The mitochondrial phosphate carrier reconstituted in liposomes is inhibited by doxorubicin

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The phosphate carrier has been isolated from beef heart mitochondria in the presence of cardiolipin and reconstituted in asolectin vesicles. It has been found that 100 μM doxorubicin and 100 μM Br-daunomycin inhibit the unidirectional phosphate uptake in the reconstituted liposomes to the same extent as *N*-ethylmaleimide. The inhibition by Br-daunomycin is not due to covalent interaction with the carrier. The specific interaction between doxorubicin and cardiolipin is responsible for the inhibition of the phosphate carrier. Br-daunomycin interacts with 3 mitochondrial proteins of apparent M_r ~45000, ~35000 and ~30000.

Doxorubicin *Phosphate carrier* *Proteoliposome* *Br-daunomycin* *Mitochondria*

1. INTRODUCTION

The P_i-carrier catalyzes the electroneutral transport of P_i through the inner mitochondrial membrane (review [1]). The transport is inhibited by SH-reagents like NEM, mersalyl and *p*-chloromercuribenzoate. The carrier has been isolated using non-ionic detergents and chromatography on hydroxylapatite/celite [2] and on mersalyl-ultrogel [3]. The purified fraction contains 4–5 protein bands having M_r ranging from 34500 to 30000 [4]. Purification of the carrier to homogeneity has been achieved by using an organomercurial agarose column [5]. It has also been demonstrated that the P_i-carrier has an essential requirement for cardiolipin [6]. Cardiolipin is necessary for full activity of the carrier and to prevent its inactivation during the extraction from mitochondria with Triton X-114.

Abbreviations: P_i, inorganic phosphate; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine

The anthracycline antibiotic doxorubicin (fig.1) apparently segregates membrane cardiolipin in a separate phase, making it inaccessible to cytochrome *c* oxidase incorporated into cardiolipin-containing liposomes [7]. Doxorubicin forms specific complexes with negatively charged phospholipids [8]. The association constant of the complex doxorubicin–cardiolipin has been calculated to be 1.6×10^6 M^{-1}.

Here, the effect of doxorubicin and Br-daunomycin on the transport activity of the P_i-carrier reconstituted in asolectin liposomes has been investigated. Both drugs inhibit the unidirectional P_i-uptake to the same extent as NEM.

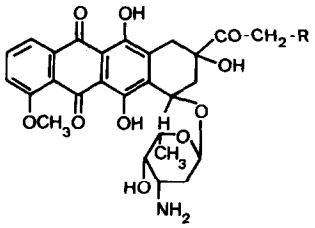

Fig.1. Molecular structure of doxorubicin and Br-daunomycin.

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2. MATERIALS AND METHODS

Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad (Richmond CA); Celite 535 (Johns Meville) from Roth (Karlsruhe); cardiolipin from P-L Biochemicals (Milwaukee IL); soybean asolectin from Associated Concentrates (New York NY); Triton X-114, Amberlite XAD-2 and Dowex AG 1-X8 from Fluka AG (Buchs); NEM from Sigma (St Louis MO); mersalyl from Mann Res. Labs (New York NY); doxorubicin and Br-daunomycin were generous gifts of Farmitalia-Carlo Erba (Milano).

Beef heart mitochondria were prepared as in [9], and stored frozen at −80°C. They were solubilized at 20 mg/ml in a buffer containing 20 mM KCl, 10 mM Tris, 10 mM Pipes, 1 mM EDTA, 1 mg cardiolipin/ml, 2.5% Triton X-114 and enough KOH to bring the pH to 6.5, using the procedure in [10] with slight modifications. After 30 min the suspension was centrifuged for 30 min at 100000 × *g*. The supernatant was adsorbed on a hydroxylapatite/celite (1:1, w/w) column (Pasteur pipette, containing ~600 mg dry material) and eluted with 1 ml detergent-free extraction buffer diluted 1:1 (v/v) with doubly distilled water. The pH was adjusted to 8 with KOH. Asolectin liposomes were prepared essentially as in [2], in a buffer containing 50 mM KCl, 20 mM Tris, 1 mM EDTA and enough HEPES to raise the pH to 8 at 2°C. 100 μl of the column eluate were mixed with 1 ml sonicated asolectin, frozen in liquid N_2, thawed in water and sonicated for 20 s. Triton X-114 was removed using Amberlite XAD-2 beads in a batch system for 90 min at 2°C. The unidirectional P_i-uptake was started by the addition of aliquots of HCl adequate to adjust the external pH from 8 to 6.5, and of 1 mM KP_i, to 250 μl of reconstituted liposomes [11]. The reaction was stopped by the addition of mersalyl. Free P_i was removed as in [2]. The column eluate was extracted with 1 ml chloroform and 150 μl of the water phase were analyzed colorimetrically for P_i [12]. All operations were performed at 5°C. Protein was determined by a biuret procedure [13] or as in [14] in the presence of 0.5% sodium dodecylsulfate. Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed as in [15].

3. RESULTS AND DISCUSSION

Table 1 shows the P_i-uptake mediated by the P_i-carrier in reconstituted asolectin liposomes. Asolectin contains up to ~25% of the total lipid P_i as cardiolipin, an amount which is adequate for full stimulation of P_i-uptake. The uptake shows a rapid initial phase, which reaches saturation within 2 min under the experimental conditions (not shown). The uptake is limited by the dissipation of the pH gradient, owing to the electroneutral nature of the P_i-carrier (P_i/H^+ symport or P_i/OH^- antiport). Addition of 10 μg NEM/mg protein to mitochondria prior to their solubilization inhibits the reconstituted P_i-carrier. If added after reconstitution, the effect of NEM becomes less evident, possibly due to the higher lipid to protein ratio in liposomes. Mersalyl was also able to inhibit the P_i-carrier, albeit at higher concentrations than in intact mitochondria. From these inhibition experiments it may be concluded that the orientation of the P_i-carrier in the reconstituted liposomes is uniform or, alternatively, that the essential SH-groups are accessible from both sides of the carrier. Table 1 shows that 10 μM doxorubicin inhibit the uptake of P_i to the same extent as NEM does. Since it has been shown that doxorubicin forms stable complexes with cardiolipin [8], and since the P_i transport activity is very sensitive to the presence of cardiolipin in the immediate vicinity of

Table 1

Inhibition of the unidirectional P_i-uptake mediated by the P_i-carrier reconstituted in asolectin liposomes

Addition	P_i-uptake ($\mu mol \cdot 15\ s^{-1} \cdot mg\ prot.^{-1}$)
None	39
NEM (10 μg/μg protein)	12
Mersalyl (100 μg/μg protein)	13
Doxorubicin (100 μM)	8
Br-daunomycin (100 μM)	12
NEM (10 μg/mg protein)[a]	10

[a] NEM was added to the mitochondria prior to solubilization

The uptake was stopped after 15 s; experimental conditions were as in section 2

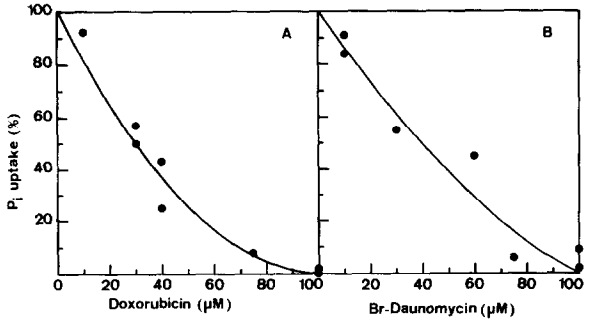

Fig.2. Concentration-dependent inhibition of the P_i-uptake in reconstituted liposomes by doxorubicin (A) and Br-daunomycin (B). The inhibitors were added to the liposomes immediately prior to starting the uptake reaction, which was stopped after 15 s; conditions are described in section 2.

the P_i-carrier, it is very probable that this inhibition is due to the binding of cardiolipin by doxorubicin. The possibility that the inhibition by doxorubicin reflects the binding of the drug to the P_i-carrier itself has been tested using the highly reactive brominated doxorubicin analogue Br-daunomycin (fig.3). When added to the reconstituted liposomes, the analogue inhibits the uptake of P_i to the same extent as doxorubicin (table 1, fig.2). However, colorimetric tests indicate that the P_i-carrier isolated from mitochondria treated with Br-daunomycin is free of the inhibitor, and is fully active after reconstitution in liposomes. In addition, it can still be fully inhibited by doxorubicin, Br-daunomycin, NEM or mersalyl (not shown). This supports the tentative conclusion reached before that doxorubicin and Br-daunomycin react specifically with cardiolipin molecules essential for the P_i-carrier.

It is known that the P_i-carrier has a pronounced hydrophobic character, and is one of the few proteins which binds the hydrophobic probe TID in the inner mitochondrial membrane (unpublished). It would thus not be surprising if it would form aggregates and lose activity, when solubilized in the absence of cardiolipin. A possible role of cardiolipin could thus be the maintenance of the active carrier structure, which has been proposed to be a dimer [16]. It may also be noted here that the

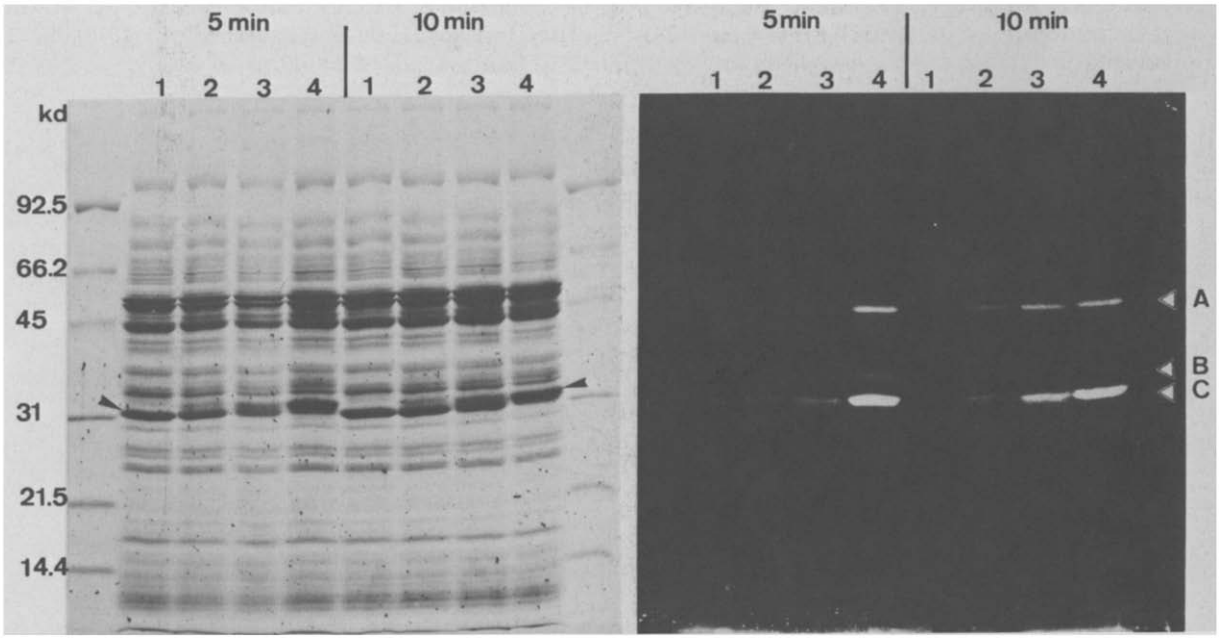

Fig.3. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of mitochondria incubated with increasing amounts of Br-daunomycin (50, 100, 200, 300 μM, respectively, lanes 1–4) for 5, 10 min at room temperature in isoosmotic medium at pH 7.4. Left panel: Coomassie blue stain. Right panel: fluorography. The labeled bands have apparent M_r of ~45000 (A), ~35000 (B) and ~30000 (C). The ADP/ATP translocator is labeled by the arrows.

P_i-carrier is a basic protein (pI ≃ 8, unpublished). Its affinity for cardiolipin could thus be explained by the formation of ion pairs between positively charged amino groups on the carrier molecules and negatively charged phosphate residues of cardiolipin.

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