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The Effects of Cytochalasin-B on the Membranes of Enzymatically Active Mitochondria*

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Abstract

Cytochalasin-B, one of a series of structurally related metabolites obtained from the mold *Helminthosporium dematioideum*, has been shown to alter several partial reaction parameters associated with mitochondrial oxidative phosphorylation. State-3 (active) respiratory rates, respiratory control ratios and overall ATPase activity were all inhibited by cytochalasin-B at concentrations between 0.2 and 1.0 mM. However, the efficiency of coupled oxidative phosphorylation, evaluated by the ADP/O ratio, was not significantly affected. Therefore, the metabolite does not appear to act at enzymic loci directly affiliated with the coupling mechanism of oxidative phosphorylation. The mode of action of cytochalasin-B may be conceived as restricting diffusion by means of macromolecular conformational changes occurring within the mitochondrial membranes. Electron micrographs of paired, 10 min mitochondrial incubations, in the presence and absence of 1.0 mM cytochalasin-B, clearly display a structural alteration within these organelles, such that upon the binding of cytochalasin-B the matrix area decreases and the inner membrane cristae develop condensed, tubular distortions.

Introduction

The structurally related series of fungal metabolites known as cytochalasins have displayed a number of confusing but fascinating biological affects on whole cell systems [1, 2, 3]. Among the principal

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observations made on cells treated with cytochalasins have been alterations in gross morphology and patterns of cell movement, in addition to inhibition of metabolite transport.

Controversy exists [4] as to whether the direct site of action of the cytochalasins are microfilament structures located within the cytosol matrix in aposition with the cell membrane [5], or alternatively, can be identified with a component integral to the plasma membrane itself [6]. Proponents of the former hypothesis [7, 8] present evidence for a host of inhibitory cytokinetic and morphogenetic changes in diverse cell populations upon exposure to one of the structural homologs, cytochalasin-B. More recent studies favor the latter theory [9, 10] by demonstrating cytochalasin-B's ability, in low concentration, to inhibit the transport of sugar derivatives across the plasma membrane. Such investigations, which have been extended to include inhibition of nucleoside transport [11, 12], support the proposal that cytochalasin-B acts on the plasma membrane itself, perhaps by specifically inhibiting the functioning of a membrane-affiliated carrier moiety requisite for metabolite translocation.

The present studies demonstrate that the effects of cytochalasin-B are not limited to the plasma membrane of intact cells, but may now be extended to include those enzymatically active intracellular membranes of isolated mitochondria. The data indicate that cytochalasin-B elicits a perturbation of several partial reactions and processes affiliated with oxidative phosphorylation.

Cytochalasin-B clearly inhibits the rate of oxygen consumption during State-3 respiration, lowers the respiratory control ratio, and decreases the rate of ATPase activity in both the presence or absence of uncoupling concentrations of 2,4-dinitrophenol. However, cytochalasin-B does not appreciably alter the efficiency of coupled oxidative phosphorylation as measured by ADP/O ratios.

Our preliminary results support the view that the primary locus of action of cytochalasin-B would be some transport function manifested by the membrane systems of mitochondria, and not, directly, the mechanism involved with the coupling of oxidative phosphorylation. It should be noted that microfilament structures, as variously described in the literature, are not associated with isolated mitochondria, and thus the processes of oxidative phosphorylation inhibited by cytochalasin-B need not depend on the presence of microfilaments [13].

Biochemical analysis of cytochalasin-B on mitochondrial oxidative phosphorylation was followed by correlative transmission electron microscopy. A comparison of isolated mitochondrial structure both in the presence and absence of metabolically effective concentrations of cytochalasin-B display a very discernable perturbation in gross mitochondrial morphology when the metabolite is included in the incubation medium.

Methods

Long-Evans rat liver mitochondria were prepared, resuspended, and assayed by methods previously reported [14], employing a YSI Model 53 oxygen monitor. Respiratory kinetics and the indirect evaluation of oxidative phosphorylation efficiency were obtained by the graphic method of Chance and Williams [15]. The kinetics of mitochondrially-catalyzed ATPase activity were analyzed in triplicate via phosphate release, according to the assay method of Fiske and SubbaRow [16] on a Varian Model 635 spectrophotometer.

ATP, ADP (Grade I) and oligomycin were from Sigma, DMSO from Fisher, and cytochalasin-B from Aldrich. High specific-resistance deionized water was employed throughout the work.

Cytochalasin-B was made up as a DMSO stock. Aliquots yielding final concentrations between approximately 0.2 or 0.33 mM (100-167 μg/ml) and 1.0 mM (500 μg/ml) were used for each oxidative phosphorylation or ATPase incubation. The controlled temperature for all enzymatic studies was 28°.

For electron microscopic evaluation, the mitochondria were preincubated with stirring for 10 min at 24°C prior to fixation in a medium consisting of: 0.05 ml mitochondria resuspended in 0.25 M Sucrose *plus* 1 mM EDTA; 5 mM $MgCl_3$; 250 mM Sucrose; and 10 mM phosphate buffer, pH 7.4. Two sets of incubations were performed. Both contained, in addition to the above medium with or without cytochalasin-B, a final concentration of 5% DMSO. For the incubation in the presence of cytochalasin-B, the metabolite was added as the DMSO solution to a final concentration of 1 mM. Immediate fixation was carried out in 1% glutaraldehyde (Fisher, Biological Grade, 50%) at room temperature for 1 h, followed by continued fixation in the cold overnight. After fixation, both sets of incubations were stirred and gently spun down through a brief phosphate buffer rinse, then post-fixed in 1% OsO_4 (in 0.1 M phosphate buffer pH 7.4) for 1 hr at room temperature. The preparation was dehydrated in dry ethanol by infinite dilution, and infiltrated and embedded in Epon 812 (Shell Corp). The thin sections were stained with uranyl acetate and lead citrate (in phosphate buffer). At no time was the fixed mitochondrial preparation tightly pelleted by centrifugation. A Phillips 300 instrument was employed for these electron micrograph scans.

Results and Discussion

Freshly prepared mitochondria were examined polarographically for their ability to display coupled oxidative phosphorylation under conditions where either cytochalasin-B was added together with ADP to

mitochondria in suspension, or where the mitochondria were permitted a 5 min exposure to cytochalasin-B prior to ADP addition. The data from several liver preparations, and the incubation conditions, are given in Tables I and II. These incubations proceeded in the presence of final DMSO concentrations of 6.67%. True control runs in the absence of DMSO did not elicit a substantial difference from those where DMSO was present, insofar as the enzymic parameters of oxidative phosphorylation measured here.

Thus, the following data were obtained for true control incubations in the absence of DMSO, and may be compared with those in Tables I and II.

TABLE I. Effects of Cytochalasin-B on some Parameters of Coupled Oxidative Phosphorylation

Parameter	Additions: 250 μM ADP	Additions: 250 μM ADP plus Cytochalasin-B	% Inhibition or Decrease
A. With 0.33 mM Cytochalasin-B			
1. Respiratory Control Ratio	3.34	2.10	37.13
2. ADP/O Ratio	1.82	1.68	7.69
3. State-3 kinetics (n atoms 0/min/mg protein)	86.67	69.51	19.79
B. With 1.0 mM Cytochalasin-B			
1. Respiratory Control Ratio	4.45	2.47	41.48
2. ADP/O Ratio	1.55	1.35	12.90
3. State-3 kinetics (n atoms 0/min/mg protein)	94.19	55.12	44.49

Conditions for Table I: Medium contained 10 mM phosphate buffer, pH 7.4; 100 mM sucrose; 5 mM $MgCl_2$; 2.5 mM succinate; 2.0 nM rotenone; 5% DMSO. Total volume = 3.0 ml. Cytochalasin-B added as DMSO solution, yielding 6.67% final DMSO. Data for A are mean values of 3 mitochondrial preparations, 4.05 mg protein/incubation. Data for B are mean values of 2 mitochondrial preparations, 3.39 mg protein/incubation.

(A) Without preincubation: Respiratory Control ratio, 3.54; ADP/O ratio, 1.86; State-3 kinetics, 83.05×10^{-3} μ atoms 0/min/mg protein.

(B) With 5 min preincubation: Respiratory Control ratio, 2.83; ADP/O ratio, 1.63; State-3 kinetics, 103.53×10^{-3} μ atoms 0/min/mg protein.

Clearly, those functions of oxidative phosphorylation affiliated with the rate of electron transfer along the respiratory chain are most

significantly affected by the presence of cytochalasin-B, while the coupling mechanism itself remains relatively unchanged with respect to phosphorylation efficiency.

The data for respiratory control are particularly interesting in this regard. Without cytochalasin-B pretreatment, the mitochondrial respiratory control appears more severely reduced than upon preconditioning the organelles for 5 min prior to ADP addition. Inasmuch as the respiratory control ratio measures comparative electron transfer kinetics during and subsequent to coupled oxidative phosphorylation, the result of the immediate exposure of already-present mitochondria to cytochalasin-B may be a manifestation of membrane structure perturbation—a "pulse" effect—affording an instantaneous minimum in enzymatic activity elicited by the membranes under these conditions. The 5 min preincubation with cytochalasin-B would thus allow the structurally altered enzymatically-active membrane sufficient "relaxation time" to establish a comparatively more stable and more rapid steady-state. Nonetheless, both incubation conditions yield overall inhibition of the respiratory control ratio in the presence of the metabolite.

TABLE II. Effect of 5 min Preincubation in 5% DMSO ± 0.33 mM Cytochalasin-B

Parameter	Preincubation Conditions Prior to 250 μM ADP Addition		% Inhibition or Decrease
	With DMSO	With DMSO + Cytochalasin-B	
1. Respiratory Control Ratio	3.00	2.44	18.70
2. ADP/O Ratio	1.55	1.42	8.39
3. State-3 kinetics (n atoms 0/min/mg protein)	101.92	81.53	20.00

Conditions for Table II: Conditions as for Table I. Data are mean values of 4 mitochondrial preparations, 4.52 mg protein/incubation.

The other parameters given in Tables I and II are functions of coupled oxidative phosphorylation only, and in this regard there is complete consistency as to cytochalasin-B-effected activity decrease *versus* control, whether or not the mitochondria are pre-exposed to the fungal metabolite prior to triggering respiratory chain-linked phosphorylation.

Cytochalasin-B also demonstrated a significant inhibition of ATPase activity. Tables III and IV present these results in pairs, under a variety of already established conditions for mitochondrial ATPase assay.

TABLE III. 0.2 mM Cytochalasin-B-effected ATPase Inhibition

Conditions	Pi released nmoles/mg protein/ml incub.			% Inhibition of Control			Mean % Inhibition
	Time Interval (min)			Time Interval (min)			
	5	10	15	5	10	15	
A. No Preincubation							
1. DMSO (as control)	150.9	309.6	463.7	–	–	–	–
2. DMSO + Cytochalasin-B	138.5	228.9	414.8	8.2	26.1	10.5	14.9
3. DMSO + 2,4-DNP + (as control)	607.5	1081.2	1404.5	–	–	–	–
4. DMSO + 2,4-DNP + Cytochalasin-B	514.8	914.4	1237.2	15.3	15.4	11.9	14.9
5. DMSO + OLIGO + 2,4-DNP (as control)	228.6*	339.8*	442.8*	–	–	–	–
6. DMSO + OLIGO + 2,4-DNP + Cytochalasin-B	191.5*	284.2*	372.7*	16.2	16.4	15.8	16.1
B. With 10 min Preincubation							
1. DMSO (as control)	799.9*	1137.0*	1378.1*	–	–	–	–
2. DMSO + Cytochalasin-B	626.2*	962.2*	1277.2*	21.7	14.8	6.8	14.4

*Time intervals are 6 min; 12 min; 18 min.

Conditions for Table III: For incubations with and without preincubation, Medium contained 5 mM $MgCl_2$; 100 mM Sucrose; 20 mM Tris buffer, pH 7.4 in a total volume of 5.0 ml. T = 28°. Cytochalasin-B added as DMSO solution to a final concentration of 0.2 mM, and a final DMSO level of 5% (v/v). Mean mitochondrial protein was 0.933 mg/ml incubation (7 preparations). Where appropriate, 1 μM oligomycin and 50 μM 2,4-DNP were added.

A. no preincubation: 6 mM ATP added prior to mitochondrial enzyme addition. Data represent mean values from 5 rat-liver preparations.

B. 10 min preincubation: 6 mM ATP added 10 min after mitochondrial enzyme addition. Data represent mean values from 2 rat-liver preparations.

TABLE IV. 1.0 mM Cytochalasin-B-effected ATPase Inhibition

Conditions	Pi released nmoles/mg protein/ml incub.			% Inhibition of Control			Mean % Inhibition
	Time Interval (min)			Time Interval (min)			
	6	12	18	6	12	18	
1. DMSO (as control)	157.9	283.6	509.7	—	—	—	—
2. DMSO + Cytochalasin-B	53.8	75.4	127.4	65.9	73.4	75.0	71.4
3. DMSO + 2,4-DNP (as control)	656.9	1108.2	1233.9	—	—	—	—
4. DMSO + 2,4-DNP + Cytochalasin-B	423.5	630.6	723.6	35.5	43.1	41.3	39.9

Conditions for Table IV: Conditions as for Table III, part A. Mean mitochondrial protein was 0.920 mg/ml incubation (2 preparations). Where appropriate, 50 μM 2,4-DNP was added.

It is significant that at lower concentrations, cytochalasin-B promoted a cumulatively constant mean ATPase inhibition, regardless of subsequent additions to the incubations—additions which are believed to affect the oxidative phosphorylation coupling mechanism itself [17, 18]. Thus, the presence of the classical uncoupling agent 2,4-DNP alone displayed the same 15% inhibition of ATPase with the metabolite, as did coupled control incubations without 2,4-DNP. Similarly, incubations with oligomycin *plus* 2,4-DNP, agents whose sites of activity have been hypothetically localized in the chemical coupling mechanism theory [19], can manifest further inhibition of ATPase activity upon cytochalasin-B addition. This further incremental inhibition by cytochalasin-B is very close to that obtained with incubations where only 2,4-DNP was added together with cytochalasin-B.

Attempting pre-exposure of mitochondria to low concentrations of cytochalasin-B, prior to ATP addition, again yielded kinetic inhibition of ATPase nearly identical to those runs without pre-incubation.

When higher concentrations of cytochalasin-B were employed (1 mM), the data obtained, while in qualitative agreement with those for low metabolite levels (0.2 to 0.33 mM), do not allow straightforward correlation between dosage and inhibitory response. For example, Table I displays more than a 40% decrease in control State-3 rates of oxygen consumption in the presence of 1 mM cytochalasin-B. Yet, one-third of this higher amount of cytochalasin-B decreases active State-3 respiration by roughly 20% of the control rates; clearly a non-linear effect.

Further difficulty in quantitative characterization of the high metabolite concentration is manifest with ATPase experiments. Table IV shows that 1 mM cytochalasin-B distinctly inhibits ATP hydrolysis by mitochondrial suspensions, but does so without the consistency so obvious when 0.2 mM cytochalasin-B is employed (Table III). Thus, the high level of metabolite inhibits ATPase activity over 70% for control (5% DMSO) incubations over an eighteen minute time course, but inhibits only 40% when 50μM 2,4-DNP is present together with cytochalasin-B.

We feel that the data obtained with 1 mM levels of cytochalasin-B, while not anomalous or artifactual insofar as clearly demonstrating the inhibitory effect on enzymatically active whole mitochondria, are, nonetheless incapable of critical analysis at this time. Cytochalasin-B is rather insoluble in aqueous media above 0.1 mM, even in the presence of 5% DMSO. At 1 mM concentrations, the metabolite precipitates heavily, thereby permitting only a small and indeterminate fraction of the solute to effect the whole mitochondrial membranes during the incubation time examined. It should be noted that all previous work with whole cells in the presence of any of the cytochalasin congeners rarely includes data from incubations in which the cytochalasin level exceeds 10 μg/ml (approx 20 μM) [see, for example, ref 20].

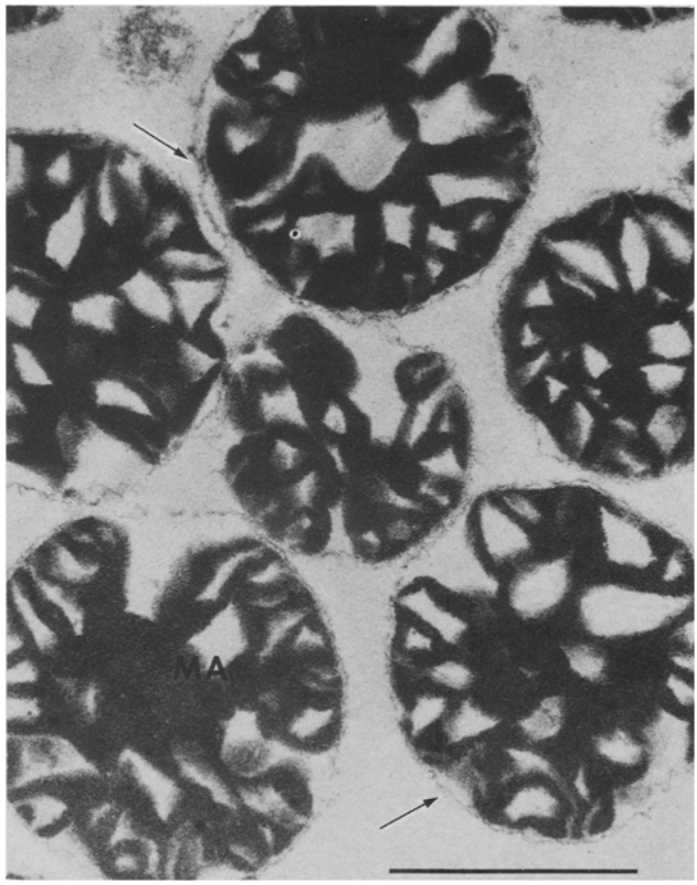

Figure 1: Isolated rat-liver mitochondria, incubated as a Control for 10 min prior to fixation, with medium and conditions described in Methods. Magnification = 65,000. Bar represents 10,000 Å. The electron-dense matrix (MA) displays an orthodox conformation, and is surrounded by a generally uniform outer membrane (arrows) in close aposition with the peripheral area of the matrix. The matrix itself, which is immediately bounded by the inner membrane (not visible here), presents a sponge-like appearance.

Figs. 1 and 2 demonstrate the correlation between the structural alteration of the mitochondrial membranes and the kinetics of oxidative phosphorylation and ATPase data presented above.

The preparation containing 1 mM cytochalasin-B (Fig. 2) shows that the cristae and the densely staining matrix material become distorted,

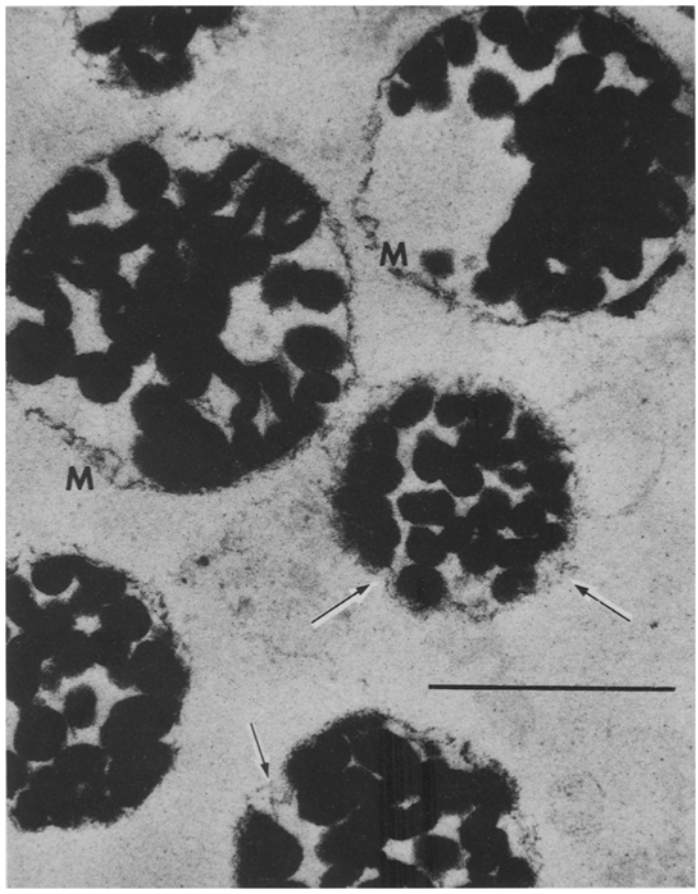

Figure 2: Isolated rat-liver mitochondria, incubated with 1 mM cytochalasin-B, as in Figure 1 and Methods. Magnification = 65,000. Bar represents 10,000 Å. The matrix has condensed to form contracted spheres and tubules, and the outer membrane (M) has become highly corregated and, in several instances, almost non-existent (arrows).

disconnected, 2-dimensional spheres, which give the appearance of having shrunken away from the outer membrane and rounded up into a presumably 3-dimensional tubular geometry. The outer membrane itself assumes a more highly irregular shape, and, in several instances, only vaguely hints at its presence at all. The control preparation (Fig. 1) presents a recognizable and normal pattern for isolated rat-liver mitochondria, displaying the sponge-like orthodox conformation of cristae-enclosed matrix. In addition, the outer limiting membrane is more

consistently present in the control, even though both mitochondrial preparations employed 5% DMSO in the incubation medium.

If the kinetics of oxidative phosphorylation and affiliated partial reactions are perturbed by a metabolite that possesses the ability to drastically alter the structural geometry of mitochondria as well, one may ask where the principle locus of cytochalasin-B activity lies. Since the inner mitochondrial membrane has been shown to possess the respiratory chain as well as most, if not all, required factors for coupled oxidative phosphorylation, we can at least argue that, based on the evidence presented in this paper, the primary locus of cytochalasin-B activity, at concentrations below 1 mM, must exclude those enzymic reactions directly concerned with oxidative phosphorylation. Instead, cytochalasin-B disturbs some of the other components associated with the structural integrity of the membranes, and this action is reflected in altered kinetics of oxidative phosphorylation.

Recent data from this laboratory [21] appear to implicate the membrane-affiliated adenine nucleotide carrier moiety, that has received extensive attention by Klingenberg and co-workers [22, 23], as the principle target of cytochalasin-B effects discussed above, and this relationship between cytochalasin-B and adenine nucleotide translocation will be communicated shortly.

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