Pulse radiolysis study of rapid electron transfer from electron adduct and reducing OH adduct of dAMP to riboflavin and flavin adenine dinucleotide (FAD)

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Rapid electron transfer from electron adduct and reducing OH adduct of 2'-deoxyadenosine-5'-monophosphate acid (dAMP) to riboflavin (RF) and flavin adenine dinucleotide (FAD) was studied using the time-resolved pulse radiolysis techniques. Both spectroscopic and kinetic analyses showed that transient absorption spectrum of electron adduct or OH adduct of dAMP formed at first, and then changed to that of radical anion of RF or FAD after several microseconds of pulse. The evidence indicated that electron transfer from electron adduct and reducing OH adduct of dAMP to RF or FAD did occur. From buildup or bleaching kinetics of radical anions of RF and FAD, the rate constants for electron transfer were determined, respectively.

Keywords Electron adduct of dAMP, reducing OH adduct of dAMP, riboflavin (RF), FAD, electron transfer, pulse radiolysis

Introduction

The carcinogenic mutagenic and lethal effects in biological system exposed to ionizing radiation are thought to be the predominant result of the cellular DNA damage.[1,2] The most important types of the multiple radiation-induced lesions that occur in DNA are strand breaks, cross-links, and modifications of sugars and bases.[3,4] Therefore, there has been a continuing interest to unravel the mechanism of biological damage caused by the oxidizing and the reducing species that result from ionizing radiation.

Two components are believed to be responsible for DNA damage, the direct effect in the situation of ionizing radiation absorbed by the DNA itself and the indirect effect in the situation of DNA attacked by active radicals generated by the absorption of ionizing radiation in the water surrounding the DNA. The hydrated electron (e^-_{aq}) and hydroxyl radical ($^{\cdot}OH$), the major primary products from the radiolysis of water, were considered as the main cause of DNA damage. Since the ribose phosphate moiety is less reactive with $^{\cdot}OH$ (rate constants ca. 10^9 $dm^3/(mol\cdot s)$) and almost unreactive with respect to e^-_{aq} (rate constants $< 10^7$ $dm^3/(mol\cdot s)$),[3,5] it is accepted generally that e^-_{aq} and $^{\cdot}OH$ radical would be trapped predominantly by one of the nucleic acid bases: the pyrimidines and the purines.

Radical anions of the bases have been identified by ESR on irradiation of DNA in various forms at low temperatures.[6] Although all of the nucleic acid bases have the same reactivity with electron, the electron localizes preferentially on the cytosine bases in double-stranded DNA and thymine in single-stranded DNA whereas the hole localizes on guanine in both forms. The initial localization of electron and hole on the DNA would depend mostly on the electron affinities and ionization potentials of the individual DNA bases, respectively.[7,8]

Concerning purines, the electron adducts of adenine nucleosides and nucleotides have already been investigated in detail under various experimental conditions. It was shown that similar to the electron adducts of

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cytosine and its derivatives,[4] the radical anions of adenine and its derivatives are rapidly protonated by water on the nitrogens (to dAMP, it is to give dAMP(N)H$^{\cdot}$) even in basic solutions.[9,10] The dAMP(N)H$^{\cdot}$ radical is a strongly reducing radical, which can transfer an electron to the oxidant *p*-nitroacetophenone (PNAP) with the rate constant 5×10^9 dm^3/(mol·s) at pH ≤ 11 and methyl viologen (MV^{2+}) with the rate constant 2.5×10^9 dm^3/(mol·s) at pH 7.[3,9,10]

The interaction of OH radical with adenine system mainly by addition to the double bond was investigated by Steenken, O'Neill and coworkers.[3, 11-13] The addition of OH radical to dAMP in aqueous solutions produces three major radicals, *i.e.*, dAMP4OH$^{\cdot}$, dAMP5OH$^{\cdot}$, and dAMP8OH$^{\cdot}$. The site of radical addition has been shown to strongly influence the redox properties of the resulting radicals determined by reaction with *N*, *N*, *N'*, *N'*- tetramethyl -*p*-phenylenediamine (TMPD) or tetranitromethane (TNM), respectively.[11,12] In general, it was suggested that OH radical adducted to C4 be oxidative, whereas to C5 and C8 be reductive. Besides, dAMP4OH$^{\cdot}$ and dAMP5OH$^{\cdot}$ can be converted into a strongly oxidizing radical by dehydration reaction, leading to neutral *N*-centered radicals dAMP(-H)$^{\cdot}$ as long as there is one hydrogen at N-6.[3,11]

It has been suggested that the oxidizing adduct be fast repaired via electron transfer process by enzymes and antioxidants, such as thiols, hydroxycinnamic acid derivatives,[13-15] then the anion of OH adduct can regenerate the parent molecule of bases. Whereas the reducing adduct can be oxidized to form the cation of OH adduct by oxidants which can react with water to form steady products and cause DNA damage.[16]

Riboflavin (RF) and flavin adenine dinucleotide (FAD), widely distributed in human tissues and fluids in free and conjugated forms and called as the "crossover point" from two-electron to one-electron reaction mechanisms in biology redox systems, are important endogenous cellular photosensitizers *in vivo* and *in vitro*.[17,18] Photoexcitation of RF and FAD may potentially occur in the organs and tissues permeable to light, such as the skin or eye. Moreover, the excited triplet states of RF and FAD can also be generated enzymatically in a "photochemical without light" type reaction mediated by peroxidases,[19] and make DNA and other cell-matrix components damage causing inflammation and accelerating aging. The photosensitization process of RF and FAD has been studied in detail by our group and others, the reaction mechanism was elucidated clearly by combination of thermodynamics with time-resolved spectra and steady experiment results. Both oxidized radicals and triplet states of RF and FAD were suggested to be the reaction intermediates.[20,21]

In our previous studies, the interaction of RF and FAD with (protonated) electron adducts and reducing OH adducts of pyrimidines were studied using pulse radiolysis techniques.[22,23] It has been demonstrated that RF and FAD have radiosensitivity on pyrimidine damage. To gain further insight into the effect of flavin in ionizing radiation, in this work, the interaction of RF and FAD with electron adduct and reducing OH adduct of dAMP were studied by use of pulse radiolysis. By direct observation of electron transfer process, the rate constants were determined. The results show clearly that RF and FAD have radiosensitivity on purine damage.

Experimental

Materials

Riboflavin (RF) was obtained from Huamei Biochemical Co. (Shanghai, China) and used as received. Flavin adenine dinucleotide (FAD) and 2'-deoxyadenosine-5'-monophosphate acid (dAMP) were purchased from Sigma Chemical Co. and used without further purification. *Tert*-BuOH was redistilled twice before use. NaOH, $HClO_4$ and phosphate were analytic grade reagents.

Pulse radiolysis experiments

Pulse radiolysis experiments were performed utilizing a 10 MeV linear accelerator which delivers an electron pulse with a duration of 8 ns. The analysing light beam passed perpendicularly through a 2 cm quartz cuvettes . The transmitted light entered a monochromator equipped with a R955 photomultiplier. The signals were collected using an HP54510B 300 MHz transient recorder and then processed with a PC-586 personal computer. The dosimetry of electron pulse was determined by a thiocyanate dosimeter using $G[(CNS)_2^{\cdot-}] = 6.0$ in a 1.0×10^{-2} mol/dm^3 KCNS solution saturated with nitrous oxide by taking $\varepsilon_{480} = 7600$ dm^3/(mol·cm) for $(CNS)_2^{\cdot-}$. The details of the setup were described in

the previous paper.[24] In this work, the dose per electron pulse was10 Gy.

Unless otherwise indicated, all solutions were made freshly with triply distilled water, buffered with 4.0×10^{-3} mol/dm^3 phosphate, and protected from light at all time. The pH value of the solution was adjusted by adding NaOH, $HClO_4$ or phosphate solution. The solutions were deaerated with high purity nitrogen or nitrous oxide bubbling for 20 min prior to the experiments. All experiments were performed at room temperature (~20℃).

Results

Electron transfer reaction of electron adduct of dAMP *with* RF *and* FAD

e^-_{aq} was produced by irradiation of aqueous solution containing *tert*-butyl alcohol as OH radical scavenger. e^-_{aq} is a powerful reductant, which reacts with dAMP at nearly diffusion-controlled rate.[3,5]

Fig. 1(A, B) shows the transient absorption spectra after pulse radiolysis of 4.0×10^{-3}mol/dm^3 dAMP, 0.1 mol/dm^3 *tert*-BuOH and 1.0×10^{-4} mol/dm^3 RF or FAD aqueous solution, saturated with N_2 at pH 9. The reaction rate constants of e^-_{aq} with dAMP, RF and FAD have been determined as 4.2×10^9, 2.1×10^{10} and 1.8×10^{10} dm^3/(mol·s), respectively,[3,5,22,25] thus, the fraction of electron adduct of dAMP was calculated to be >98 % for 4.0×10^{-3} mol/dm^3 dAMP aqueous solutions containing 1.0×10^{-4} mol/dm^3 RF and FAD, respectively. The transient absorption spectra at 1.0 μs after electron pulse is stemming from the electron adduct of dAMP predominantly. At 30.0 μs after electron pulse, a new absorption peak at the 520 nm with a bleaching at 440 nm grows, which should be assigned to the absorption band of radical anion of RF or FAD arising from electron transfer reaction from the electron adduct of dAMP to RF or FAD.[22,23,25]

Fig. 2 shows the change of absorption at 520 nm and 440 nm with time after the pulse, which is mainly attributed to the radical anion of RF (formation and bleaching). Based on synchronization between two curves, it is obvious that electron transfer from electron adduct of dAMP to RF does occur. By varying RF and FAD concentrations (concentrations in the range 0.02—0.12×10^{-3}mol/dm^3), the rate constants for the electron transfer have been determined from the pseudo-first-order formation kinetics observed at 520 nm or bleaching observed at 440 nm in N_2-saturated solution and listed in Table 1.

$$H_2O \sim\sim\longrightarrow e^-_{aq},\ {}^{\cdot}OH,\ H,\ \cdots$$

$$OH + (CH_3)_3COH \longrightarrow {}^{\cdot}CH_2(CH_3)_2COH + H_2O$$

$$e^-_{aq} + dAMP \longrightarrow dAMP^{\cdot-} \longrightarrow dAMP(N)H^{\cdot}$$

$$dAMP(N)H^{\cdot} + RF/FAD \longrightarrow dAMP(H_2O)(N)H^+ + RF^{\cdot-}/FAD^{\cdot-}$$

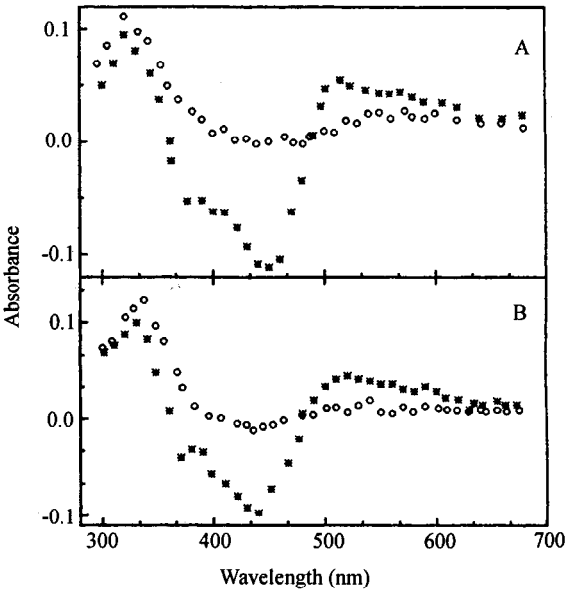


Fig. 1 Transient absorption spectra from pulse radiolysis of N_2-saturated aqueous solution containing 4.0×10^{-3} mol/dm^3 dAMP, 0.1 mol/dm^3 *tert*-BuOH, 4.0×10^{-3} mol/dm^3 phosphate and 1.0×10^{-4} mol/dm^3 (A) RF and (B) FAD at pH 9 recorded at: (○) 1.0 μs; (*) 30.0 μs after electron beam pulse.

Table 1 Rate constants forinteraction of RF or FAD with electron adduct of dAMP (unit: dm^3/(mol·s))

Substrate	RF	FAD
$k_{dAMP(N)H^{\cdot}}$	1.8×10^9	2.5×10^9

In the same way the rate constants for the electron transfer reaction from electron adduct of dGMP to RF or FAD have also been determined to be 1.6×10^9 and 1.9×10^9 dm^3/(mol·s), respectively.

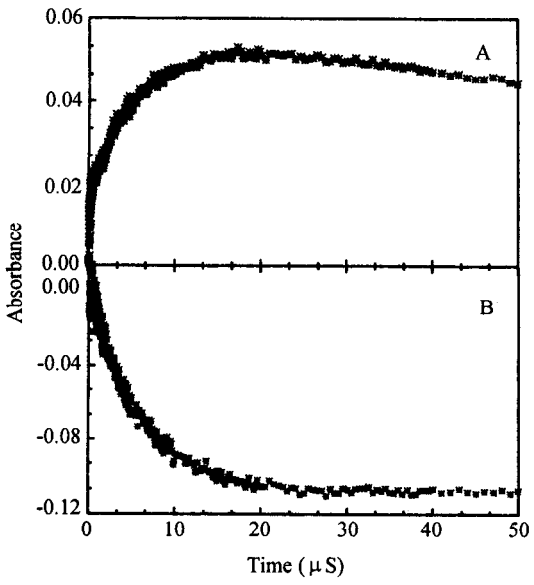


Fig. 2 (A) Growth trace of the transient absorbance observed at 520nm and (B) bleaching curve recorded at 440 nm obtained from pulse radiolysis of N_2-saturated aqueous solution containing 4.0×10^{-3} mol/dm^3 dAMP, 0.1 mol/dm^3 *tert*-BuOH, 4.0×10^{-3} mol/dm^3 phosphate and 1.0×10^{-4} mol/dm^3 RF at pH 9.

Electron transfer reaction of reducing OH *adduct of* dAMP *with* RF *and* FAD

The reaction rate constants of OH radical with RF, FAD and dAMP have been determined as 1.1×10^{10}, 6.4×10^9 and 4.1×10^9 dm^3/(mol·s) at neutral solution, respectively.[5,23] Thus, the fraction of OH radical adduct of dAMP was calculated to be > 98% for 4.0×10^{-3} mol/dm^3 dAMP aqueous solutions containing 1.0×10^{-4} mol/dm^3 RF or FAD, respectively. The transient absorption spectra recorded at 1.0 μs and 25.0 μs after pulse radiolysis of 4.0×10^{-3} mol/dm^3 dAMP aqueous solution containing 1.0×10^{-4} mol/dm^3 RF or FAD saturated with N_2O at pH 9 is shown in Fig. 3 (A, B). The transient absorbance at 1.0 μs after electron pulse is predominantly due to OH adduct of dAMP. Accompanying the decay of the absorption band in wavelength region 390—540 nm, a new absorption peak at 520 nm with a bleaching at 440 nm grows, which has been assigned to the absorbance of radical anion of RF or FAD,[22,25] arising from electron transfer reaction from reducing OH adduct of dAMP *via* following reactions:

$$e^-_{aq} + N_2O + H_2O \longrightarrow {}^{\cdot}OH + N_2 + OH^-$$

$$^{\cdot}OH + dAMP \longrightarrow [dAMPOH]^{\cdot}_{ox} + [dAMPOH]^{\cdot}_{red}$$

$$[dAMPOH]^{\cdot}_{red} + RF/FAD \longrightarrow [dAMPOH]^{+}_{red} + RF^{\cdot -}/FAD^{\cdot -}$$

$$[dAMPOH]^{+}_{red} \longrightarrow Products \longrightarrow DNA\ damage$$

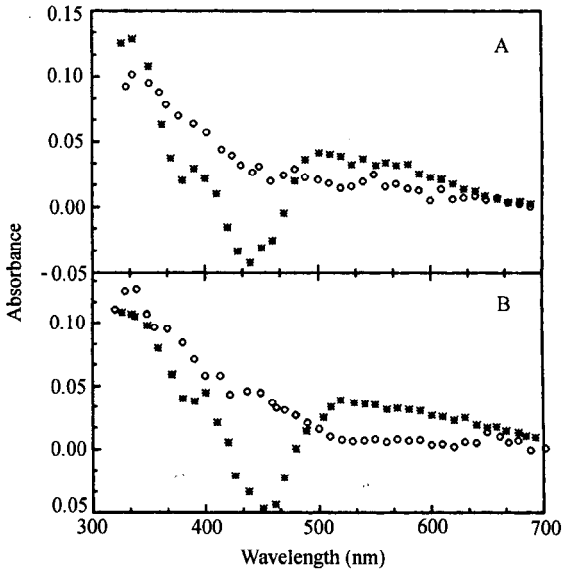


Fig. 3 Transient absorption spectra obtained in the pulse radiolysis of 4.0×10^{-3} mol/dm^3 dAMP and 1.0×10^{-4} mol/dm^3 (A) RF and (B) FAD aqueous solution saturated with N_2O at pH 9 recorded at (○) 1.0 μs, (*) 25.0 s, respectively.

The rate constants for interaction of RF and FAD with reducing OH adduct of dAMP were determined to be 2.3×10^9 and 1.8×10^9, dm^3/(mol·s) from the dependence of the first-order formation at 520 nm or bleaching of radical anion at 440 nm on the concentration of RF and FAD, respectively.

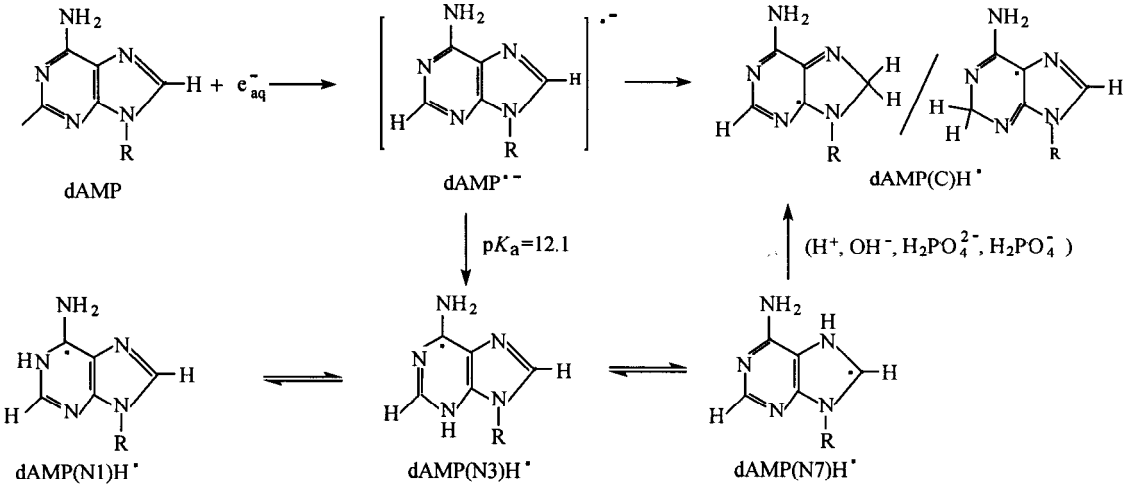
Discussion

Electron transfer mechanism of electron adduct of dAMP *by* RF *and* FAD

When reacting with purine nucleosides and nucleotides, e^-_{aq} has a high preference for interaction with the base moiety of the molecule rather than with (deoxy)-ribose(phosphate) moiety. In agreement with expecta-

tion based on the low rate constants for reaction with ribose 5′-phosphate monoanion,[5] it reacts with dAMP to form the radical anion of dAMP. The radical anion is rapidly protonated by water to give neutral radicals in < 5 ns.[3,9,10] Since nitrogen has a greater electron affinity than carbon, it is reasonable to assume that the negative charge in the radical anion reside mainly on the nitrogens as indicated by the mesomeric structures. As a result of the high charge density at the nitrogens, they are rapidly protonated ($k > 1.4 \times 10^8\ s^{-1}$) to give the *N*-protonated radicals of type dAMP(N)H$^{\cdot}$, which can exist in the tautomeric forms dAMP(N1)H$^{\cdot}$, dAMP(N3)H$^{\cdot}$, and dAMP(N7)H$^{\cdot}$.[10] In comparison with protonation on nitrogen(s), protonation on carbon(s) (λ_{max} = 355 nm) is much slow, a well-known phenomenon, caused by the high bond reorganization energies, by no means restricted to radicals. Under catalysis by OH^-, phosphate or acids, dAMP(N)H$^{\cdot}$ can be converted into dAMP(C)H$^{\cdot}$. For *C*-protonated species having appreciably more spin density than the nitrogen, the dAMP(C)H$^{\cdot}$ is a weaker reductant than the *N*-protonated one, and is difficult to be oxidized by oxidants. The reaction sequence could be expressed as Scheme 1.

Scheme 1

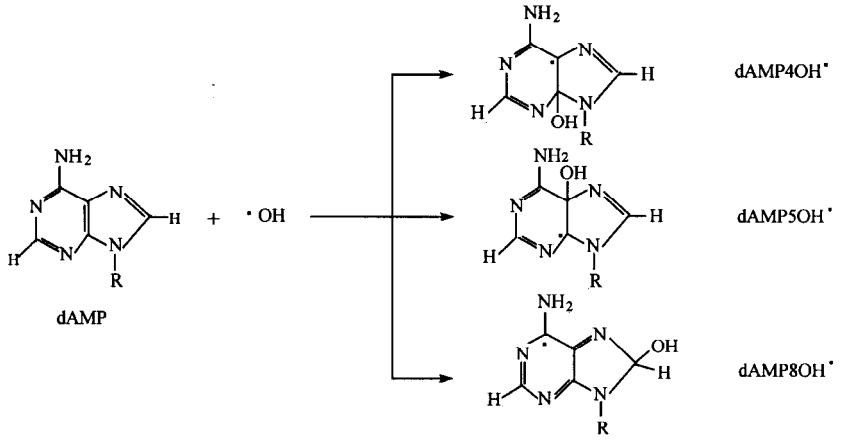


Under the experimental condition, the e^-_{aq} produced by the radiation reacts predominately with the adenine moiety to give dAMP(N)H$^{\cdot}$. As shown by Hissung *et al*. using conductivity detection, addition oxidants to the system leads to the formation of radical anion of oxidant and protonated dAMP which includes the elements of water, *i.e.*, dAMP(H_2O)(N)H^+.[9] Before regeneration (*i.e.*, repair) of the dAMP, the protonated dAMP form has undergone spontaneous structural changes which would fix the electron-induced damage of the adenine moiety.

Free radical process of OH *adduct of* dAMP *by* RF *and* FAD

Hydroxyl radical reacts with nucleic acids mainly by addition to the double bond of the bases. The addition of OH radical to dAMP in aqueous solution produces three major radicals (see Scheme 2), *i.e.*, dAMP4OH$^{\cdot}$, dAMP5OH$^{\cdot}$ and dAMP8OH$^{\cdot}$.[11,12] dAMP4OH$^{\cdot}$, with appreciable unpaired spin density located on N-6 centered radical, is considered to be similar to β-oxoalkyl radicals which have oxidizing properties.[3] Also it is due to the unpaired spin density on N-1 and N-3, therefore dAMP4OH$^{\cdot}$ is a very weak oxidant which can be fast repaired via electron transfer from antioxidants. However, dAMP5OH$^{\cdot}$ and dAMP8OH$^{\cdot}$ are analogous with that of α-monoalkyloxyalkyl radicals which have reducing properties since the carbon carrying the unpaired electron is substituted by electron donating amino group,[26] accordingly, dAMP5OH$^{\cdot}$ and dAMP8OH$^{\cdot}$ may be fast oxidized by oxidants of RF or FAD involving electron transfer followed by the formation of carboncation of OH adduct.[16,23]

Scheme 2



Our experimental results have demonstrated that the rapid electron transfer from electron adduct and reducing OH adduct of dAMP to RF and FAD can occur. In short, kinetic studies have provided novel confirmations as well as the development of charge transfer sensitization mechanism which is the main constituents of the "three transfer" mechanisms of radioprotection and radiosensitization proposed in our laboratory previously.[27]

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