquid solution. The effect of KNO_3 can be ascribed to changes in the ionic strength, similarly as in the case of liquid samples, and, in addition, to the electron-withdrawing properties of the compound. Because the selected time of irradiation was sufficiently long to monomerize all of the dimers in complexes, the value $0.46 \div 0.38$ for the amount of thymine present

in frozen aqueous solutions irradiated for a long period indicates a relatively high concentration of isolated molecules in frozen solution, too.

On the basis of the results obtained it follows that aromatic amino acids and thymine dimer form a molecular complex, the excitation of which is followed by photosensitized cleavage of the dimer. The complex appears to be a charge-transfer one, at least partially, because the cleavage can be effected by irradiation with light of a longer wavelength region than is the region corresponding to the absorption bands of individual components. Since such complexes undergo ionic dissociation after excitation [26] we assume that the monomerization of thymine dimers is a radical reaction.

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