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Journal of Inorganic Biochemistry 78 (2000) 269–273

JOURNAL OF Inorganic Biochemistry

Zinc tetraruthenated porphyrin binding and photoinduced oxidation of calf-thymus DNA

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Received 19 July 1999; received in revised form 7 February 2000; accepted 9 February 2000

Abstract

The photooxidation of calf-thymus DNA has been investigated in the presence of a supramolecular tetraruthenated zincporphyrin (ZnTRP) sensitizer. A strong interaction of ZnTRP with DNA has been observed, exhibiting a gradual transition from a non-specific electrostatic binding mode to a more specific one at high DNA concentrations. Formation of $O_2(^1\Delta_g)$ has been detected from its near-infrared emission, after the excitation of ZnTRP in dioxygen-containing solutions. In the presence of DNA and dioxygen, ZnTRP promotes efficient photocatalytic oxidation of the 2′-deoxyguanosine sites, via their direct reaction with $O_2(^1\Delta_g)$, as in a previous work on the ZnTRP-photoinduced oxidation of the free nucleosides.

Keywords: Tetraruthenated porphyrin; Photooxidation; PDT sensitizer; DNA

1. Introduction

Recent work from our laboratories has focused on the plasmid pBR322 and DNA interactions with a free-base tetraruthenated porphyrin species (TRP), revealing the occurrence of light-induced single-strand breaks and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) formation in the presence of molecular oxygen [1]. The special sensitizer employed combines the remarkable redox and photochemical/photophysical properties of the porphyrins and ruthenium(II)–polypyridine compounds, in addition to their ability to bind DNA via intercalation and outside association [2–19]. From the supramolecular point of view, the peripheral ruthenium–polypyridine groups interact with the porphyrin center, promoting energy and electron transfer processes, and enhancing the photodynamic properties of the molecule [20].

Two types of mechanisms have been associated with the photosensitized reactions involving DNA [21]. The so-called Type I mechanism involves direct electron transfer or hydrogen abstraction of the 2′-deoxyguanosine (dGuo) bases by the excited sensitizer, while Type II proceeds via their reaction with singlet oxygen, $O_2(^1\Delta_g)$, yielding 8-oxodGuo and 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine. Recently, the photodynamic properties of TRP and its zinc-metallated derivative, ZnTRP (Fig. 1), have been evaluated in comparison with methylene blue and riboflavin, using 2′-deoxyguanosine as a model compound [22,23].

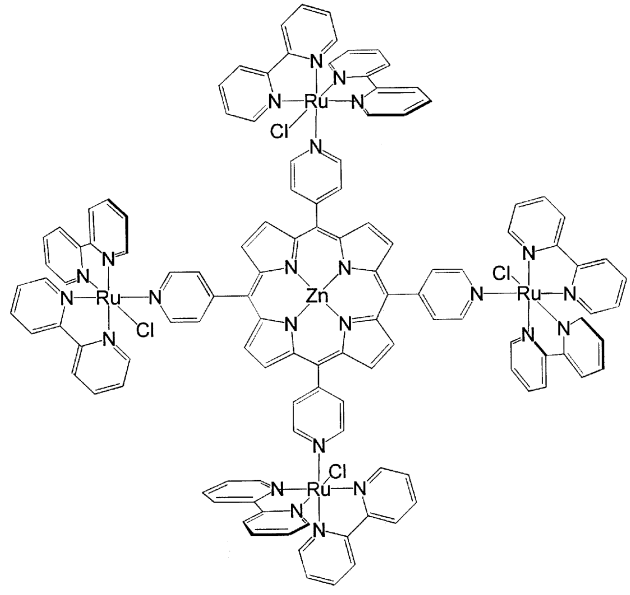

Fig. 1. Structure of the μ-{*meso*-5,10,15,20-tetra(4-pyridyl)porphyrin}-tetrakis-{bis-(bipyridine)chlororuthenium(II)} supermolecule, ZnTRP.

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0162-0134/00/$ - see front matter
PII S0162-0134(00)00053-2

Riboflavin is a typical Type I photosensitizer, while methylene blue exhibits a Type II behavior. The selectivity measured by the ratio of the amount of photoproducts generated by Type II/Type I mechanisms was 0.4 for riboflavin, and 2.3, 3.6 and 5.6 for TRP, methylene blue and ZnTRP, respectively, showing that ZnTRP is the most specific Type II photosensitizer of the series. This interesting result obtained with the dGuo model compound has prompted the present investigation on the molecular interaction and photodynamic properties of the ZnTRP–DNA system.

2. Materials and methods

2.1. Chemical

ZnTRP was obtained as previously reported [24]. 2′-Deoxyguanosine was purchased from Pharma-Waldorf (Geneva, Switzerland); calf-thymus DNA (CT-DNA), nuclease P1, alkaline phosphatase (Sigma, Missouri, St Louis) and all other reagents were analytical grade and used as supplied. All solutions were prepared using bidistilled deionized MilliQ (Millipore, Milford, MA) water and kept in the dark at 4 °C.

2.2. Fluorescence and electronic absorption measurements

Electronic absorption and luminescence spectra were recorded on a Hewlett-Packard model 8453A diode array spectrophotometer and a Photon Technology Inc. model LS100 spectrofluorimeter, respectively. The solutions were prepared by adding variable volumes of distilled water and CT-DNA stock solution into 0.10 mL of ZnTRP solution, in order to attain 3.00 mL total. In this way, the concentration of DNA was varied in the 0–60 μM range. The solutions were mixed in a test-tube shaker and left to stand for 30 s before starting the measurements. UV–Vis and luminescence spectra were collected in sequence with the same sample, at room temperature. The use of phosphate buffer was precluded by the formation of turbid solutions.

2.3. Singlet oxygen light emission

Time-resolved near-infrared luminescence of $O_2(^1\Delta_g)$ was obtained by collecting the 1270 nm light emitted at right angles [1], using a liquid-nitrogen-cooled germanium photodiode (EG&G Judson model J16D-M204-R05M-60), after excitation at 532 nm with a frequency doubled Q-switched Nd-YAG laser (Spectron Laser System).

2.4. Photosensitization

Irradiation of the CT-DNA solutions was performed with a 500 W tungsten halogen lamp, emitting 1.75×10^{17} quanta seg^{-1} cm^{-2}, as measured with a photometer from Biospherical Instruments Inc., model QSL-100 (San Diego, CA).

2.5. HPLC detection of 8-oxodGuo

The amount of 8-oxodGuo present in DNA was measured by HPLC with electrochemical detection (HPLC-ECD) using a Shimadzu model LC-10AD pump with a Phenomenex Spherex 5 C18 column (250 × 4.6 mm), a Waters model 484 UV detector at 254 nm and a Shimadzu model L-ECD-6A amperometric detector kept at 0.6 V. An EZChrom chromatography data system was used to record outputs and calculate peak areas. The mobile phase consisted of potassium phosphate buffer (0.05 M, pH 5.5) containing 10% methanol and pumped at a flow rate of 1 mL min^{-1}. Standard curves were constructed for both 8-oxodGuo and for dGuo which were used for the quantification of total DNA. About 400 μg of CT-DNA suspension were digested to nucleotides with 25 μg of nuclease P1 at 37 °C for 24 h. Then, 25 μL of 1 M Tris–HCl, pH 7.4, were added to the samples, followed by 2.5 units of *E. coli* alkaline phosphatase. After 120 min at 37 °C the solution was analyzed by HPLC-ECD.

3. Results and discussion

3.1. Interaction of ZnTRP with DNA

The binding of ZnTRP to CT-DNA was monitored from the corresponding DNA-induced changes on the UV–Vis absorption and luminescence spectra in aqueous solution. The addition of increasing amounts of DNA (in the range 0.0 to 17.9 μM) to the ZnTRP solution led to a pronounced hypochromic effect on the Soret band at 426 nm (Fig. 2), in parallel with a bathochromic shift to 438 nm. However, when the concentration of DNA reached the 17.9 to 127 μM range, the Soret band remained at 438 nm and only a systematic increase of the absorption was observed, evidencing a change in the interaction mode between those species.

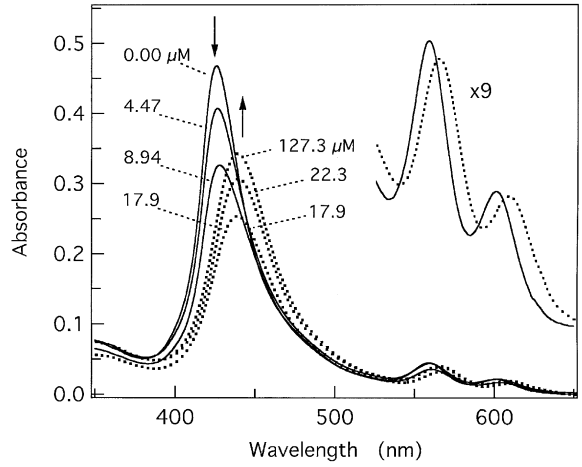

Fig. 2. Spectrophotometric titration of a 1.3 μM ZnTRP aqueous solution with increasing CT-DNA relative concentrations. The visible region for the initial and final spectra has been expanded 9 times and is shown in the inset.

The binding constants for the low and high DNA concentration ranges were determined [8] by applying Eq. (1) on the spectrophotometric titration curves:

$$\frac{[\mathrm{DNA}]}{(\varepsilon_A-\varepsilon_F)}=\frac{[\mathrm{DNA}]}{(\varepsilon_B-\varepsilon_F)}+\frac{1}{K(\varepsilon_B-\varepsilon_F)} \quad (1)$$

where ε_A, ε_B and ε_F are respectively the absorbance/[ZnTRP] ratio and the absorptivities of the bound and free ZnTRP. Accordingly, the binding constant, K, is given by the ratio of the slope and the intercept of the $[\mathrm{DNA}]/(\varepsilon_A-\varepsilon_F)$ versus [DNA] plot.

The absorbance changes observed in the spectrophotometric titration curves of ZnTRP by DNA are consistent with the existence of two distinct interaction mechanisms, in the low and high DNA concentration range, respectively. The corresponding association constants were calculated as $K_1=(3.0\pm0.5)\times10^4$ and $K_2=(5.0\pm0.5)\times10^5\ \mathrm{M}^{-1}$, and can be compared with those found [1] for TRP ($K_1=2.5\times10^4$ and $K_2=1.5\times10^5\ \mathrm{M}^{-1}$). A stronger specific electrostatic interaction of ZnTRP with DNA in the high concentration range (Fig. 2) is evidenced, with respect to the TRP analog.

The luminescence spectrum of ZnTRP exhibited a stronger emission band at 621 nm and a side band at 660 nm assigned to the Q_{0-0} and Q_{0-1} porphyrin transitions, respectively, as shown in Fig. 3.

In the low DNA concentration range ($<15\ \mu\mathrm{M}$), the luminescence intensity at 621 nm decreased systematically to about one-third of the initial value and the resulting spectral profile became very broad. This type of behavior differs from that previously reported for the TRP–DNA system [1], in which case, only a systematic increase in the luminescence has been detected.

In the high DNA concentration range (up to 255 μM), a systematic increase of the luminescence emission at 630 nm was observed. This process was accompanied by an inversion in the spectral pattern, such that the 660 nm band became more intense. The luminescence spectra are consistent with the observed changes in the UV–Vis spectra as a function of the DNA concentration, confirming the occurrence of two distinct binding modes in the ZnTRP–DNA system. This type of behavior has also been reported for a number of systems [9,11,12], and interpreted in terms of a switch from distinct binding modes as the concentration of DNA increases.

In analogy to porphyrin derivatives containing bulky [12,16,17,19,25] substituents, ZnTRP is not expected to intercalate in the conventional way between the DNA bases. The predominant interaction in this case should be of electrostatic nature. It should be noted that, at low DNA concentrations, the porphyrin species is available in relatively high proportion, and a less specific DNA outside binding seems more plausible, e.g. via electrostatic interactions with the $[\mathrm{Ru(bipy)_2Cl}]^+$ group located at the corner of the square supermolecule. Presumably, in this case, the porphyrin center would be less perturbed by the outside association with DNA, remaining susceptible to the vibrational and collisional

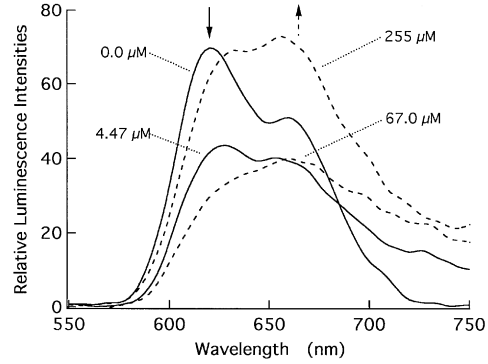

Fig. 3. Fluorescence spectra of ZnTRP as the concentration of CT-DNA increases from 0 to 4.47 μM and from 67.0 to 255 μM.

effects on the locally associated ion pairs. As a consequence, the electronic spectra are not substantially affected, in contrast to the light emission properties that are strongly susceptible to vibrational and collisional effects.

On the other hand, as the DNA concentration increases, the interaction with ZnTRP would become more specific, since the porphyrin species would be preferentially associated with stronger binding sites. Illustrative molecular models indicate that two vicinal $[\mathrm{Ru(bipy)_2Cl}]^+$ groups can simultaneously approach the existing grooves in DNA, providing a lateral insertion mode for the square ZnTRP species. As a consequence the porphyrin center would be partially inserted into the DNA grooves, giving rise to hydrophobic effects, as reflected in the changes on the Soret band, and in the luminescence spectra.

A recent work has shown that specific interactions involving porphyrin and DNA can also be distinguished from the less specific ones, by the occurrence of significant changes in the Q_{00} and Q_{01} bands [26]. The Q_{00} and Q_{01} bands are bathochromically shifted at high DNA concentrations, as can be seen in Fig. 2 (inset), in agreement with our expectation. According to the molecular models, one cannot exclude the possibility of some interaction between the phosphate groups and the Zn(II) center. This type of interaction involving phosphate groups and Zn(II) ions is quite common in coordination chemistry, and would also favor the insertion of the ZnTRP species into the DNA grooves, explaining the observed differences between the TRP and ZnTRP species in the presence of DNA, e.g. in the electronic and luminescence spectra and in the photocatalytic activity.

3.2. Photochemical activity of ZnTRP

2′-Deoxyguanosine is the critical DNA target component in both type I and II photosensitization mechanisms, consisting in a good model compound for understanding the photoinduced oxidation of DNA. Type I mechanism is known to convert dGuo into oxazolone and imidazolone products [22,27] through a mechanism that involves an electron transfer from the excited photosensitizer to DNA. Type II mechanism involves energy transfer from the excited sensitizer to dioxygen, yielding $O_2(^1\Delta_g)$ reactive species which convert

dGuo into the oxidation products 8-oxodGuo and 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine. From the quantification of the different oxidation products, $O_2(^1\Delta_g)$ species has been postulated as the reactive species in dGuo photooxidation mechanisms mediated by the tetraruthenated porphyrins [27].

In this work, we provide a direct evidence of the formation of $O_2(^1\Delta_g)$ in the photoexcitation of ZnTRP in air saturated acetonitrile solutions, from its characteristic phosphorescence emission decay at 1270 nm, as shown in Fig. 4. The

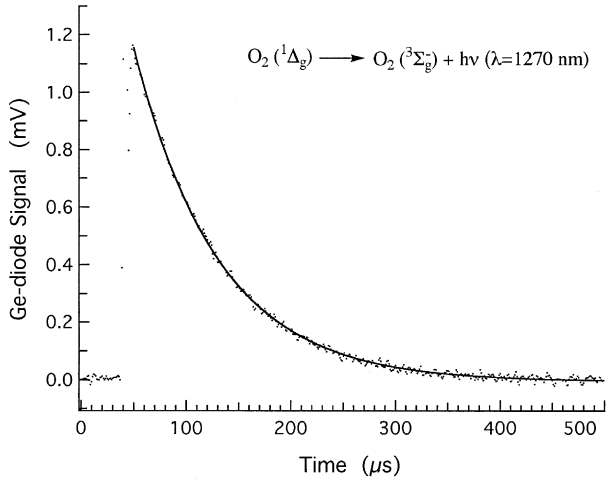

Fig. 4. Phosphorescence decay of $O_2(^1\Delta_g)$ at 1270 nm, at room temperature, after pulse excitation of 2 μM ZnTRP in normally aerated acetonitrile.

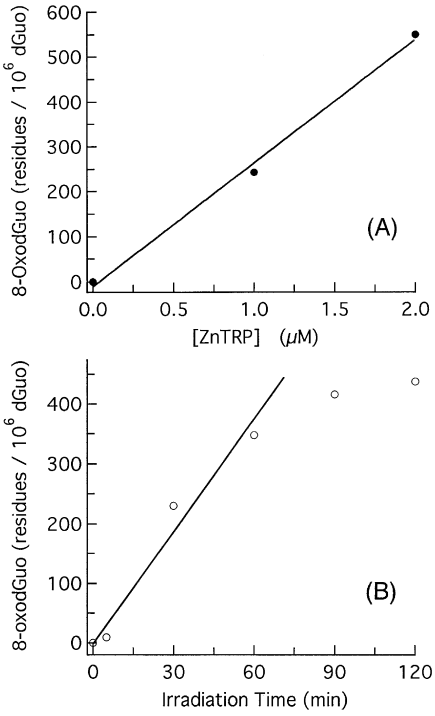

Fig. 5. (A) Linear increase of the amount of photogenerated 8-oxodGuo residues in CT-DNA (400 μg in 50 mM phosphate buffer pH 7.4), upon 60 min irradiation, as a function of ZnTRP concentration. (B) Kinetics of photooxidation of CT-DNA in the presence of 2 μM ZnTRP, monitored by measuring the amount of 8-oxodGuo produced as a function of time.

calculated lifetime was $\tau = (8.0 \pm 0.1) \times 10^{-5}$ s, in excellent agreement with the literature [1,28].

The efficiency of ZnTRP as a photosensitizer in the photooxidation of DNA was investigated by monitoring the formation of 8-oxodGuo residues as a function of time and ZnTRP concentration, using the procedure detailed in Section 2.

The amount of 8-oxodGuo increased linearly with the concentration of ZnTRP when the time of exposure and irradiation intensity were kept constant (Fig. 5(A)), as expected for a photooxidation process promoted by the porphyrin sensitizer.

The amount of 8-oxodGuo residues increases as a function of the irradiation time, exhibiting a deviation from linearity after 60 min, as can be seen in Fig. 5(B). It should be noted that, in all the cases, the reactions are far from completion. The pronounced deviations seem to reflect the occurrence of additional reactions consuming the oxidation product. As a matter of fact, it has been reported that the 8-oxodGuo product is two orders of magnitude more reactive with singlet oxygen than the guanosine bases themselves [29]. In this way, to overcome this point, the kinetics should be treated by the initial rates method.

The following scheme can be proposed:

$$\mathrm{ZnTRP} + h\nu \rightarrow \mathrm{ZnTRP}^*$$

$$\mathrm{ZnTRP}^* + O_2(^3\Sigma_g^-) \rightarrow \mathrm{ZnTRP} + O_2(^1\Delta_g)$$

$$O_2(^1\Delta_g) \rightarrow O_2(^3\Sigma_g^-) + h\nu' + \mathrm{heat}$$

$$O_2(^1\Delta_g) + \mathrm{DNA} \rightarrow \mathrm{DNA\text{-}photooxidation\ products}$$

In this scheme, the kinetics between $O_2(^1\Delta_g)$ and DNA is the rate determining step, and the preceding reactions are rather fast, ensuring a steady state condition for $O_2(^1\Delta_g)$. Therefore, the initial rates can be expressed as

$$d[\text{8-oxodGuo}]/dt = k[\mathrm{DNA}][O_2(^1\Delta_g)]$$

where the initial rate constant is $k_{obsd}[\mathrm{DNA}] = 5 \times 10^{-5}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. This kinetic constant includes the steady-state concentration of $O_2(^1\Delta_g)$.

For comparison purposes, a similar photoxidation kinetics was carried out using the dGuo nucleoside instead of CT-DNA, under identical conditions. In this case, a similar deviation from linearity was observed above 60 min. The kinetic constant calculated from the initial rate was $1.6 \times 10^{-6}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, i.e., 31 times slower than for CT-DNA. This difference can only be ascribed to the strong binding of ZnTRP to DNA, promoting a more efficient oxidative damage via the formation of $O_2(^1\Delta_g)$, in a close proximity to the dGuo targets.

4. Conclusions

ZnTRP is a promising PDT sensitizer, that acts as a Type II photooxidation catalyst for DNA, generating $O_2(^1\Delta_g)$ in the presence of light and dioxygen. The Zn ion coordinated

to the porphyrin ring can play an additional role, by promoting a strong association with DNA via electrostatic and/or coordination interactions with the phosphate groups. The kinetics of the photooxidation reaction is consistent with the direct attack of the photogenerated O_2 ($^1\Delta_g$) species, to dGuo bases.

Acknowledgements

The support from the FAPESP, CNPq and PRONEX (Brazil), and the J.S. Guggenheim Memorial Foundation (H.E.T.) is gratefully acknowledged.

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