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Characterization of the oligomycin-sensitivity properties of the F_1F_0-ATPase in mitochondria from rats infected with the liver fluke *Fasciola hepatica*

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Abstract

The F_1F_0-ATPase activity of liver mitochondria isolated from rats infected with *Fasciola hepatica* at 3 and 4 weeks post-infection showed a marked loss of sensitivity to oligomycin and to *N,N'*-dicyclohexylcarbodiimide. A loss of sensitivity to diethylstilbestrol was also demonstrated at 4 weeks post-infection. Recovery was apparent in most cases by 6 weeks post-infection. No significant difference in latent ATPase activity was observed between mitochondria from control and infected livers at any stage of the infection. The mitochondria from infected livers were therefore considered to have a full complement of the F_1 moiety of the F_1F_0-ATPase complex. Purification of the mitochondrial ATPase from 4-week infected livers resulted in a very low yield of an oligomycin-insensitive complex. This was due to a failure to enrich specific activity during purification. The evidence presented indicates that infection with *Fasciola hepatica* gives rise to alterations in the function of the host liver mitochondrial ATPase, namely loss of inhibitor sensitivity and apparent structural alterations of the ATPase complex.

Key words: ATPase; ATPase, F_1F_0-; Oligomycin; Mitochondrion; Liver; (*F. hepatica*)

1. Introduction

Major lesions in host liver bioenergetic metabolism arise during infection with *Fasciola hepatica*. The uncoupling of rat liver mitochondria during infection was first reported by Van den Bossche et al. [1]. Subsequently, studies in our laboratory showed mitochondrial respiration in vitro to be completely uncoupled at 2, 4 and 6 weeks post-infection [2]. This uncoupled respiration showed no response to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or to oligomycin and suggested an inability of mitochondria to synthesize ATP during infection. Recent work has shown that mitochondria isolated from livers at 4 weeks post-infection are unable to synthesize ATP in vitro and that respiration in isolated hepatocytes is also aberrant in 3-week infected animals [3]. The period from 2 to 6 weeks post-infection in the rat corresponds to the acute phase of infection when the growing parasites migrate through the liver parenchyma prior to becoming resident in the bile duct. It is during this time that maximal physical damage is evident in the liver [4].

Rule et al. [2] found ATPase activity in mitochondria isolated at 3 and 4 weeks post-infection to be insensitive to oligomycin. This indicates that the structural integrity of the mitochondrial F_1F_0-ATPase may be compromised in the infected state. Prior to this, Van den Bossche et al. [5] had used cytochemical methods to show that ATPase activity was increased in infected liver slices, but did not test sensitivity to ATPase inhibitors.

In view of the fact that a lesion in the assembly of the F_1F_0-ATPase could give rise to aberrant proton flux through the complex, with associated loss of ATP synthesizing ability, it was considered important to study this possibility further. In the present communication we extend our work with oligomycin as well as

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DES, diethylstilbestrol; DTT, dithiothreitol; OSCP, oligomycin-sensitivity-conferring-protein; F_6, coupling factor 6; IMVs, inner membrane vesicles; MPG, *N*-(2-mercaptopropionyl)-glycine; PMSF, phenylmethylsulfonyl fluoride.

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other inhibitors of the F_1F_0-ATPase, *N,N'*-dicyclohexylcarbodiimide (DCCD) [6] and diethylstilbestrol (DES) [7] and discuss our purification of the complex from mitochondria from infected hosts.

2. Materials and methods

2.1. Animals

Outbred male Wistar rats were bred and housed in the Faculties Animal Care Facility, Australian National University. Rats were 4–6 weeks of age at the time of infection. Age-matched uninfected rats were used as controls. Rats were kept in pairs in plastic cages and provided with commercial rat chow and water ad libitum.

2.2. Infections

Metacercariae of *Fasciola hepatica* maintained in the snail *Lymnaea viridis*, were obtained from Dr J.C. Boray, Elizabeth Macarthur Agricultural Research Institute, Camden Park, New South Wales. Metacercariae were assessed as viable if refractile secretory granules were present when examined under a light microscope [8]. Lightly etherized rats were infected with 30 viable metacercariae in approximately 0.2 ml water via a stomach tube.

2.3. Isolation of mitochondria

Rats were anaesthetized with sodium pentobarbitone (approximately 50 mg/kg body weight). Livers were removed and mitochondria prepared as described by Rule et al. [2]. When additions were made to the isolation medium, they were added just before use to give the following final concentrations: phenylmethylsulfonyl fluoride (PMSF) (200 μM), leupeptin (1 μM), EDTA (100 μM), pepstatin (1 μM), dibucaine (400 μM), dithiothreitol (2 mM), *N*-(2-mercaptopropionyl)-glycine (MPG) (2 mM, 200 μM).

2.4. Measurement of ATPase activity

Mitochondria from control and infected livers were twice freeze-thawed in liquid nitrogen. ATPase activity was measured at 37°C in a 1.5 ml reaction mixture (pH 7.4) containing 5 mM $MgSO_4$, 15 mM Hepes/KOH, 5 mM ATP and 2 μM CCCP. When required, oligomycin (1 μg/mg protein), DCCD (1.25 μg/mg protein) and DES (25–300 μM) were included. Freeze-thawed mitochondria (1.5 mg/ml) were pre-incubated for 2 min in the above medium. A zero time sample of 0.25 ml was removed into an equal volume of ice-cold 20% (w/v) trichloroacetic acid to which an amount of ATP was added to correspond to the initial concentration in the reaction medium. The reaction was started by the addition of 5 mM ATP, with samples being removed into trichloroacetic acid as above at 0.5, 1 and 2 min. After the precipitated protein was separated by centrifugation for 2 min in an Eppendorf microfuge, the levels of inorganic phosphate in the supernatant were measured using the method of Baginski et al. [9] modified for use in a microtitre plate.

2.5. Isolation of the F_1F_0-ATPase

The protocol of McEnery et al. [10] was followed to purify the F_1F_0-ATPase from control and infected livers, with the exception that adaptations to centrifugation times were made due to unavailability of some rotors. The Beckman 80Ti replaced the Sorvall T-865.1 and the Beckman SW-28 replaced the Sorvall SS-28.

2.6. Protein determination

Membrane protein was determined by a modification of the method of Lowry et al. [11] as described in [2]. Soluble protein was determined by the method of Bradford [12]. Bovine serum albumin was used as standard in both cases.

2.7. Chemicals

CCCP, oligomycin, DES, MPG and dibucaine were obtained from Sigma, St. Louis, MO, USA; ATP, PMSF, pepstatin and dithiothreitol were from Boehringer Mannheim, Germany.

2.8. Statistics

Results are expressed as mean $\pm$ S.E. The statistical significance P was determined using Student's t-test.

3. Results and discussion

The pattern of oligomycin inhibition of ATPase activity in mitochondria isolated up to 10 weeks post-infection is shown in Fig. 1a. The total activity in the absence of inhibitor remained constant throughout the period under consideration and at no stage was there any significant difference in latent ATPase activity between preparations from control and infected animals. Van den Bossche et al. [5] reported enhanced ATPase activity at 13 weeks post-infection, but examination of the methods employed suggests this reflected a loss of respiratory control rather than an increase in total enzyme activity.

ATPase activity first showed insensitivity to oligomycin at 3 weeks post-infection. At this stage, activity

remaining in the presence of oligomycin was 1.06 ± 0.08 μmol phosphate/min/mg protein (16% inhibition), compared with control values of 0.09 ± 0.02 μmol phosphate/min/mg protein (92% inhibition; $P < 0.001$). Significant insensitivity was retained at 4 weeks post-infection with inhibition reaching an average level of 30% (infected) compared with 87% (age-matched controls; $P < 0.001$).

The above findings imply that mitochondria from infected animals are not deficient in F_1 at any stage with respect to control animals. Also, it follows that the ability of the catalytic subunits to hydrolyse ATP has not been compromised by the infection. It is therefore unlikely that proteolytic or oxidative damage to the active site is contributing to the lack of ability to synthesize ATP in mitochondria isolated from 4-week infected animals [3].

At 6 weeks post-infection recovery of sensitivity to oligomycin was apparent, with levels of inhibition re-

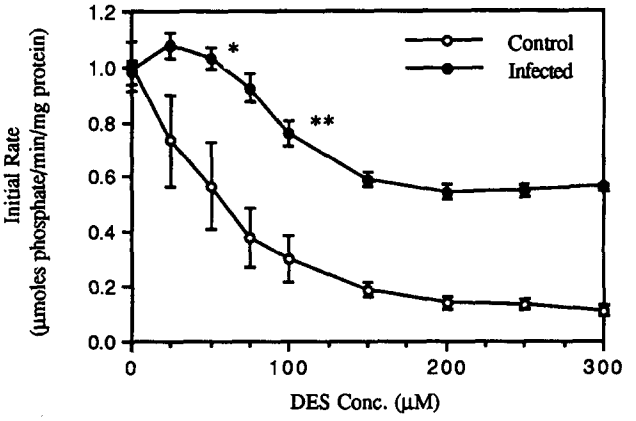

Fig. 2. Inhibition by DES of ATPase activity in mitochondria isolated from control and infected livers at 4 weeks post-infection. Mitochondria were isolated and the initial rate of ATPase activity was determined with and without DES (0–300 μM) as described in Section 2. The volume of DES in methanol was the same in all cases (10 μl). Values are mean ± S.E. for $n = 4$ (* $P < 0.05$, ** $P < 0.001$ compared with corresponding control values).

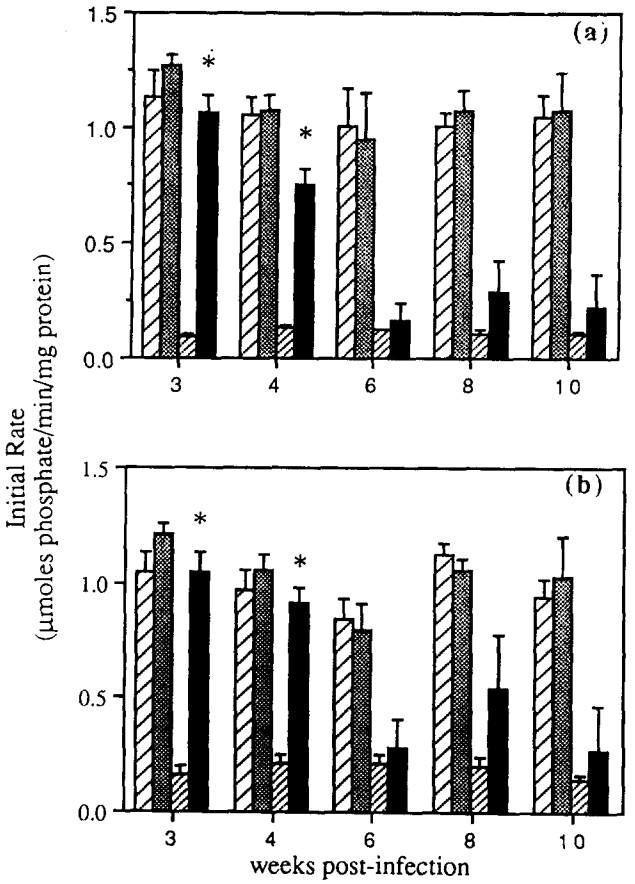

Fig. 1. Effect of oligomycin (a) and DCCD (b) on the ATPase activity of mitochondria isolated from the livers of control and infected rats at 3, 4, 6, 8 and 10 weeks post-infection. (▨) control; (▩) infected; (▩) control + inhibitor; (■) infected + inhibitor. Mitochondria were isolated and the intial rate of ATPase activity was determined with and without oligomycin (1 μg/mg protein) or DCCD (1.25 μg/mg protein) as described in Section 2. Values are mean ± S.E. for $n \geq 3$ for each experimental group. (* Values are significantly different from control + inhibitor, $P < 0.001$).

turning to control values in most cases. This recovery was sustained through 8 to 10 weeks post-infection, its onset coinciding with the appearance of parasites in the bile duct. This confirms and extends previous findings and, along with the attenuation of respiration observed at 4 weeks post-infection, is an example of a major reversible change occurring in host liver bioenergetic metabolism [2].

A similar pattern of insensitivity to inhibitors emerged when mitochondria were incubated in the presence of DCCD (Fig. 1b). Pre-incubation with DCCD reduced ATPase activity at 3 and 4 weeks post-infection by only 13% and 14%, respectively, but abolished more than 80% of activity in control preparations. ATPase inhibition by DES was also tested at 4 weeks post-infection. Results depicted in Fig. 2 show that in contrast to the strong inhibitory effects of DES on the ATPase activity of control mitochondria (maximal inhibition approximately 90% with half-maximal inhibition requiring 50 μM DES), the maximum inhibition reached with infected material was only 45%, with double the concentration of DES (100 μM) being necessary for half-maximal inhibition in this case. It is possible that DES may produce non-specific effects when long incubation periods or high concentrations are used [13,14], but a significant difference between control and infected rates of ATP hydrolysis was apparent even at 50 μM DES ($P < 0.05$), with incubation periods as short as 2 min.

Oligomycin, DCCD and DES are all reported to bind to the F_0 moiety of the ATPase complex [15,7]. They are able to prevent proton flux through F_0 and their binding is communicated to F_1 by an as yet undetermined mechanism which results in loss of activity at the catalytic sites [16]. At the concentration used,

DCCD is thought to bind preferentially to a site distinct from, but interrelated with, the oligomycin binding site [16]. Kinetic studies [18] are supportive of a distinct site, while pre-incubation with oligomycin has been shown to interfere to some extent with [^{14}C]DCCD binding [19,20]. Evidence for the overlapping of the two binding sites is emerging from studies with ATPase mutants [21]. On the other hand, DES, which has been shown to have no influence on [^{14}C]DCCD labelling of F_0, is thought to act at a site structurally distinct from oligomycin and DCCD [22]. It therefore seems unlikely that the lesion under consideration is localized to the inhibitor binding sites, but rather it represents a problem in communication to F_1 of inhibitor interaction with F_0.

Studies in our laboratory with hepatocytes have discounted the possibility that the respiratory aberrations observed in mitochondria isolated from infected livers are due to direct preparative damage [3]. However, this does not eliminate the possibility that loss of inhibitor-sensitivity by the ATPase occurred during isolation. For this reason, the proteinase inhibitors PMSF (200 μM), leupeptin (1 μM), EDTA (100 μM) and pepstatin (1 μM) or the phospholipase A_2 inhibitor dibucaine (400 μM) were included in the isolation medium, but their presence had no effect on oligomycin-sensitivity.

Oligomycin-sensitive thiol groups have been demonstrated in the mitochondrial ATPase [23] and modification of F_0 thiols has been shown to inhibit binding of F_1 to F_0 [24]. We attempted to increase oligomycin sensitivity in vitro by incubation with the thiol protective agents dithiothreitol (DTT) and *N*-(2-mercaptopropionyl) glycine (MPG), but there was no effect. This implies that if the observed loss of oligomycin-sensitivity is the result of thiol modification, then it is not readily reversible. The inclusion of DTT in the isolation medium had no effect on either the sensitivity to oligomycin or the uncoupled state of the mitochondria.

To determine whether a structural change in the complex was responsible for this lesion, we attempted to isolate an intact oligomycin-insensitive mitochondrial ATPase complex from infected liver using the same purification procedure that has been used successfully to purify the oligomycin-sensitive enzyme from normal liver [7]. The results of the major steps in the procedure are discussed below. A summary of specific activities and sensitivity to oligomycin at each stage of the purification is presented in Table 1.

3.1. Step 1: Preparation of mitochondria

From the above biochemical data it has already been proposed that 'infected' mitochondria have a full complement of the F_1 moiety. This was supported by electrophoretic evidence (data not shown). The presence of bovine serum albumin in the isolation medium (as required by the high-yield method of Bustamente et al. [25]) did not affect ATPase activity and insensitivity to oligomycin. The yield of mitochondria (assessed as mitochondrial protein/g of liver) from infected livers was typically less than 50% of that obtained from normal livers (results not shown). Whether this was a result of the presence of fewer intact mitochondria in the infected liver and/or a demonstration of their fragile nature (and hence lability during preparation) was not determined.

Table 1
Purification of ATPase from liver mitochondria of 4-week infected rats and age-matched controls

Purification step	Control		Infected	
	ATPase activity	% Inhibition	ATPase activity	% Inhibition
Mitochondria	1.19 ± 0.06 (5)	92	1.13 ± 0.07 (8)	35
Inner membrane vesicles	4.37 ± 0.48 (5)	96	1.11 ± 0.10 (7)	32
Washed membranes	5.93 (2)	96	0.84 ± 0.08 (4)	15
Isolated ATPase	6.82 (1)	92	not detectable	
Concentrated ATPase [a]	35 (1)	92	9.8 (1)	35

Mitochondria were isolated according to the high yield procedure of Bustamente [26]. Inner membrane vesicles were prepared from freshly isolated mitochondria using the method of Wehrle et al. [27]. These were washed three times in phosphate buffer [7] to yield 'washed membranes'. ATPase assays were carried out at the protein concentrations of 1.5 mg/ml (mitochondria); 0.5 mg/ml (inner membrane vesicles and washed membranes); 15 μg/ml (purified control ATPase). ATPase activity is expressed as μmol phosphate/min/mg protein. Oligomycin was used as inhibitor at a concentration of 1.5 μg/ml. Average values are presented; S.E.s are included where the number of preparations is sufficient. The number of independent preparations in each case is shown in parentheses.

[a] Concentrated purified ATPase activity (control and infected) was measured over a period of 10 min at a protein concentration of 20 μg/ml.

3.2. Step 2: Inner membrane vesicles (IMVs)

Preparation of IMVs by digitonin treatment led to a 4-fold increase in the specific activity of the control preparation, whereas the activity of the infected material failed to become enriched (Table 1). Electron microscopic evidence showed a higher abundance of F_1 portions projecting from negatively stained IMVs from control mitochondria compared with those from infected livers (results not shown). The ATPase activity remaining in the infected material at this stage of the purification was still insensitive to oligomycin (32% inhibition) relative to control values (96% inhibition).

We were unable to detect ATPase activity in the supernatants from these preparations of IMVs. In a subsequent preparation, using a more sensitive ATPase assay [10], we were able to show that there appears to be a greater ATPase activity in the supernatants following digitonin treatment of 'infected' mitochondria (9% of total ATPase activity in the control preparation compared with 25% in the 'infected' preparation). These observations support a hypothesis of leakage of activity from the membranes during purification.

3.3. Step 3: Washed membranes

Following the three wash steps, the control membranes showed a further increase in specific activity while the activity in the 'infected' membranes fell (Table 1). Only 15% of the activity remaining in the infected material was sensitive to oligomycin.

3.4. Step 4: Purified F_1F_0-ATPase complex

After sucrose gradient centrifugation, the amount of protein recovered from the infected material represented only approximately 15% of control values (results not shown). The ATPase activity of this protein fraction was too low to obtain an initial rate measurement, but following concentration of the enzyme, activity became detectable over a 10-min period. The specific activity of the ATPase prepared from 'infected' mitochondria was 28% of the control preparation and retained its oligomycin-insensitivity (Table 1). Electrophoresis of the final product (not shown) showed bands in the 'infected' preparation that corresponded with those of the control ATPase preparation, but at lower enrichment and accompanied by more intense contaminant bands.

The evidence presented above shows that the purification procedure results in the isolation of an intact oligomycin-insensitive complex, but at a very low yield. Since oligomycin insensitivity persisted in the final isolated ATPase complex, the possibility exists that this may represent the structural state in vivo at 3 and 4 weeks post-infection. That is, that communication between F_1 and F_0 is faulty, possibly engendering loss of F_1 from the membrane during purification.

Two subunits are important in modulating sensitivity of the F_1F_0-ATPase to inhibitors. Oligomycin-sensitivity-conferring protein (OSCP) is mandatory for oligomycin sensitivity [26] and DCCD sensitivity [27], while the 25 kDa PVP protein of bovine heart ATPase has been shown recently to modulate inhibitor sensitivity, acting in concert with OSCP or F_6 [28]. The structure or attachment of all or some of these subunits may be affected at 3 and 4 weeks post-infection.

Oxidative stress arising from the host's inflammatory response has been implicated in the development of metabolic lesions during infection by *F. hepatica* [3,29–31]. The alterations in ATPase function and apparent integrity that we have observed could be the result of oxidative damage to critical ATPase subunits and/or mitochondrial inner membrane lipids. Exposure of submitochondrial particles in vitro to $^{\cdot}OH$ and O_2^- has been shown to diminish ATPase activity, though oligomycin sensitivity was not tested [32]. We did not detect any loss of ATPase activity during infection; this could therefore indicate a different mechanism of damage in vivo. Peroxidation of mitochondrial phospholipids during liver fluke infection has yet to be assessed, but would appear likely, considering reports of extensive peroxidation of membranes assessed in whole liver and microsomal preparations [29,30]. Also, loss of ATPase sensitivity to oligomycin due to in situ changes in membrane phospholipids has been reported [33]. We have recently demonstrated a marked loss of several major phospholipid species from the inner mitochondrial membrane during infection (manuscript in preparation). Further work on the roles of oxidative damage and membrane lipid depletion or peroxidation in the development of the changes to the ATPase complex during liver fluke infection is under way.

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