Free Rad. Res. Comms., Vol. 11, Nos. 1-3, pp. 127-136
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ONE-ELECTRON REDUCTION OF DAUNORUBICIN INTERCALATED IN DNA OR IN A PROTEIN : A γ RADIOLYSIS STUDY

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(Received January 19, 1990; in final form April 17, 1990)

The one-electron reduction of daunorubicin, an anthracycline antibiotic, intercalated in DNA or in the apoprotein of the riboflavin binding protein, was studied by γ radiolysis. The two reduction mechanisms appear very similar to the one found for the non-intercalated drug. Hydrogen peroxide, which oxidizes non-intercalated hydroquinone daunorubicin with two electrons in one step (C. Houée-Levin, M. Gardès-Albert and C. Ferradin, *FEBS lett.*, **173**, 27-30, (1984)), reacts with daunorubicin hydroquinone in DNA but not in the protein. It appears thus that the site accessibility to hydrogen peroxide in DNA is better than in the protein. Biological consquences are discussed.

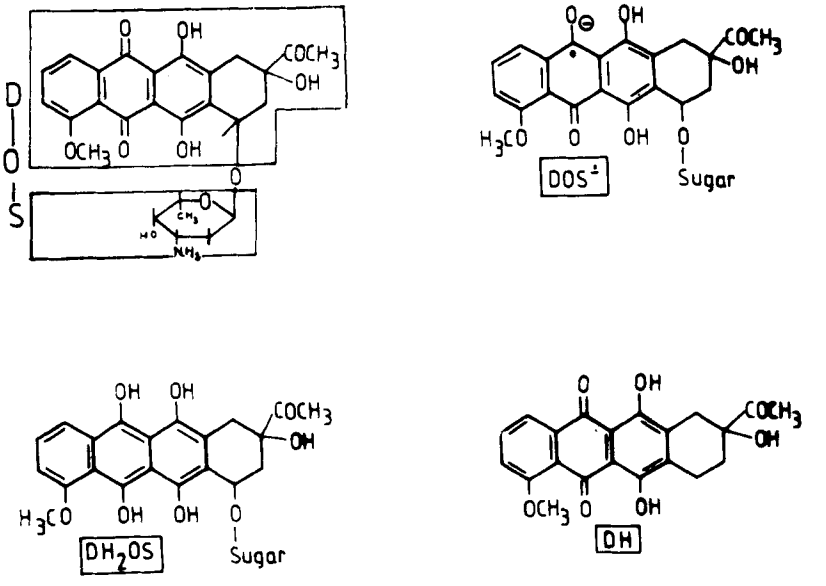
KEY WORDS: Daunorubicin-DNA complex, daunorubicin-protein complex, one-electron reduction, intramolecular electron transfer, γ - radiolysis.

INTRODUCTION

Daunorubicin (daunomycin) (DOS) is an anthracycline antitumor antibiotic. It binds to DNA by intercalation between base pairs,[1] with a dissociation constant of $\sim 10^{-6}$ [2] and disrupts the normal functions of the cells leading to its death.[3,4,5] For instance, it can cause sister chromatid exchange and chromosome aberrations. The intercalative binding of the drug would be at the origin of single and double strand breaks possibly due to alterations of the catalytic activity of DNA topoisomerase 11.[4,6]

Anthracyclines also inhibit enzymes and especially flavoenzymes.[7] It has been suggested that anthracyclines might act as antagonists of the flavoprotein coenzymes.[8] Flavoproteins such as the apoprotein of riboflavin binding protein (apoRBP) can complex anthracyclines and especially daunorubicin, with a K_d value approximately equal to 0.5 μM.[7]

Numerous studies have demonstrated that daunorubicin was activated biologically by reduction.[9] During reductive activation, daunorubicin accepts one or two electrons to provide semiquinone ($\cdot DOS^-$) or hydroquinone (DH_2OS) transient forms.[10,11] The latter disppears by losing its sugar moiety as a result of a complex mechanism.[10,11] The final product is 7-deoxy daunomycinone (DH) (Structures of these compounds are given in scheme 1). It has been proposed that the last transient has alkylating properties,[12] but real evidence of this ability is lacking. On the other hand, it is known that anthracyclines can be at the origin of reactive oxygen species formation. The interactions of the reduced transients of daunorubicin with these reactive oxygen species have been well documented.[9,13,14,15] In addition to the redox cycles of the



Scheme I Structure of daunorubicin and of its reduced forms.

daunorubicin/one-electron reductant/oxygen system, we have demonstrated that daunorubicin hydroquinone was oxidized by hydrogen peroxide with a two-electron transfer in one step, that is without production of oxygen free radical.[10]

In this work, we study the mechanism of the one-electron reduction of daunorubicin intercalated in DNA or in the apoprotein of the riboflavin binding protein. The reductants are $\cdot COO^-$ free radicals produced by γ-radiolysis, since our previous works have shown that they reduce efficiently the antibiotic in aqueous solution, giving the semiquinone form of the drug[10,11]. The reaction of these free radicals with DNA is negligibly slow.[16] The only target of $\cdot COO^-$ ions in proteins is disulfide bonds. Although apoRBP contains 9 disulfide bridges, their reduction by $\cdot COO^-$ free radicals is slower than the one of the antibiotic.[17] Thus one can expect that the action of $\cdot COO^-$ free radicals would concern specifically the intercalated drug.

MATERIALS AND METHODS

Materials for Irradiation

Daunorubicin-HCl was a generous gift of Rhône-Poulenc. 7-deoxy daunomycinone was prepared by radiolytic reduction of a solution of daunorubicin.[9] The precipitate was isolated by centrifugation and dried. Calf thymus DNA was provided by Sigma. It was dissolved by magnetic stirring during 24 hours. Apo riboflavin binding protein from Leghorn chicken egg white was a gift of V. Favaudon. Other reagents were from Rhône-Poulenc (Normapur). Water was triply distilled (resistivity $> 6\,M\Omega.cm$) or purified through a millipore MilliQ system. All glassware for irradiation was thoroughly cleaned up and rinsed with triply distilled water. After washing it was heated at 400°C for several hours to burn off impurities.

Thiol group concentration of natured proteins was determined with Ellmann's reagent, 5-5'-dithiobis (2-nitrobenzoic acid) (Fluka) which undergoes a thiolate disulfide exchange to yield 2-nitromercaptobenzoic acid. This product concentration was measured by absorbance at 412 nm (ε_{412} = 13 600 $mol^{-1}.dm^3.cm^{-1}$) against an appropriate blank.[18]

Complex Formation

The daunorubicin-DNA complex was made up by mixing equal volumes of drug and DNA solutions. The structure of the complex is well known[1,2] and strongly depends on the ratio r of the drug bound to nucleotide (r = [DOS]/[DNA]). For ratios lower than 1/10, intercalation is of only one type, that is, the antibiotic cycles are stacked between two base pairs.[2] We have worked in such conditions and have obtained the well-known absorption spectrum of the antibiotic intercalated in DNA (spectrum 0 of Figure 1). We have verified that Beer's Law was followed for [DOS] between 2×10^{-5} and 10^{-4} mol.l^{-1} and r between 1/10 and 1/20, in the wavelength region 350–600 nm, where only the antibiotic has a non-negligible absorption spectrum.

The drug-protein DOS_{apoRBP} complex was also made up by mixing equal volumes of drug and apoRBP solutions. The concentrations of the drug and of the protein in the mixing were equal. In these conditions, the drug occupies exclusively the flavin binding site. We verified that Beer's law is followed for [DOS_{apoRBP}] between 2×10^{-5} and 10^{-4} mol.l^{-1} in the wavelength region 350–500 nm where the protein has no absorption spectrum.

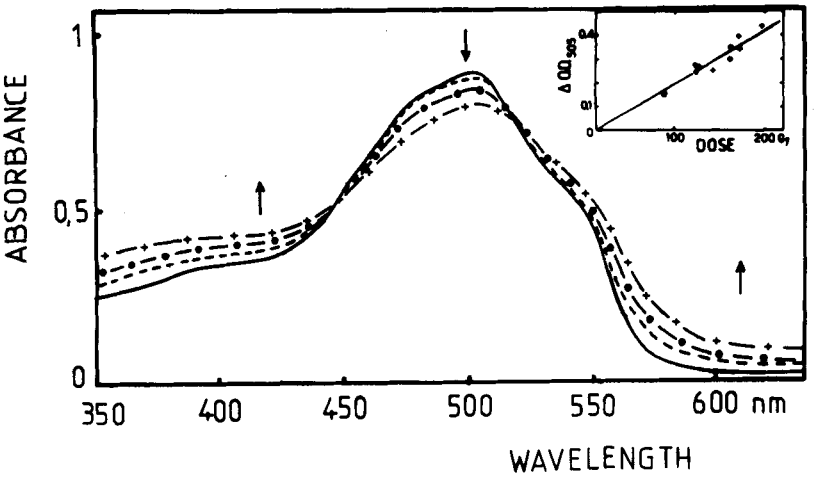


FIGURE 1 Reduction of daunorubicin intercalated in DNA by COO^- free radicals. Absorption spectra of DOS_{DNA} solutions were recorded immediately after irradiation, [DOS] = 8×10^{-5} mol.l^{-1}, [DNA] = 10 [DOS], [HCOONNa] = 0.1 mol.l^{-1}, phosphate buffer 6×10^{-2} mol.l^{-1}, pH 7, N_2O, optical path 1 cm, dose rate 6×10^{-2} $Gy.s^{-1}$.

——not irradiated; – – – 53.1Gy; -●- 107.2Gy; + – + 160Gy.

In inset : variation of the difference absorbance at 505 nm in the supernatants or irradiated solutions (see the text). Same experimental conditions as above, [DNA] = 10 [DOS]

● [DOS] = 4.1×10^{-5} mol.l^{-1}, ○ [DOS] = 7.95×10^{-5} mol.l^{-1}, + [DOS] = 8.3×10^{-5} mol.l^{-1}

The 7-deoxy daunomycinone -apoRBP complex (DH_{apoRBP}) was made by mixing a methanolic solution of aglycone (2.4×10^{-2}mol.l^{-1}) with an aqueous solution of protein. The final complex concentration (measured by absorbance of 502 nm using $\varepsilon_{502} = 9{,}300\,\text{mol}^{-1}.\text{l}.\text{cm}^{-1}$ [8]) was equal to 2.7×10^{-5} mol. l^{-1}.

Irradiation Technique

Steady state γ irradiations were performed in a ^{60}Co irradiator. The dosimetry was made by the Fricke's method assuming $G(Fe^{3+}) = 1.62\,\mu$mol. J^{-1} and $\varepsilon(Fe^{3+}) = 2{,}160\,\text{mol}^{-1}.\text{l}.\,\text{cm}^{-1}$ at 304 nm (22°C). The dose rate was equal to 6×10^{-2} Gy.s^{-1}. It is well known that in the conditions of steady state irradiations, it is not possible to see the radical intermediates formed by the one-electron transfer. On the other hand, it is possible to measure the yields of diappearance or of formation of stable products. It is also possible to study reactions of various compounds such as hydrogen peroxide, with intermediates, using their influence on the radiolytic yields.

Aqueous solutions of complex for irradiation contained formate ions (0.1 mol.l^{-1}). Solutions were deoxygenated and saturated by bubbling slowly nitrous oxide (CFPO, France, purity > 99.9%) prior to irradiation (ca.20 min. for a volume of 5 cm^3). It is known that under these conditions e^-_{aq} free radicals are scavenged by N_2O giving ·OH radicals. ·OH and ·H radicals react with formate to give ·COO− anions with a well−known yield of 0.62 μmol.J^{-1}.

RESULTS

Daunorubicin/DNA Complex

Reduction by ·COO-free radicals Danunorubicin/DNA aqueous solutions ([DOS] between 4×10^{-5} and 10^{-4}mol.l^{-1}, $r = 1/10$ or $1/20$) were irradiated in the presence of formate ions (0.1 mol.l^{-1}) (phosphate buffer 6×10^{-2} mol.l.$^{-1}$, pH 7). Absorption spectra of solutions of irradiated complex recorded immediately after irradiation are given in Figure 1 ([DOS] $= 8 \times 10^{-5}$mol.l^{-1}, $r = 1/10$). Absorbances at the maximum decrease with increaing doses. Isobestic points at ~445 nm and ~515 nm are observed, which do not exist any more for doses higher than ~160 Gy (results not shown). These spectra belong to daunorubicin and to its two-electron reduced forms since it is know that neither DNA nor its eventual reduced forms absorb in this wavelength region.

If irradiated solutions are kept (in the dark, at ~4°C or at ambient temperature), a red precipitate appears very slowly (40 to 48 hours after irradiation). In irradiated solutions of daunorubicin without DNA, this precipitate is immediately observed. NMR analysis of the powder showed only one compound, 7 deoxy daumomycinone (DH) (scheme 1). Absorption spectra of the supernatants were identical to the one of the non-irradiated DOS_{DNA} complex.

The reduction yield $G(-DOS_{DNA})$ was measured in the supernatants by difference absorbance at 505 nm between the unirradiated solution and the supernatants (insert of Figure 1), we find

$$G(-DOS_{DNA}) = (0.26 \pm 0.02)\ \mu\text{mol.J}^{-1}$$

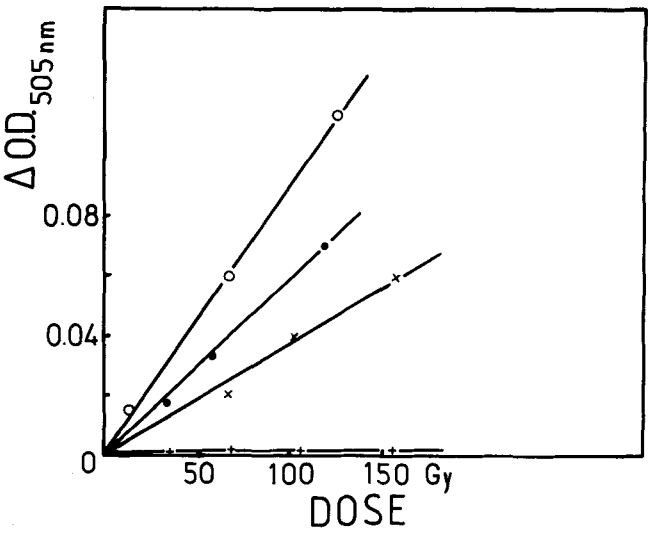


FIGURE 2 Influence of hydrogen peroxide on the reduction yield of daunorubicin intercalated in DNA, measured as the difference absorbance at 505 nm. [DOS] = 5×10^{-5}mol.l^{-1}, [DNA] = 10[DOS], [HCOONa] = 0.1 mol.l^{-1}, phosphate buffer 6×10^{-2}mol.l^{-1}, pH 7, N_2O, optical path 1 cm, dose rate 6×10^{-2} Gy.s^{-1}

○ no H_2O_2 · [H_2O_2] = 10^{-5}mol.l^{-1} × [H_2O_2] = 5×10^{-5} mol.l^{-1}; + [H_2O_2] = 10^{-4} mol.l^{-1}

Reaction of hydrogen peroxide We verified that H_2O_2 did react neither with daunorubicin nor with its aglycone form in DNA. When aqueous solutions of complex are irradiated in the presence of H_2O_2 G(-DOS) decreases with increasing H_2O_2 (Figure 2). It is likely that hydroquinone daunorubicin is oxidized by H_2O_2 similarly to what happens without DNA.[10]

Daunorubicin/Apoprotein of the Riboflavin Binding Protein

Solutions of daunorubicin-apoprotein of the riboflavin binding protein were irradiated in the same conditions as for DOS_{DNA} solutions. The resulting absorption spectra of irradiated solutions are reported in Figure 3. They are different from the ones of Figure 1. Two peaks appear at 505 and 530 nm. No precipitate was observed. The absorption spectrum of the irradiated solution ([DOS_{apoRBP}] = 4.5×10^{-5}mol.l^{-1}, 200Gy) was identical to the one of DH_{apoRBP}. It is thus concluded that the final product is 7 deoxydaunomycinone in apoRBP which is very stable.

The reduction yield G(-DOS_{apoRBP}) was measured by difference absorbance at 540 nm because the difference absorptivity is the highest at this wavelength (Figure 4) ($\Delta\varepsilon_{540}$ = 1,050 mol^{-1}.l.cm^{-1}). We find

$$G(-DOS_{apoRBP}) = (0.3 \pm 0.02)\ \mu\text{mol. J-1}$$

No effect of H_2O_2 was detected.

The quantity of thiol group coming from disulfide attack by ·COO- free radicals was measured in the same solutions (Figure 4). The initial yield G(SH) is equal to 0.034 μmol.J^{-1}. This yield increases with decreasing daunorubicin.

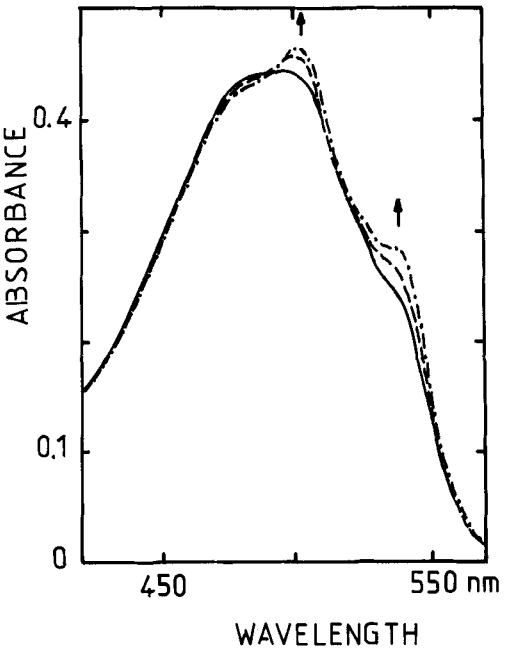


FIGURE 3. Reduction of daunorubicin intercalated in the apoprotein of the riboflavin binding protein. [DOS] = [apoRBP] = 4.5×10^{-5} mol.l^{-1}. Phosphate buffer 6×10^{-2} mol.l^{-1}, pH 7, [NaHCOO] = 0.1 Mol.l^{-1}, N_2O, optical path 1cm; dose rate 6×10^{-2}Gy.s^{-1}.

— : non irradiated

- - : 64 Gy

- ● - : 98 Gy

INTERPRETATION

Daunorubicin — DNA Complex

The kinetic scheme is very similar to the one proposed for the reduction of daunorubicin non intercalated[10]

$$DOS_{DNA} + \cdot COO^- \longrightarrow \cdot DOS^-_{DNA} + CO_2 \quad (1)$$

$$\cdot DOS^-_{DNA} + \cdot COO^- + 2\,H^+ \longrightarrow DH_2OS_{DNA} + CO_2 \quad (2)$$

$$DH_2OS_{DNA} \longrightarrow DH_{DNA\ +\ sugar} \quad (3)$$

$$DH_2OS_{DNA} + H_2O_2 \longrightarrow DOS_{DNA} + 2\,H_2O \quad (4)$$

$$DH_{DNA} \longrightarrow DH \quad (5)$$

Reaction (1) is the one-electron reduction of the drug which initiates the whole process. Evidence for this reaction sequence taking place in DNA has been dis-

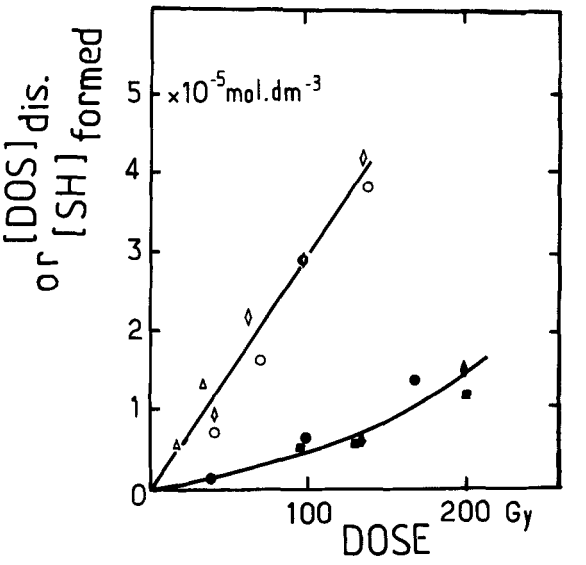


FIGURE 4. Variation of the concentration of daunorubicin disappeared ($[DOS]_{dis}$) (open symbols) and of the concentration of sulfhydryl groups appeared ($[SH]_{formed}$) (filled symbols) in irradiated solutions of daunorubicin-apoRBP complex, with the dose. Phosphate buffer 6×10^{-2} mol.l^{-1}, [HCOO] = 0.1 mol.l^{-1}, N_2O, pH 7, dose rate 6×10^{-2} Gy.s^{-1}.

○, ● $[DOS_{apoRBP}] = 4 \times 10^{-5}$ Mol.l^{-1}; △, ■ $[DOS_{apoRBP}] = 4.5 \times 10^{-5}$ Mol.l^{-1},

◇, ◆ $[DOS_{apoRBP}] = 5 \times 10^{-5}$ Mol.l^{-1}

cussed.[19] The semiquinone free radical is formed inside DNA and in the conditions of γ radiolysis, it is probably reduced again by one more $\cdot COO^-$, giving the hydroquinone form, which seems more likely than disproportionation, since it is doubtful that the semiquinone free radicals intercalated in DNA, can diffuse and meet another free radical. Indeed, all the rate constants involved in this process are certainly much faster than the complex dissociation rate constant, which is $\sim 2 s^{-1}$ for DOS_{DNA}[20] and perhaps of the same order of magnitude for the intermediate complexes since the dissociation seems limited mostly by DNA breathing.[19,20]

The hydroquinone transient is reoxidized to the initial antibiotic molecule by H_2O_2 with a two-electron transfer in one step (reaction (4)) or loses its sugar moiety (reaction (3)). The rate constant of reduction of daunorubicin intercalated in DNA by $\cdot COO$- free radicals is not known. However, the rate constant of reaction of H_2O_2 with $\cdot COO$- is very low

$$k(H_2O_2 + \cdot COO^-) < 2 \times 10^6 \text{ mol}^{-1}.l.s^{-1}$$ [21]

Knowing the rate constants of reduction of daunorubicin alone by $\cdot COO$- ($2 \times 10^9 mol^{-1}.l.s^{-1}$)[11], and intercalated in apoRBP ($2.4 \times 10^8 mol^{-1}.l.s^{-1}$), we can expect that the competition kinetics is in favour of the reduction of daunorubicin.

This indicates that hydrogen peroxide can enter into DNA and react with intercalated compounds with a rate higher than the glycosidic breakage one.

Calculation of the drug reduction yield using this kinetic scheme gives

$$G(\text{-DOS}_{DNA}) = \frac{G(\cdot COO\text{-})}{2} - G_{H202} = 0.24\ \mu mol.J^{-1}$$

which is in good agreement with the experimental G-value.

Daunorubicin-Protein Complex

The kinetic scheme is

$$DOS_{apoRBP} + \cdot COO\text{-} \dashrightarrow \cdot DOS^{-}_{apoRBO} + CO_2 \quad (6)$$
$$\dashrightarrow DOS_{apoRBP\cdot -} + CO_2$$

$$\cdot DOS^{-}_{apoRBP} + \cdot COO \xrightarrow{2H^+} DH_2OS_{apoRBP} + CO_2 \quad (7)$$

$$\cdot DOS^{-}_{apoRBP} \xrightarrow{2H^+} DH_2OS_{apoRBP+} \quad (8)$$

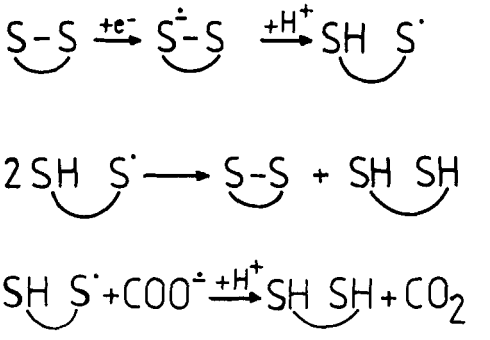
$$DH_2OS_{apoRBP+} + \cdot COO^{-} \dashrightarrow DH_2OS_{apoRBP} + CO_2 \quad (9)$$

$$DH_2OS^{-}_{apoRBP} \dashrightarrow DH_{apoRBP} + \text{sugar} \quad (10)$$

$$2\ DOS_{apoRBP\cdot -} \dashrightarrow DOS_{apoRBP} + DOS_{apoRBP(SH)2} \quad (11)$$

Similar transients as in DNA (such as the semiquinone and the hydroquinone drugs) are formed in the protein, in a process ending up by 7 deoxyaglycone formation in the protein (reaction (10)). However, ·COO- anions can also reduce disulfide bond(s) in competition with the drug (reaction (6)), since sulfhydryl groups are created by γ radiolysis. $DOS_{apoRBP\cdot -}$ symbolizes the disulfide radical anion resulting from the reduction of one of the 9 disulfide bonds of the protein.

A pulse radiolysis study of the semiquinone free radical decay inside this protein has shown that it involved an intramolecular electron transfer (reaction (8)), which should lead to an electron "hole" in the protein. In the conditions of γ radiolysis either this process does not take place and the semiquinone transient is reduced by a ·COO- entity (reaction (7)), or more probably, this electron "hole" is reduced to its initial form by the reducing free radical (reaction (9)).



Scheme II A proposed mechanism for the one-electron reduction of a dissulfide bond in a protein, initially, when [DOS] = [apoRBP].

Reaction (11) is not an elementary reaction, but it represents the process going from disulfide anion to the sulhydryl group formation in the protein. Such reduction mechanisms of small disulfide compounds in the presence of formate have been the subject of numerous studies,[22] but to our knowledge, this reduction mechanism in proteins has never been studied in the conditions of steady state reduction. Examination of the initial reduction yields given by the initial slopes of the curves of Figure 4, indicates that initially this process can be made of a minimal set of reactions (scheme II) symbolized by the stoichiometry of reaction (11). These reactions happen during the reduction of dithiothreitol in the presence of formate ions.[22] Indeed, using this minimal reaction scheme and reactions (6) to (10), one obtains

$$G(\cdot COO\text{-}) = 2G(-DOS_{apoRBP}) + G(SH) = 0.62\ \mu mol.J^{-1}$$

which is in very good agreement with the experimental value ($0.63\ \mu mol.J^{-1}$).

However, it is obvious that the yield G(SH) increases considerably with decreasing $[DOS_{apoRBP}]$ where $G(-DOS_{apoRBP})$ does not change. Preliminary experiments concerning the reduction of the apoprotein alone by ·COO- ions indicated that it is a chain reaction, with a mechanism very similar to the one reported for small molecules,[22] with pH-dependent propagation steps. We are currently studying this mechanism.

DISCUSSION AND CONCLUSION

In this work, we have studied the reduction mechanism of daunorubicin intercalated in DNA or in a protein, apoRBP. In both cases, the antibiotic is stacked between planar residues, two base pairs in DNA and two aromatic aminoacids in the protein.[23] We found that in both situations, the drug can be attacked by reductants of small size totally or partially unreactive with the "host" macromolecule and that the reduction is quantitative in both cases. The reduction mechanism is similar and involves the same intermediates. In steady state conditions the transfer of the second electron to daunorubicin semiquinone can be explained very simply by a reaction of the intercalated free radical with ·COO- ion continuously produced. However, as for DOS_{apoRBP}, we have shown that in the conditions of pulse radiolysis, the second-electron reduction was provided by the oxidation of an unidentified residue in the protein, in an intramolecular reaction[17]. It is possible that DNA plays a similar role, i.e. transfer an electron from a base to the drug.

The most interesting difference is the difference of accessibility of the site to hydrogen peroxide. It appears that DNA is much less protected than the active site of apoRBP, perhaps because of the "breathing" movements. One can thus postulate that H_2O_2 is able to react with many different compounds inside DNA including metal impurities, leading to oxygen free radical formation in the double helix.

Acknowledgements

We are indebted to "Rhône-Poulenc Recherches" (Dr. Lavelle) for gifts of daunorubicin. We thank V. Favaudon for the preparation of the protein and for many stimulating discussions.

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