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Intramolecular Electron Transfer in Proteins. Radiolysis Study of the Reductive Activation of Daunorubicin Complexed in Egg White Apo-Riboflavin Binding Protein†

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ABSTRACT: Daunorubicin, an anthracycline antitumor antibiotic, can be complexed in egg white apo-riboflavin binding protein. The reduction of this complex was studied by γ-radiolysis and pulse radiolysis using $COO^{\bullet -}$ free radicals as reductants. The final products are 7-deoxydaunomycinone intercalated in the protein and thiol groups coming from the reduction of disulfide bonds of the protein, in the respective proportions of 90% and 10%. One-electron reduction of the complex gives daunorubicin semiquinone radical and a disulfide anion. The rate constants of the reactions of $COO^{\bullet -}$ ions with the complex and with the disulfide bond in the protein alone are respectively equal to 2.4×10^8 $mol^{-1} \cdot L \cdot s^{-1}$ and 6.4×10^7 $mol^{-1} \cdot L \cdot s^{-1}$. Daunorubicin semiquinone decays by a first-order process, the rate constant of which is independent of the initial protein and radical concentrations. Without protein, daunorubicin semiquinone undergoes a disproportionation–comproportionation equilibrium [Houée-Levin, C., Gardès-Albert, M., Ferradini, C., Faraggi, M., & Klapper, M. (1985) *FEBS Lett. 179*, 46–50]. We propose that a protein residue reduces the semiquinone by an intramolecular path. This creates an electron hole in the protein which may alter its function. This reduction process is very different from the reduction mechanism of riboflavin binding protein by the same reductant [Faraggi, M., Steiner, J. P., & Klapper, M. H. (1985) *Biochemistry 24*, 3273–3279]. These results suggest a new deleterious pathway to explain the antitumor and/or cytotoxic effect of this drug.

Flavoproteins are a very important class of proteins which carry out a wide variety of different biochemical processes including electron transfer. Numerous studies have been devoted to a better understanding of how electrons are transmitted at flavoenzyme active sites through the protein matrix (Moore & Williams, 1976; Simondsen et al., 1982; Merrill et al., 1981; Klapper & Faraggi, 1983; Faraggi & Klapper, 1984; Tegoni et al., 1984; Tollin et al., 1984; Faraggi et al., 1985; Ghisla & Massey, 1986; Anderson et al., 1986). One of the most extensively studied flavoproteins is the chicken egg white riboflavin binding protein (RBP),[1] because of its

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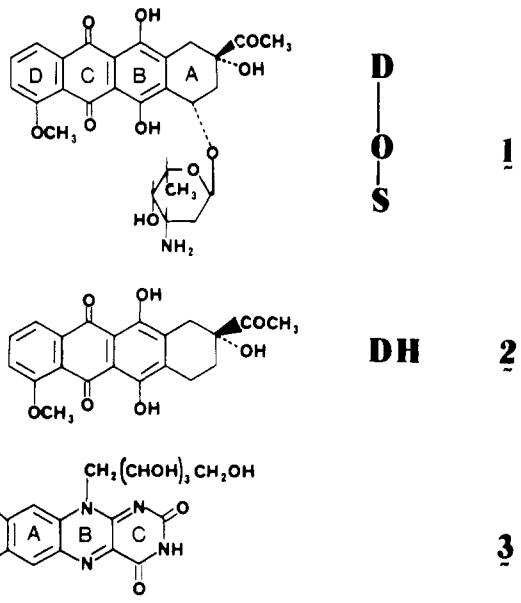
Chart I: Structures of Daunorubicin (1), 7-Deoxydaunomycinone (2), and Riboflavin (3)



abundance, stability, and ease of isolation. Its amino acid sequence is known (Hamazume et al., 1984; White & Merrill, 1988). It has nine disulfide bridges and no thiol groups. The details of the interaction between riboflavin (Chart I) and this protein are reasonably well understood (Nishikimi & Kyogoku, 1973; Blankenhorn, 1978; Choi & McCormick, 1980; Merrill et al., 1981; Hamazume et al., 1984; Wessiak et al., 1984). The active site is hydrophobic and involves tyrosine and tryptophan residues, which probably interact with the resonant part of the flavin by stacking (Blankenhorn, 1978; Choi & McCormick, 1980). It seems that riboflavin buried in the active site of RBP can be reduced by chemical reductants (Spencer et al., 1977) and by free radicals such as e_{aq}^- and $COO^{\bullet -}$ entities produced by pulse radiolysis (Klapper & Faraggi, 1983; Faraggi & Klapper, 1984; Faraggi et al., 1985), and the resulting flavin semiquinone is stabilized in its neutral form [e.g., Merrill et al. (1981) and Muller (1987)]. However, pulse radiolysis studies indicated that the mechanism of this one-electron reduction is very complex, including competitive disulfide anion formation and the possible intervention of an unknown group transferring its electron to the flavin (Faraggi et al., 1985).

The anthracyclines are among the most efficacious antitumor antibiotics that are reductively activated in vivo (Arcamone, 1981). Daunorubicin (**1**; DOS, daunomycin) (Chart I) is one of the most important anthracyclines in clinical use. In vitro, at the molecular level, anthracyclines inhibit enzymes and especially flavoenzymes (Taylor & Hochstein, 1978; Pan et al., 1981; Kharash & Novak, 1981). They are known to intercalate in DNA (Neidle 1979) and to initiate lipid peroxidation (Goodman & Hochstein, 1977; Winterbourn et al., 1985). These abilities contribute both to general toxicity and to neoplastic activity. During reductive activation, the quinone functional group of daunorubicin accepts either one or two electrons to provide semiquinone ($DOS^{\bullet -}$) or hydroquinone (DH_2OS) reduced states, respectively (Bachur et al., 1977; Lown et al., 1977; Houée-Levin et al., 1984, 1985, 1986). The involvement of these two species in reactive oxygen formation in vivo and in vitro has been well documented (Lown et al., 1979, 1982; Davies et al., 1983; Doroshow & Davies, 1986; Davies & Doroshow, 1986; Houée-Levin et al., 1986).

Several relations between this drug and flavoenzymes have been demonstrated. Low-potential flavin-containing enzymes are excellent in vitro reduction catalysts (Fisher et al., 1985). It has been suggested that anthracyclines might act as antagonists of the flavoprotein coenzymes (Kharash & Novak, 1981; Fisher et al., 1982). NADH:flavin oxidoreductase forms a ternary complex with NADH and daunorubicin, the anthracycline occupying the flavin binding site of the enzyme (Fisher et al., 1983). It has been demonstrated that the apoprotein of riboflavin binding protein (apoRBP) could also complex anthracycline antitumor antibiotics (especially daunorubicin; Fisher et al., 1982). The K_d values are approximately equal to 0.5 μM (Fisher et al., 1982). The anthracyclines occupy the flavin binding sites in this complex, their D–B rings overlapping the region normally occupied by the riboflavin (**3**) A–C rings, respectively (Chart I). In these complexes, the drug can be reduced easily, and the final product is the same as the one formed by reduction in aqueous solution using chemical or biochemical reductants (Arcamone, 1981; Houée-Levin et al., 1984), i.e., 7-deoxydaunomycinone (**2**; DH) (Chart I) (Fisher et al., 1982).

We present here a kinetic study of the reduction of daunorubicin complexed in egg white apo-riboflavin binding protein, performed by the methods of γ-radiolysis and pulse radiolysis. Our aims are the following. First, on the basis of the numerous possible interactions between flavoproteins and daunorubicin, a better knowledge of the reduction mechanism of the drug–protein complex is worthwhile. Second, there is general interest in electron transfer over long distances through proteins (Grossweiner, 1976; Klapper & Faraggi, 1979; Isied, 1984; Marcus & Sutin, 1985). One of the main methods in this field consists of modifying a structurally well characterized protein and measuring electron-transfer rates across the peptide framework (Isied, 1984; Faraggi & Klapper, 1988). In this sense, the daunorubuicin–apoRBP complex is similar to a protein in which the electron acceptor, riboflavin, has been replaced by an antibiotic, the redox properties of which have been the subject of numerous studies in our laboratory (Houée-Levin et al., 1984, 1985, 1986). The methods of pulse radiolysis are well adapted to this kind of study, as explained by Steiner et al. (1985). However, steady-state radiolysis also provides fruitful information concerning electron transfer across DNA in daunorubicin–DNA complexes (Rouscilles et al., 1988). Thus, in this work, we used both methods. The reducing species were $COO^{\bullet -}$ free radicals, since (i) we have studied the reduction mechanism of the drug by γ-radiolysis and pulse radiolysis using the same reductant (Houée-Levin 1984, 1985, 1986) and (ii) the reactions of these free radicals with proteins are functional group specific since they involve primarily a disulfide bridge(s) with negligible participation of other groups such as histidine [see, for example, Steiner et al. (1985)].

Part of these results has been presented elsewhere in a preliminary form (Sekaki et al., 1988).

Materials and Methods

Materials for Irradiation. Daunorubicin hydrochloride was a generous gift of Rhône-Poulenc. γ-Radiolysis studies have shown in some samples the existence of traces of impurities which could be destroyed by preirradiation of the solutions (ca. 50 Gy). Thus treated, aqueous daunorubicin solutions were reduced by $COO^{\bullet -}$ free radicals with the same yield as chemically pure DOS solutions (Houée-Levin et al., 1984).

[1] Abbreviations: DOS, daunorubicine; $DOS^{\bullet -}$, daunorubicine semiquinone; DH_2OS, daunorubicine hydroquinone; DH, 7-deoxydaunomycinone; RBP, riboflavin binding protein.

7-Deoxydaunomycinone was prepared by radiolytic reduction of a solution of daunorubicin (Houée-Levin et al., 1984). The precipitate was isolated by centrifugation and dried. All other reagents were from Rhône-Poulenc (Normapur) or Merck (PA). Sodium formate either was Rhône-Poulenc Normapur or was made by mixing formic acid (Merck Suprapure) with sodium hydroxide (Merck PA). This latter mixing is known to minimize the amount of metallic impurities which can catalyze free-radical reactions. In both cases our results were identical. Nitrous oxide was provided by CFPO. Its purity was better than 99.99%. Water was triply distilled (resistivity > 6 MΩ·cm) or purified through a Millipore Milli-Q system. All glassware for irradiation was thoroughly cleaned and rinsed with triply distilled water. After washing, it was heated at 400 °C for several hours to burn off impurities.

Apo-riboflavin binding protein was a gift of V. Favaudon. It was prepared from Leghorn chicken egg white according to the procedure of Rhodes (1959). The purified protein was stored frozen at −20 °C. Its concentration was determined by absorbance at 280 nm [ϵ_{280} = 42 850 $mol^{-1}\cdot L\cdot cm^{-1}$, in agreement with Blankenhorn (1978) and Nishikimi and Kyogoku (1973)]. The 7-deoxydaunomycinone–apoRBP complex was made by mixing a methanolic solution of aglycon (2.4×10^{-2} $mol\cdot L^{-1}$) with an aqueous solution of protein. The final complex concentration (measured by absorbance at 502 nm with ϵ_{502} = 9300 $mol^{-1}\cdot L\cdot cm^{-1}$) was equal to 2.7×10^{-5} $mol\cdot L^{-1}$.

The complexation rate was studied on a fast-kinetics stopped-flow spectrophotometer (Durrum Instrument Co., Model D-110) with a mixing time of 4 ms.

Thiol group concentration of natured proteins was determined with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (Fluka), which undergoes a thiolate disulfide exchange to yield 2-nitro-5-mercaptobenzoic acid. This product concentration was measured by absorbance at 412 nm (ϵ_{412} = 13 600 $mol^{-1}\cdot L\cdot cm^{-1}$) against an appropriated blank (Ellmann, 1959).

Steady-state γ-irradiations were performed in a ^{60}Co irradiator. The dosimetry was made by the Fricke's method assuming $G(Fe^{3+})$[2] = 1.62 $\mu mol\cdot J^{-1}$ and $\epsilon(Fe^{3+})$ = 2160 $mol^{-1}\cdot L\cdot cm^{-1}$. Solutions were deoxygenated and saturated by bubbling N_2O prior to irradiation (ca. 20 min for a volume of 5 cm^3). Pulse radiolysis experiments were performed on a Febetron 707 whose setup has been described (Marignier & Hickel, 1985). Each experimental point represents an average of three to four independent measurements.

In γ-radiolysis as well as in pulse radiolysis, irradiation of a nitrous oxide saturated solution containing formate ions produces $COO^{\bullet-}$ free radicals by the following reaction sequence:

$$H_2O \rightsquigarrow e_{aq}^-,\ OH^\bullet,\ H^\bullet,\ H_2O_2,\ H_2$$

$$e_{aq}^- + N_2O \rightarrow OH^\bullet + N_2 + OH^-$$

$$OH^\bullet\ (\text{or } H^\bullet) + HCOO^- \rightarrow COO^{\bullet-} + H_2O\ (\text{or } H_2)$$

It is known that the yield of $COO^{\bullet-}$ ions [$G(COO^{\bullet-})$] is equal to 0.622 $\mu mol\cdot J^{-1}$. In γ-radiolysis, the maximum dose rate was equal to 6×10^{-2} $Gy\cdot s^{-1}$, which equals a $COO^{\bullet-}$ delivery rate of 3.85×10^{-8} $mol\cdot L^{-1}\cdot s^{-1}$. In pulse radiolysis, the average dose was delivered in less than 20 ns and was between 10 and 60 Gy (5×10^{-6} and 3×10^{-5} $mol\cdot L^{-1}$ $COO^{\bullet-}$) per pulse.

[2] In radiation chemistry, yields are usually named as *G* values, *G*(X) being the initial yield of formation of X.

Results

Evaluation of the Kinetic Parameters of Daunorubicin–Apo-Riboflavin Binding Protein Complex Formation. Daunorubicin and apoRBP solutions were rapidly mixed in a stopped-flow rapid-kinetic device. The final concentration of each species in the final mixture was equal to 2.5×10^{-5} $mol\cdot L^{-1}$. Observation was made by absorbance at 460 nm, the reference solution being a daunorubicin solution (2.5×10^{-5} $mol\cdot L^{-1}$) ($\Delta\epsilon$ = 650 $mol^{-1}\cdot L\cdot cm^{-1}$). At the end of the mixing time, the complex was already formed. Thus, a limit of the rate constant k_{assoc} of the reaction

$$DOS + apoRBP \underset{k_{dissoc}}{\overset{k_{assoc}}{\rightleftharpoons}} (DOS, apoRBP)$$

was obtained by assuming that at the end of the mixing time the concentration of the uncomplexed species was equal to their initial concentration C_0 divided by 10. The following relation is thus obtained:

$$(10/C_0 - 1/C_0)k_{assoc} < 4 \text{ ms}$$

This gives lower limits for k_{assoc} of $\geq 9 \times 10^7$ $mol^{-1}\cdot L\cdot s^{-1}$ and for k_{dissoc} of

$$k_{dissoc} = K_d k_{assoc} \geq 45\ s^{-1}$$

The association rate constant for daunorubicin is of the same order of magnitude as those for flavin analogues (ca. 3×10^8 $mol^{-1}\cdot L\cdot s^{-1}$) (Becvar & Palmer, 1982).

Steady-State γ-Radiolysis. Aqueous solutions of the daunorubicin–apoRBP complex were irradiated by γ-rays under steady-state conditions. We verified first, by comparing absorption spectra of irradiated solutions to the spectrum of 7-deoxydaunomycinone–apoRBP complex, that the final product of daunorubicin reduction was this quinonic aglycon in the complex, similar to the findings of Fisher et al. (1982) for the reduction of the complex with dithionite.

The yield of reduction of the drug in the complex [*G*(−DOS)] was measured by absorbance at 540 nm. At this wavelength, the difference absorptivity is maximal ($\epsilon_{DOS} - \epsilon_{DH}$ = −1050 $mol^{-1}\cdot L\cdot cm^{-1}$). This yield is equal to 0.3 $\mu mol\cdot J^{-1}$.

The quantity of thiol groups formed by disulfide breakage was measured in the same solutions. The initial yield of −SH group formation *G*(SH) is equal to 0.034 $\mu mol\cdot J^{-1}$, thus approximately 10 times lower than the yield of DOS reduction.

One-Electron Reduction by Pulse Radiolysis. The reaction of the apoprotein alone with $COO^{\bullet-}$ was studied by pulse radiolysis ($[apoRBP]_0$ between 2×10^{-4} and 10^{-3} $mol\cdot L^{-1}$). The resulting absorption spectrum recorded 100 μs after the pulse is reported in Figure 1 (spectrum C). It shows a maximum between 400 and 430 nm similar to the disulfide anion spectrum (Hoffmann & Hayon, 1972). No shoulder around 360 nm is seen, which is known to be the histidyl radical absorption maximum (Bisby et al., 1976; Steiner et al., 1985). The rate constant k'_1 of the reaction of a disulfide bond(s) with $COO^{\bullet-}$ was determined under pseudo-first-order conditions and is equal to 6.4×10^7 $mol^{-1}\cdot L\cdot s^{-1}$.

As for the reduction by pulse radiolysis of the daunorubicin–apoRBP complex (from 5×10^{-5} to 2×10^{-4} $mol\cdot L^{-1}$), the complex to radical concentration ratios were generally kept at 10 or higher for kinetic studies, or at 5 for absorption spectra to minimize multiple radical reactions with one protein molecule. Figure 1 shows the difference absorption spectrum (referenced to unirradiated solutions) recorded 22 μs after the pulse, when absorbances at all the maxima have reached a plateau lasting ca. 100 μs (spectrum A). This spectrum is compared to the one of daunorubicin semiquinone (Houée-

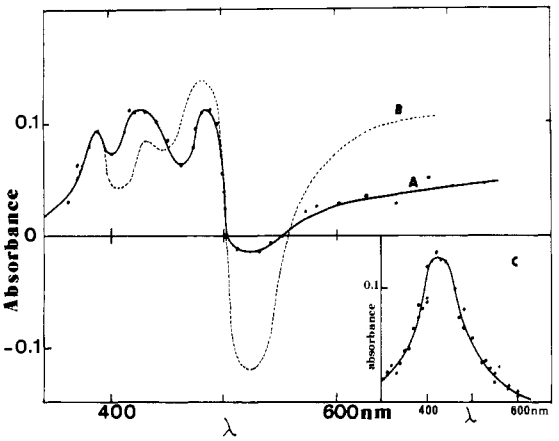


FIGURE 1: Pulse radiolysis reduction of the daunorubicin–apoRBP complex by $COO^{\bullet-}$ free radicals. (A) Difference absorption spectrum of the daunorubicin semiquinone free radical intercalated in the protein. [DOS] = [apoRBP] = 10^{-4} mol·L^{-1}; [phosphate] = 6×10^{-2} mol·L^{-1}, pH 7; [$HCOO^-$] = 0.1 mol·L^{-1}; N_2O; dose is 30 Gy, 22 μs after the pulse; optical path is 2.5 cm. (B) Difference absorption spectrum of daunorubicin semiquinone in aqueous solution without protein (Houée-Levin et al., 1985), normalized at 380 nm. (C) Difference absorption spectrum of the apoRBP free radical. [apoRBP] = 2.32×10^{-4} mol·L^{-1}; [phosphate buffer] = 6×10^{-2} mol·L^{-1}, pH 7; [$HCOO^-$] = 0.1 mol·L^{-1}; N_2O; dose is 20 Gy, 100 μs after the pulse; optical path is 2.5 cm.

Levin et al., 1984), normalized at 380 nm (spectrum B). Both spectra have maxima at the same wavelengths (380, 420, 480, and >700 nm) and a minimum around 540 nm, but the maximum at ca. 420 nm is higher in the case of the complex, as it is in the case of daunorubicin semiquinone intercalated in DNA (Rouscilles et al., unpublished results). This spectrum is thus attributed to daunorubicin semiquinone in the complex with a slight contribution of the disulfide free radical.

The kinetics of the reaction of the complex with $COO^{\bullet-}$ free radicals were studied under pseudo-first-order conditions at all wavelengths between 350 and 750 nm. The observed pseudo-first-order rate constant is independent of the wavelength and directly proportional to the initial complex concentration. The rate constant k_1 for the reaction of $COO^{\bullet-}$ with the complex is thus equal to the slope of this straight line, 2.4×10^8 mol^{-1}·L·s^{-1}, which is 3.75-fold higher than k'_1.

The contribution of the disulfide radical anion to the one-electron reduction of the daunorubicin–protein complex is certainly small since (i) the absorption spectrum of the free radical is very similar to the one recorded for the daunorubicin–DNA complex, (ii) the free radical formation obeys the same kinetic law at all the wavelengths, and (iii) the –SH group formation yield is approximately 10 times lower than the DOS reduction yield in γ-radiolysis experiments.

Free-Radical Decays. The kinetics of the disulfide anion decay were measured at all wavelengths between 380 and 500 nm in apoRBP solutions irradiated by pulse radiolysis ($[apoRBP]_0 = 2 \times 10^{-4}$ to 10^{-3} mol·L^{-1}). The final absorbances are equal to 0 in this region. The reaction is first-order with a rate constant k_2 equal to 10.5 s^{-1}.

As for the DOS–apoRBP complex, spectrum A of Figure 1 disappears on the millisecond time scale. Figure 2 shows the resulting difference spectrum, 16 ms after the pulse (experimental conditions are the same as stated above). This spectrum is stable up to the hundreds of millisecond time scale. Only one peak at 420 nm and a minimum around 540 nm are seen. This difference spectrum is identical with the one for daunorubicin hydroquinone formation in aqueous solution, normalized at 420 nm (Houée-Levin et al., 1984) (Figure 2). Thus, we conclude that daunorubicin hydroquinone is formed

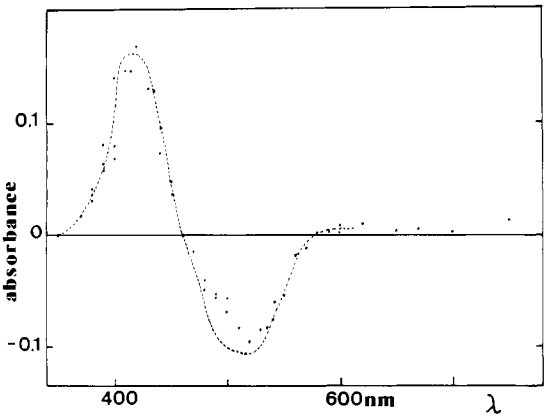


FIGURE 2: Difference absorption spectrum of daunorubicin hydroquinone intercalated in apoRBP (●) compared to the spectrum of daunorubicin hydroquinone in aqueous solution without protein (—) (Houée-Levin et al., 1985), normalized at 420 nm. [DOS] = [apoRBP] = 10^{-4} mol·L^{-1}; [phosphate] = 6×10^{-2} mol·L^{-1}, pH 7; [$HCOO^-$] = 0.1 mol·L^{-1}; N_2O; dose is 30 Gy, 16 ms after the pulse; optical path is 2.5 cm.

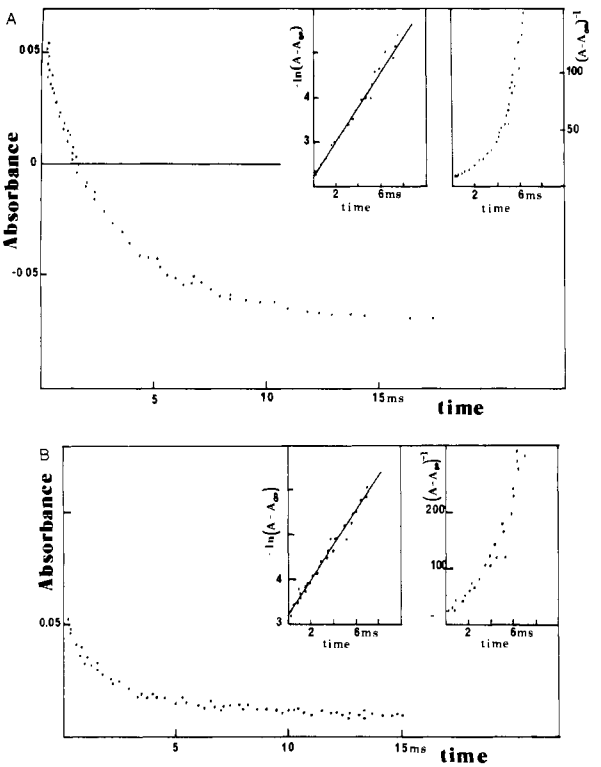
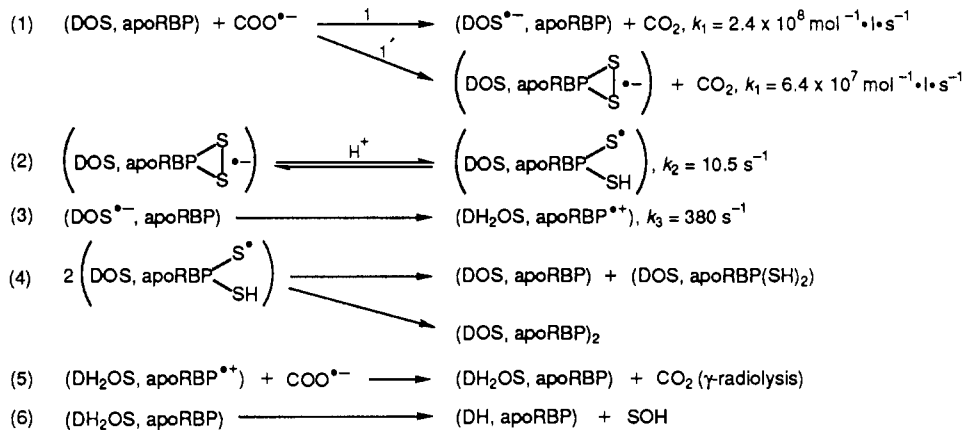


FIGURE 3: Kinetics of daunorubicin semiquinone reduction into its hydroquinone form, studied in two different initial conditions and at two different wavelengths. (Insets) First- and second-order plots of the experimental points. [Phosphate] = 6×10^{-2} mol·L^{-1}, pH 7; [$HCOO^-$] = 0.1 mol·L^{-1}; optical path is 2.5 cm; N_2O. (A) [DOS] = [apoRBP] = 10^{-4} mol·L^{-1}; dose is 37 Gy; 490 nm. (B) [DOS] = [apoRBP] = 2×10^{-2} mol·L^{-1}; dose is 41 Gy; 380 nm.

in the complex. The kinetics of the reaction corresponding to the transformation of spectrum A of Figure 1 into the one of Figure 2 were studied for several initial complex concentrations (from 5×10^{-5} to 2×10^{-4} mol·L^{-1}) and at several wavelengths between 450 and 750 nm. As examples, two experimental curves obtained for two different initial conditions of concentration and of dose and at two different wavelengths are reported in Figure 3, with their first- and second-order plottings. We can see that it is a first-order reaction at all wavelengths independent of the complex initial concentration, with a rate constant k_3 equal to 380 s^{-1}. The process corresponding to rate constant k_2 was not detected in the complex

Scheme I



free-radical decay, probably because of the low amount of disulfide anion.

Thus, daunorubicin semiquinone in the complex is not stabilized by the protein-like riboflavin semiquinone (Merrill et al., 1981). On the contrary, it is reduced to its hydroquinone state as shown by the absorption spectra of Figures 1 and 3. In anoxic aqueous solution, the noncomplexed semiquinone transient undergoes a disproportionation–comproportionation equilibrium (Houée-Levin et al., 1984)

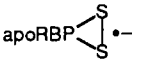
$$2DOS^{\bullet-} \rightleftharpoons DOS + DH_2OS,\ K_{eq} \sim 25$$

which can be observed easily by pulse radiolysis. In the presence of the protein another process takes place.

The kinetics of the glycosidic cleavage undergone by the hydroquinone drug in the complex was followed on the seconds time scale (same experimental conditions as above; results not shown). The mechanism of this cleavage seems to be the same as in aqueous solution, involving three first-order reactions with rate constants of the same order of magnitude as in aqueous solution (Houée-Levin et al., 1986).

Interpretation and Discussion

We propose kinetic Scheme I to explain the results. Two sites of the complex are reduced, the antibiotic which gives its semiquinone form and a disulfide bridge into its radical anion form noted



Since the complex concentration was 10-fold higher than the $COO^{\bullet-}$ one, the probability of two free radicals reacting with the same complex molecule is small. Thus, the two free-radical sites are created independently. These very simple results are markedly different from the ones reported by Faraggi et al. (1985) for the reduction of RBP by the same reductant: no second-order component is seen in these first-order processes, and no intermediate other than the drug semiquinone (and the disulfide anion) is seen. The presence of daunorubicin instead of riboflavin modifies the reaction mechanism.

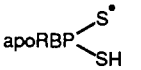
The second-electron reduction of the drug is a first-order reaction (k_3 = 380 s^{-1}), independent of the radical concentration in the range investigated and with no second-order component during more than three half-lives, in the limit of the experimental uncertainty. We propose it is an intramolecular reduction of the drug by an electron coming from a group belonging to the protein (reaction 3). Such intramolecular processes are observed as first-order transformations (Isied, 1984; Prutz et al., 1981, 1982) and with rate constants of the same order of magnitude as k_3 (Prutz et al., 1980; Kipke et al., 1988; Faraggi et al., 1988). Furthermore, we can estimate the stoichiometry of the transformation

$$(DOS^{\bullet-}, \text{apo RBP}) \rightarrow (DH_2OS, \text{apo RBP})$$

using the extinction coefficients of the corresponding forms free nonintercalated (Houée-Levin et al., 1984) and assuming that they do not change much with intercalation. For instance, the radical concentration corresponding to Figure 1 is roughly equal to 1.2 × 10^{-5} mol·L^{-1}, and the hydroquinone concentration corresponding to Figure 2 is equal to 10^{-5} mol·L^{-1}. Though these values are approximate, they suggest that the stoichiometry is not one of a dismutation process.

Finally, the daunorubicine hydroquinone loses its sugar moiety SOH to give 7-deoxy aglycon DH bound to the protein in a complex process symbolized by reaction 6.

The protein radical anion decay is interpreted by reactions 2 and 4. It is known that with small cyclic disulfide molecules such as dithiothreitol or lipoamide, the reduction of the disulfide bridge in the presence of formate is a chain reaction with a very complex mechanism (Elliot et al., 1984). The reaction mechanism of the reduction of protein disulfide bonds is poorly known. However, our present experimental results can be explained by a minimal set of reactions (reactions 2 and 4). Thus, we propose that the disulfide radical anion is in equilibrium with its cleaved form (reaction 2) noted



Preliminary experiments indicate that some protein dimer is formed by radiolysis reduction of the apoprotein (Favaudon, unpublished results). Thus, we propose that the thiyl radical in the protein disproportionates and/or dimerizes (reaction 4). The rate-limiting reaction is usually said to be the disulfide anion bond cleavage, corresponding to the first-order reaction with rate constant k_2 observed in pulse radiolysis.

Using kinetic Scheme I the following relations are found between the yields measured in γ-radiolysis irradiations for antibiotic reduction, $G(-DOS)$, and sulfhydryl group formation, $G(SH)$, both in the complex, and the yield of $COO^{\bullet-}$ formation:

$$G(COO^{\bullet-}) = 2G(-DOS) + G(SH) = 0.62\ \mu mol \cdot J^{-1} \quad (7)$$

$$G(-DOS)/G(SH) = k_1/2k'_1 \quad (8)$$

Experimentally we find for relation 7

$$2G(-DOS) + G(SH) = 0.63\ \mu mol \cdot J^{-1}$$

which is in very good agreement with the predicted value. If reaction 5 was replaced by the protein disproportionation, the

yield $G(-DOS)$ should have been doubled, which is in disagreement with experimental values. This shows that in steady-state irradiation conditions the oxidized protein is "repaired" by the reducing entities (reaction 5).

As for eq 8, it links the yields in γ-radiolysis and the rate constants of reaction of $COO^{\bullet-}$ radicals with both targets, assuming that k'_1 is the same for the disulfide group in the apoprotein alone as in the complex. The experimental values are

$$G(-DOS)/G(SH) = 8.8$$

and

$$k_1/2k_1 = 1.9$$

To explain this disagreement between both numerical values, we propose reaction 9, which is the reduction of daunorubicin

$$(DOS, \text{apo } RBP^{\bullet-}) \rightarrow (DOS^{\bullet-}, \text{apoRBP}) \qquad (9)$$

by a disulfide anion. The one-electron redox potential of the couple $SS/SS^{\bullet-}$ in this protein is not known. Similar redox potentials have been recently found equal to −1.6 V for small cyclic disulfides (Surdhar & Armstrong, 1987). If this potential has the same order of magnitude in the protein, reaction 9 is strongly energetically favored [$E^{\circ}(DOS/DOS^{\bullet-}) = -0.43$ V in aqueous solution (Houée-Levin et al., 1984)]. However, we do not know whether this reaction is intra- or intermolecular. Reaction 9 is in competition with the disulfide anion cleavage (reaction 2, with rate constant k_2), since sulfhydryl groups are formed by reduction of the complex.

Conclusions

The aim of this work was to provide better knowledge about the role of the protein, apoRBP, on the reduction mechanism of an antitumor drug, daunorubicin, intercalated in the active site of this protein. The following points have been shown.

(i) The one-electron reduction of the complex gives mostly daunorubicin semiquinone with a slight amount of disulfide anion radical. The mechanism of this first step is different from that of the one-electron reduction of RBP (Faraggi et al., 1985). No other group seems involved in this electron transfer. The active site of this protein has been said to be close to or at the surface of the protein (Becvar & Palmer, 1982), which is consistent with the nearly diffusion-controlled reaction of $COO^{\bullet-}$ radicals with the quinone buried in it.

(ii) The second-electron reduction mechanism of the drug is different whether the drug is in the protein or free in aqueous solution since the semiquinone form seems to be reduced by a protein residue to the hydroquinone state, by an intramolecular process. The reducing residue cannot be a disulfide radical anion, since, as we explained, it is unlikely that both free radicals are situated on the same complex molecule. Thus, another amino acid is implied in this reaction, which gets oxidized in this process. Few amino acids are able to react by electron and/or hydrogen atom transfer: tyrosine, tryptophan, cysteine, cystine, and perhaps histidine. Their one-electron redox potentials are reported in Table I. They seem to be independent of the nature of the side chain (Jovanovic et al., 1986). Considering their one-electron redox potentials, none of them is likely to donate an electron to the antibiotic. The source of the electron remains thus unknown.

The role of the driving force in intramolecular electron transfer is described by the Marcus' theory, summarized and reviewed in many texts [e.g., Marcus and Sutin (1985)]. The rate constant increases with the driving force, except in the so-called "inverted region" for which it is the reverse. In our case, few data are available to know whether this inverted region effect can be invoked in the explanation of this striking difference between the behavior of the protein toward daunorubicin and riboflavin semiquinones.

Table I: One-Electron Redox Potentials of the Amino Acids That Can Undergo Easily Electron Transfer

redox couple	E°_7 (V)	ref
Tyr-O•/Tyr-OH	0.85	Jovanovic et al., 1986
$Trp^{\bullet+}$/Trp	0.64	
$RS^{\bullet}$/RSH	~1.33[a]	Surdhar & Armstrong, 1987

[a] Measured for mercaptoacetic acid, β-mercaptoethanol, and penicillamine.

The rate constant of the electron transfer between two redox sites, at a given temperature, is said to be proportional to $\exp(-\beta d)$, where d is the distance between the closest atoms of each redox center and β is a factor interpreted in terms of electron tunneling through a potential barrier (Marcus & Sutin, 1985; Isied, 1984). For aromatic redox sites, a value of 12 nm^{-1} for β seems satisfactory (Marcus & Sutin, 1985). Using this value, a rate constant of 380 s^{-1} at ambient temperature would mean a separation distance between the two redox centers of ca. 5–7 Å. Unfortunately, the crystalline structure of this protein is not known, and no correlation between this distance and possible electron donors can be made.

(iii) Finally, the disulfide anion radical reduces the antibiotic probably by a relatively slow process since it is in competition with the disulfide anion cleavage ($k_2 = 10.5\ s^{-1}$). In this case, we do not know if this process is intra- or intermolecular.

We believe these results provide new information about the interaction of daunorubicin with a flavoprotein and perhaps a new mechanism of inactivation of proteins by anthracyclines, by intramolecular oxidation of a residue. Anthracyclines are easily bound to proteins, and such a mechanism might happen with other proteins.

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Registry No. DOS, 20830-81-3; RBP, 32384-98-8; DH_2OS, 73610-99-8; DH, 85404-45-1; COO^-, 2564-86-5.

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