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Interaction of adriamycin with cardiolipin-containing vesicles. Evidence of an embedded site for the dihydroanthraquinone moiety

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In the presence of cardiolipin-containing small unilamellar vesicles, the antitumor compound adriamycin loses its ability to catalyse the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase. The data strongly suggest that in the presence of cardiolipin the dihydroanthraquinone moiety is embedded in the phospholipid bilayer and thus inaccessible to the enzyme.

Adriamycin, an antibiotic of the anthracycline class, is a potent antineoplastic agent; however, its clinical use is limited due to clinical and histopathological evidence of cardiotoxicity [1]. The mechanism of anthracycline cardiac toxicity remains uncompletely understood; however, recent studies have suggested that the cardiotoxic effects of these agents may be related to the formation of semiquinone free radical intermediates in vivo [2] and/or to the association of adriamycin with cardiolipin [3]. Thus both cardiac sarcomes [4] and mitochondria [4,5] can reduce adriamycin to semiquinone, initiating a free-radical cascade and Doroshow et al. [6,7] have demonstrated that a component of mitochondrial NADH dehydrogenase actively reduces adriamycin. On the other hand it has been shown that the association of adriamycin with cardiolipin gives rise to an inactivation of the cytochrome *c* oxidase activity by exclusion of the enzyme from its cardiolipin essential environment [8].

The interactions of anthracyclines with cardiolipin-containing systems have been largely studied especially by Ruysschaert and co-workers and by Tritton, Sartorelli and co-workers. The first group reported the formation of a complex involving 2 mol adriamycin per mol cardiolipin. Using surface-potential measurements they have shown that this complex is stabilized by electrostatic interactions without penetration of the drug into the lipid lipophilic phase [9]. On the other hand, using fluorescence quenching measurements Karczmar and Tritton [10] suggested that the presence of small amounts of cardiolipin (3%) in a phosphatidylcholine matrix creates two types of binding environments for drug, one relatively exposed and the other more deeply buried in the membrane.

These reports led us to examine the ability of adriamycin to catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase, in the presence of cardiolipin-containing vesicles. In this paper, we report experiments showing that the presence of cardiolipin inhibits the ability of adriamycin to be reduced by the enzyme. This result together with other data strongly suggests that the dihydroanthraquinone moiety is embedded in the phospholipid bilayer

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Abbreviations: egg PC, L-α-phosphatidylcholine; SUV, small unilamellar vesicles; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

and thus inaccessible to the enzyme.

Purified adriamycin was kindly provided by Laboratoires Roger Bellon. Concentrations were determined by diluting stock solutions to approx. 10 μM and using $\varepsilon_{480} = 11\,500\ M^{-1}$ [11]. As anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just before use. Cytochrome *c* (type VI from horse heart), NADH (grade III), cardiac NADH dehydrogenase, L-α-phosphatidylcholine (from egg yolk type V-E), cardiolipin (from bovine heart) were purchased from Sigma Chemical Co. and superoxide dismutase from Behring. All other reagents were of the highest quality available and deionized bidistilled water was used throughout the experiments. Unless otherwise stated buffer solutions were 0.05 M Hepes (pH 7.2).

Absorption spectra were recorded on a Cary 219 spectrophotometer.

Cardiolipin and egg PC were sonicated, at room temperature, in Hepes buffer (pH 7.2) under N_2 atmosphere, for 10 min and setting 1.5 on a vibracell sonicator. In such conditions SUV are formed. The solutions currently used contained 1.25 mg/ml of cardiolipin and various amounts of egg PC ranging from 1.9 to 5 mg/ml. The molar ratio of cardiolipin to egg PC was thus varied from 1 : 8 to 1 : 3. SUV of pure egg PC were also prepared.

NADH dehydrogenase activity was determined at 25°C by modification of a method described previously [12,13] using cytochrome *c* as the electron acceptor. Adriamycin in the presence of various amounts of cardiolipin-containing vesicles of egg PC were assayed for their NADH-cytochrome *c* oxidoreductase activity by following cytochrome *c* reduction at 550 nm using the extinction coefficient for cytochrome *c* (reduced minus oxidized) of 19600. Unless otherwise stated the reaction mixture contained Hepes buffer (0.05 M, pH 7.2), cytochrome *c*, NADH, NADH dehydrogenase, adriamycin and cardiolipin-containing vesicles. The molar ratio of cardiolipin to adriamycin was varied from 0 : 1 to 4 : 1. The reaction was initiated by the addition of the enzyme. Enzymatic activity has been expressed in units, where 1 unit of activity is that amount of enzyme capable of reducing 1 μM of cytochrome *c* per min at pH 7.2 and 25°C under the reaction conditions outlined above. The production of superoxide anion in the experimental samples was calculated from the rate of cytochrome *c* reduction inhibited by superoxide dismutase (20 μg/ml).

The rate of oxygen consumption was determined at 25°C with a YSI 5331 oxygen monitoring system under the reaction conditions outlined above but without cytochrome *c*. The reaction was initiated by the addition of NADH to the reaction chamber through the access slot of the oxygen electrode plunger. The rate of oxygen consumption was calculated from a value of 256 μM for the total dissolved oxygen content of the reaction mixture.

The effect of increasing the concentration of adriamycin on the rate of superoxide formation by NADH dehydrogenase, as well as the rate of oxygen consumption was measured in the absence of SUV. As it can be seen in Fig. 1, the consumption of oxygen is increased in a dose-dependent fashion that appeared to follow saturation kinetics and, in the conditions used, the rate of oxygen consumption is a linear function of the concentration of adriamycin up to about 70 μM. In the following experiments we thus used a concentration of adriamycin lower than 70 μM; under these conditions the rate of oxygen consumption, as well as that of O_2^- formation, is proportional to the quantity of adriamycin which can act as a substrate for the enzyme (i.e. which can be reduced by the enzyme).

In order to check the modifications, if any, of the NADH, NADH dehydrogenase, cytochrome *c* system that could be due to cardiolipin containing SUV, the rate of reduction of cytochrome c^{3+} was measured (in the absence of adriamycin) in the presence of increasing concentration of cardiolipin-containing SUV. As it can be seen on Fig. 2, curve a, no modification was observed. This demonstrated that the enzyme is not inserted into the lipid matrix but remained free in the solution.

The ability of adriamycin to induce superoxide production was then measured in the presence of various amounts of cardiolipin-containing vesicles. This was performed at molar ratio of cardiolipin to egg PC varying from 1 : 8 to 1 : 3. The rate of superoxide production has been plotted as a function of the molar ratio of cardiolipin to adriamycin in Fig. 2, curve b. In that experiment the

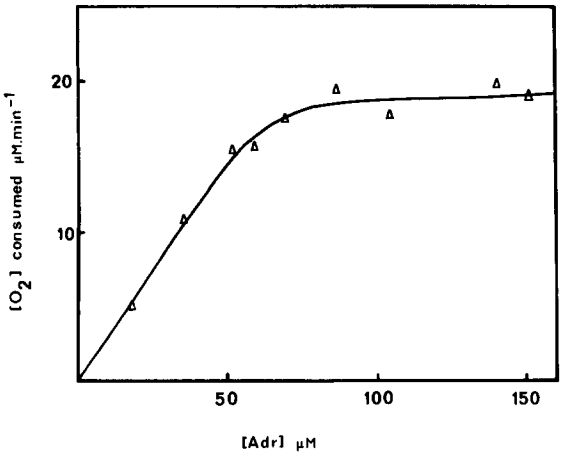

Fig. 1. Effect of adriamycin (Adr) concentration on oxygen consumption by NADH dehydrogenase. The reaction mixture contained Hepes buffer (0.05 M, pH 7.2), NADH (77 μM), NADH dehydrogenase (25 units/l) and the indicated amount of adriamycin.

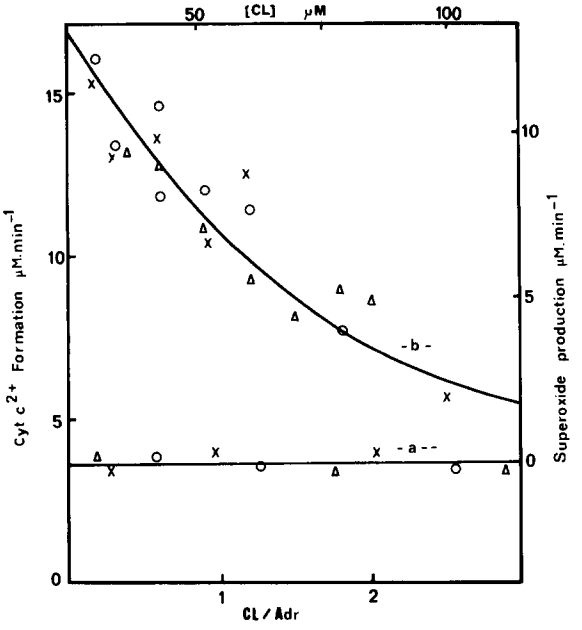

Fig. 2. Effect of cardiolipin-containing SUV on superoxide formation by NADH dehydrogenase in the absence (curve a) and in the presence of adriamycin 60 μM (curve b). The reaction mixture contained Hepes buffer (0.05 M, pH 7.2), NADH (81 μM), NADH dehydrogenase (3.7 units/l), cytochrome *c* (40 μM), 0 or 20 μg/ml superoxide dismutase and the indicated amount of cardiolipin-containing SUV. The molar ratio of cardiolipin to egg PC was 1:8 (△), 1:6 (○) and 1:3 (×). CL, cardiolipin; Adr, adriamycin.

concentration of adriamycin was kept constant at 60 μM. As it can be seen the rate of superoxide production decreased as the molar ratio of cardiolipin to adriamycin increased. Moreover, this result did not depend on the molar ratio of cardiolipin to egg PC ranging from 1 : 8 to 1 : 3.

In order to determine the rate of oxygen consumption, the same experiments were performed in the absence of cytochrome c^{3+}. As it can be seen on Fig. 3, analogous trend was observed i.e. a decrease of the rate of oxygen consumption when the molar ratio of cardiolipin to adriamycin increased. This result did not depend on the molar ratio of cardiolipin to egg PC ranging from 1 : 8 to 1 : 3. In all the foregoing experiments adriamycin was first added to the SUV and left to incubate for about one minute and then the various reactives were added. This incubation time value has been chosen after we had checked that the results were independent on it.

All these experiments have also been performed in the presence of pure egg PC vesicles and in that case no modification of the rate of superoxide production as well as oxygen consumption could be detected.

The foregoing data show that, though interaction with cardiolipin-containing SUV, adriamycin loses its ability to transfer electron from NADH to molecular oxygen, alternatively, adriamycin cannot be reduced anymore by the enzyme. Goor-

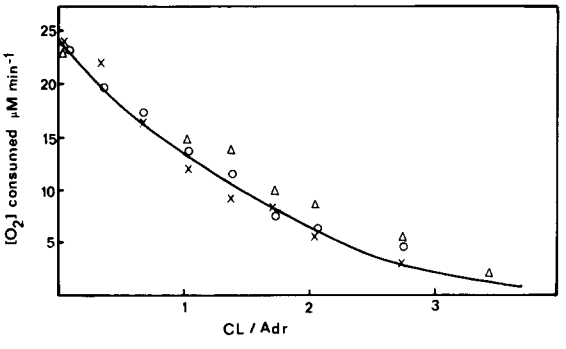

Fig. 3. Effect of cardiolipin-containing SUV on oxygen consumption by NADH dehydrogenase in the presence of adriamycin (60 μM). The reaction mixture contained Hepes buffer (0.05 M, pH 7.2), NADH (75 μM), NADH dehydrogenase (25 units/l), and the indicated amount of cardiolipin-containing SUV. The molar ratio of cardiolipin to egg PC was 1:8 (△), 1:6 (○), 1:3 (×). CL, cardiolipin; Adr, adriamycin.

maghtigh et al. [9] have demonstrated that one molecule of cardiolipin can bind two molecules of adriamycin; an electrostatic interaction between the protonated amino groups of the sugar residues and the ionized phosphate residues is the essential interaction responsible for the complex stabilization. In this complex the drug does not penetrate into the lipidic phase. At a 2:1 molar ratio of adriamycin to cardiolipin most of the molecules of drug are thus bound to the negatively charged phosphate with the dihydroanthraquinone moiety lying outside the bilayer. As it can be seen in Fig. 3, adriamycin is still able to be reduced by the enzyme since the consumption of oxygen is reduced to only about 80% of the initial value.

Our data show that a decrease of the molar ratio of adriamycin to cardiolipin gives rise to a decrease of the consumption of oxygen. This strongly suggests that another type of binding site of adriamycin to cardiolipin-containing SUV does exist in which the dihydroanthraquinone moiety is embedded in the phospholipid bilayer and thus inaccessible to the enzyme. These results compare with those of Lown et al. [14] on the adriamycin-DNA system, showing that DNA-intercalated adriamycin cannot be reduced and cannot undergo the redox cycling that allows the free drug to support the generation of superoxide.

Our data together with those of Goormaghtigh et al. [9] and those of Karczmar and Tritton [10] strongly suggest that in fact two types of binding can take place between adriamycin and cardiolipin containing SUV. The first type (I) involves the fixation of the α-amino group of the sugar residue of adriamycin to the ionized phosphate residue of cardiolipin with the dihydroanthraquinone moiety lying outside the bilayer (as it has been stated by Goormaghtigh et al. [9]). In that case adriamycin can be reduced by the enzyme as if it was free in the solution. The second type (II) involves also the fixation of adriamycin to cardiolipin through interaction or the α-amino group with the phosphate residue but, in addition, the dihydroanthraquinone is embedded in the bilayer. When the molar ratio of adriamycin to cardiolipin is high the binding site I is predominant but when this molar ratio decreases the binding site II then becomes predominant.

The last point we wish to emphasize is that since the cardiotoxicity of adriamycin seems to be related (i) to the formation of semiquinone free radical intermediate and (ii) to its association with cardiolipin, it should be kept in mind that, in fact, in the presence of cardiolipin-containing membrane, adriamycin is much less easily able to be reduced by NADH dehydrogenase and then to activate oxygen.

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