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MITOCHONDRIAL MEMBRANE MODIFICATIONS INDUCED BY ADRIAMYCIN-MEDIATED ELECTRON TRANSPORT

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Abstract—Adriamycin (ADM) was found to have a two-step mode of action on the cardiac mitochondrial membrane. (1) An interaction with cardiolipin (CL) resulted in the formation of an ADM–CL complex able to transfer electrons from NADH to cytochrome *c* (cyt.*c*) as well as coenzyme Q (CoQ). This complex formation stimulates an increased activity of NADH–CoQ oxidoreductase (complex I) and CoQ–cyt.*c* oxidoreductase (complex III). (2) Transfer of electrons through ADM resulted in the formation of a very strong complex between ADM and CL. This new complex is different and much stronger than the already known ADM–CL complex.

Adriamycin (NSC 123.127) (ADM), an anthracycline antitumor drug, is one of the most promising agents for the treatment of both acute leukemia and solid tumors [1]. Most of its side-effects are reversible. However, its clinical use is limited by the total dose of ADM that may be given [2]. Many reports suggest that ADM cardiotoxicity is related to the mitochondrial respiration [3–7] with only a slight effect on the coupling state [4, 7]. We previously demonstrated the existence of a strong association between ADM and cardiolipin (CL) [8–10], with an affinity comparable to that of the ADM–DNA nucleotide complex [11]. Since CL is a negatively charged phospholipid specific to the inner mitochondrial membrane [12, 13] and is an absolute requirement in the lipid environment of several mitochondrial enzymes [14–19], it seems likely that CL could play a role in the ADM cardiotoxicity. It was previously demonstrated that the ADM–CL complex modifies the Ca^{2+} transport [20] and the cytochrome *c* (cyt.*c*) oxidase activity in mitochondria [21].

We present here evidence that the formation of an ADM–CL complex stimulates an increased activity of NADH–CoQ oxidoreductase (complex I) and CoQ–cyt.*c* oxidoreductase (complex III).

Transfer of electrons through adriamycin induces the formation of a new very strong association between ADM and CL and a drastic modification of the mitochondrial membrane fluidity.

MATERIALS AND METHODS

Materials. Beef heart CL, complexes I and III, cyt. *c* from beef heart and NADH (grade III) were purchased from Sigma Chemical Co. (St. Louis, MO); CoQ was a Calbiochem product (Lucerne, Switzerland) and 1,6-diphenylhexatriene (DPH) an Aldrich product (Beerse, Belgium); ADM was generously supplied by the National Institute for Health (Bethesda, MD) and *N*-Ac-ADM was a gift of Prof. A. Trouet and Dr Baurin (Laboratoire de Chimie Physiologique, Universtité de Louvain, Belgium). All chemicals were of analytical grade and water was triple distilled. Fluorescence polarization measurements were performed on an Elscint MV 1a microviscosimeter. The microviscosimeter employed a mercury light source filtered at 365 nm and polarized by a Glan–Thompson polarizer. Emitted light with a wavelength longer than 418 nm only was used for polarization determination.

Methods. Mitochondria were extracted from beef heart according to the procedure of Smith [22] and were stored at −20° for several weeks without loss of activity. CoQ depletion by pentane extraction was carried out as described previously [23]. Complex I was assayed for its NADH–CoQ oxidoreductase activity by following NADH oxidation at 340 nm. Complex III was assayed for its CoQ–cyt.*c* oxidoreductase activity by following cyt.*c* reduction at 550 nm. A double-beam Shimatzu UV-190 spectrophotometer, thermostatized at 25°, was used. For complex I and III activity measurements, mitochondria were dissolved in Tris–HCl buffer (4×10^{-2} M, pH 7.5) containing 1% deoxycholate and 50 mM NaN_3.

Lipids were dissolved in ethanol. Organic solvent was evaporated under a nitrogen stream and the lipid film so obtained was further dried under vacuum for 3 h.

Multilamellar proteoliposomes were obtained by dispersing the dry lipid film with a purified enzyme suspension [24]. The proteoliposome suspension was not sonicated in order to avoid enzyme damages.

In fluorescence depolarization experiments, mitochondria (5 mg protein/6 ml) were added to a solution of ADM (10 mg/6 ml). To 3 ml of this suspension 35 mg of NADH were added. After 3 h, mitochondria were centrifuged 5 times. After the last centrifugation, mitochondria were resuspended

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in 6 ml of Tris–HCl buffer (4×10^{-2} M, pH 7.5) and DPH (0.6 μg DPH/ml mitochondrial suspension) was added from a tetrahydrofurane solution (0.6 mg/ml) and incubated for 2 h. The mitochondrial suspension so prepared was diluted 20 times with the same buffer. A blank prepared without DPH showed that, after this dilution, only DPH gave a measurable signal of fluorescence.

To test the formation of the new complex between ADM and CL, proteoliposomes were incubated with ADM (1.8×10^{-4} M) in the presence or absence of NADH (2×10^{-3} M) for 3 h. After incubation, the excesses of ADM and NADH were eliminated by centrifugation of the proteoliposomes (10 min, 800 *g*) followed by five washings. Both the electrostatic complex and the new complex between ADM and CL can be extracted by a $CHCl_3$ phase. In order to distinguish between the two types of binding, the electrostatically bound complex was first dissociated by $CaCl_2$ (6 M) [20]. Then, the new complex was extracted selectively in a $CHCl_3$ phase, whereas the free ADM molecules remained in the aqueous phase. The concn of ADM was assayed spectrophotometrically in both phases with corrections for the solvent and the $CaCl_2$ effect.

RESULTS

Effect of ADM and N-Ac-ADM on the activity of complexes I and III

The effect of increasing concns of ADM and *N*-Ac-ADM on the activity of complexes I and III is shown in Fig. 1(a) and (b). Both drugs increase the rate of electron transfer from NADH to CoQ and from CoQ to cyt.*c*. *N*-Ac-ADM, however, was less effective. As these two molecules differ in their capacity to bind CL [20], it was of interest to determine if the binding of ADM to the mitochondrial CL could play a role in this process. For this purpose, mitochondria were incubated for 10 min in the presence of ADM and *N*-Ac-ADM at various concns. The suspension was centrifuged twice to eliminate the unbound drug. The resulting pellet was resuspended and assayed for complex I activity [Fig. 1(c)]. Clearly, the association between ADM and CL allows the maintenance of the effect observed earlier. On the contrary, with *N*-Ac-ADM, no increase of complex I activity was obtained. It is likely that the increase of activity observed in Fig. 1(a) and (b) is due to the anthraquinone residue of the ADM and *N*-Ac-ADM molecules. Indeed, the anthraquinone residue is able to accept electrons [25, 26]. This hypothesis has already been proposed to explain the effect of ADM on NADPH–cytochrome P-450 reductase in microsomes [27, 28]. In the present case, it can be suggested that ADM binds to CL to form a ubiquinone-like complex. Since complexes I and III [29, 30] as well as ubiquinone [31–33] diffuse in the phospholipid bilayer and, since their interaction is mediated by their lateral diffusion rate [29, 30], an increase in the number of electron carriers could explain the increase in the rate of electron transport. Whether the ubiquinone-like ADM–CL complex interacts directly with complexes I and III [reaction (2)] or whether it interacts with CoQ only [reaction (1)] was investigated by using CoQ-depleted mitochondria.

$$\text{Complex I} \rightarrow \text{CoQ} \rightarrow \text{ADM–CL} \rightarrow \text{complex III} \quad (1)$$

$$\text{Complex I} \begin{matrix} \nearrow \text{ADM–CL} \searrow \\ \searrow \quad\quad \nearrow \\ \quad \text{CoQ} \end{matrix} \text{complex III} \quad (2)$$

CoQ-depleted mitochondria have a very low complex III activity (less than 10% of the initial activity). The activity is recovered (Fig. 2) by dispersion of CoQ_6 (10^{-4} M) in the medium. The most efficient activation obtained with adriamycin (10^{-4} M) allows us to conclude that electrons follow the pathway described by equation (2).

Effect of the ADM-mediated electron transport

ADM structure. The u.v.–visible absorption spectrum of 1,4-dihydroxy-anthraquinones and their

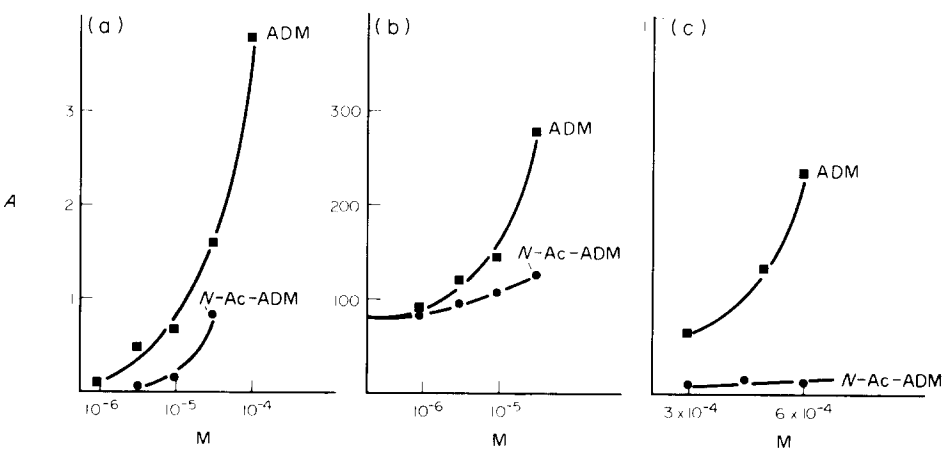

Fig. 1. Effect of adriamycin (ADM) (■) and *N*-Ac-adriamycin (*N*-Ac-ADM) (●) on mitochondrial enzyme activity (*A*). (a) NADH dehydrogenase (μmoles NADH oxidized/min/mg protein). (b) Cytochrome *c* (cyt.*c*) reductase (μmoles cyt.*c* reduced/min/mg protein). (c) NADH dehydrogenase (μmoles NADH oxidized/min/mg protein). In this experiment, unbounded ADM and *N*-Ac-ADM were eliminated by centrifugation (10 min, 800 *g*).

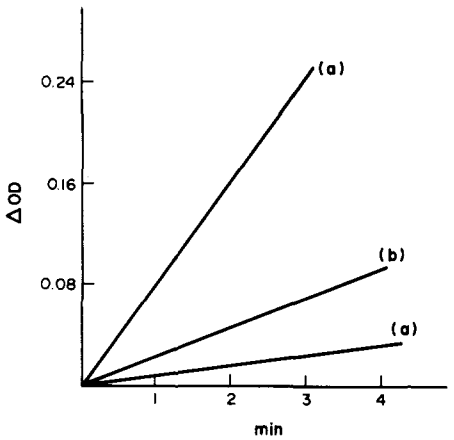

Fig. 2. Restoration of cytochrome *c* (cyt.*c*) reductase in pentane-extracted mitochondria. O.D. change (ΔOD) of cyt.*c* at 550 nm as a function of time is shown. (a) Activity of pentane-extracted mitochondria (0.5 mg protein/ml). (b) The same as (a), but in the presence of CoQ (10^{-4} M). (c) The same as (a), but in the presence of ADM (10^{-4} M).

derivatives have been assigned previously to π–π^* transitions of the quinoid system [34]. Fig. 3 illustrates the modifications of the absorption spectra of ADM bound to mitochondria. Spectrum (a) is obtained in the absence of the electron source (NADH). No influence of the incubation time has been detected . This spectrum is similar to that of unbound ADM. In the presence of NADH but in the absence of cyt.*c*, ADM acts as an electron acceptor. Its spectrum [Fig. 3(b)] is not modified for incubation times between 10 min and 3 h. If cyt.*c* is added within 10 min, the modification observed of spectrum (b) is quite reversible and the spectrum of Fig. 3(a) is obtained [spectrum (c)]. However, spectrum (d), obtained after an incubation time of 3 h before addition of cyt.*c*, shows an irreversible process: the comparison of spectra (a) and (d) shows that the quinone moiety of the ADM molecule is slowly and irreversibly modified when reduced by electron transfer from NADH. Sinha has recently shown that ADM, in the presence of reducing agents, binds covalently to nucleic acids and proteins [35–37].

Daunomycin, an ADM analog, was shown to form a new and undissociable complex with CL in conjunction with lipid peroxidation [38]. The authors suggest the formation of a covalent linkage. This possibility that ADM, reduced by the electron flow, forms a new complex with CL was investigated on functionally active proteoliposomes containing only purified CL, complex I and complex III. Results reported in Table 1 indicate clearly that the electron flow through ADM enhanced strikingly the formation of a new complex with CL, much stronger than the so-called electrostatic complex for which an association constant of 1.8×10^6 l/mole has been determined [9].

Membrane structures. A possible way to evaluate a general modification of the membrane properties consists in measuring the fluorescence depolarization (*P*) of a dye embedded in the membrane. An increase in *P* corresponds to a diminution in the lipid fluidity. *P* is easily correlated to the membrane viscosity [39–41]. It is well known that DPH is deeply buried into the hydrocarbon chains. Indeed, the *P* value measured reflects a rigid environment (Table 2). Incubation in the presence of NADH decreases the fluidity of the membrane. The loss of mobility of DPH must be correlated to a modification of its environment since the latter was added at the end of the reaction [42]. This may be due to the strong binding of ADM to CL and likely to lipid peroxidation due to the electron flow through ADM (already observed in other membrane systems [43–

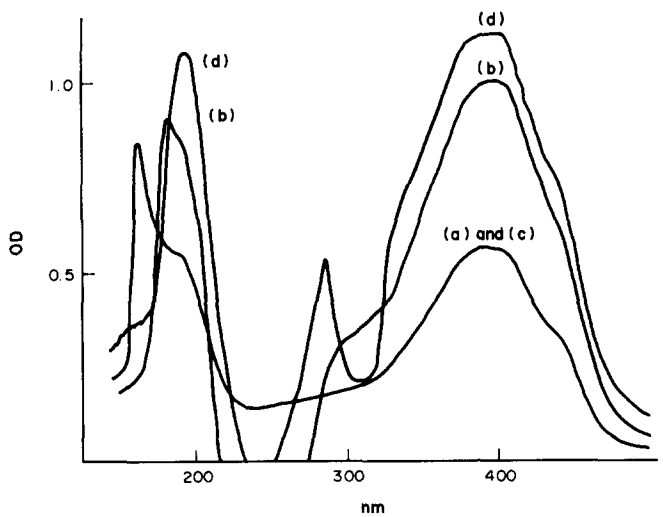

Fig. 3. U.v.–visible spectra of adriamycin (10^{-4} M). (a) In the presence of mitochondria (0.2 mg protein/ml). No spectral change was observed, even after 3 h of incubation. (b) In the presence of mitochondria (0.2 mg protein/ml) + NADH (1.66 mM). No spectral change was observed between 10 min and 3 h of incubation in the presence of NADH. (c) In the presence of mitochondria (0.2 mg protein/ml) + NADH (1.66 mM). After 10 min of incubation, mitochondria were centrifuged to eliminate NADH and cytochrome *c* (0.7 mg/ml) was added. (d) The same as in (c), but the incubation time in the presence of NADH was 3 h.

Table 1. Formation of a new complex between ADM and CL

Proteoliposomes + ADM	Proteoliposomes + ADM + NADH	ADM
0.1 ± 0.05	33 ± 5	% of ADM bound in the new complex ($CHCl_3$ phase)
95 ± 10	60 ± 8	% of ADM electrostatically bound (aqueous phase)

Multilamellar proteoliposomes were obtained by dispersing the lipid film (CL) with Tris–HCl buffer (pH 7.5) containing the purified enzymes (complexes I and III). Proteoliposomes (1.8 mg CL/ml + 0.5 mg complex I or III/ml) were incubated for 3 hr with ADM (1.8×10^{-4} M) in the presence or absence of NADH (2×10^{-4} M). The proteoliposomes were centrifuged (800 *g*, 10 min) and assayed for electrostatically bound ADM or for ADM bound in the new complex. Data for each situation are the means from three experiments.

Table 2. Membrane fluidity change observed after electron transport through ADM

	P_{DPH}
Mitochondria + ADM incubated without NADH	0.280
Mitochondria + ADM incubated with NADH	0.330

Mitochondria (5 mg protein/6 ml) were incubated with ADM (2.9×10^{-3} M). To 3 ml of this suspension, 35 mg of NADH were added. After 3 hr, mitochondria were centrifuged and washed with Tris–HCl buffer (pH 7.5). Mitochondria were then incubated with 20 μl of a tetrahydrofurane DPH solution (0.6 mg/ml) for 2 hr. After incubation, this solution was diluted 20 times. Under these conditions, only DPH gave a detectable signal of polarization (*P*). *P* is defined as:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation beam. All fluorescence polarization measurements were made at 25°.

47]) and subsequent polymerization of peroxidized CL [48, 49].

DISCUSSION

Two kinds of ADM cardiotoxicity are recognized. (a) The acute toxicity which occurs a few minutes after administration of the drug. It is characterized by the occurrence of cardiac arrhythmias and non-specific electrocardiographic changes [50, 51]. It was related to alterations in the Ca^{2+} transport [52–54]. (b) The irreversible congestive heart failure which occurs after the cumulative dose has been reached [50, 51, 55].

It is striking that we have pointed out here that ADM binds to CL in a two-step process.

In the first one, it simply binds electrostatically to CL [8–10]. The predictable consequences of this binding are: (a) An increase in the rate of the NADH oxidation, even in the absence of electron acceptors. The subsequent decrease in the $NADH/NAD^+$ ratio would therefore increase the plasma Ca^{2+} concn and decrease the Ca^{2+} concn inside the mitochondria as demonstrated by Lehninger *et al.* [56]. (b) An inhibition of the CL-mediated Ca^{2+} transport [20]. (c) An inhibition of cyt.*c* oxidase [21]. The oxido-redox state of the different elements of the respiratory chain in the presence of ADM was measured by Gosalvez *et al.* [7]. They were indeed characteristic of an inhibition of cyt.*c* oxidase associated with a high activity of complexes I and III.

In the second step, ADM is chemically modified and forms a new complex with CL which is undissociable at high ionic strength. Moreover, as demonstrated in other electron transport systems, it is likely that ADM enhances lipid peroxidation [45–47]. The consequence of the observed rigidification of the membrane is a decrease in the rate of electron transport [29, 30]. Peroxidation of lipids can induce damaging changes in the properties of the mitochondrial membrane [57] and could explain the obervation that ADM affects the respiratory control when this is measured after a certain delay [58]. In a previous work, Iwamoto supposed that ADM was an inhibitor of complex I since the electron flow was inhibited between NADH and O_2 [59].

This work and another [21] indicate that, on the contrary, complex I is not inhibited by ADM whereas cyt.*c* oxidase is well blocked in the concn range used in another work [57]. Indeed, the recommended pretreatment with CoQ_{10} [60, 61] failed in many cases to lower cardiac toxicity [62].

To conclude, the results described here support the hypothesis that CL could be a target for ADM. Indeed, its association with CL is of the same order or magnitude as its association with DNA [11]. The complexation of ADM maintains it in the neighbourhood of complexes I and III in a position which favours electron transport and further chemical modification of the molecule itself and of the membrane. *N*-Ac-ADM, which does not complex CL does not induce cardiomyopathies either [63]. Moreover, in a series of ADM derivatives, it is easier to correlate their cardiotoxic effect to their affinity for CL [12] than to their lipophilicity [64].

It should be important to study in more details the phenomena described in the present paper. The demonstration of a peroxidation induced by the electron flow through ADM and of a subsequent covalent linkage formation between the different components

of the mitochondrial membrane and ADM should contribute to the understanding of ADM-induced cardiac toxicity.

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