The mitochondrial adenosine triphosphatase of Acanthamoeba castellanii

Oscillatory accumulation of enzyme activity, enzyme protein and F₁-inhibitor during the cell cycle

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1. The mitochondrial ATPase of Acanthamoeba castellanii accumulated discontinuously in synchronous cultures prepared by a minimally perturbing size-selection technique. 2. Enzyme activity per ml of culture doubled overall during one cell cycle time of 8 h, but oscillated to give seven maxima during this period. Similar oscillations were observed in the specific activities of ATPase and of the naturally occurring inhibitor protein. 3. These variations in enzyme activity reflected changes in amount of enzyme protein as assayed by an immunological technique. 4. Large variations in \( I_{50} \) values (\( \mu g \) of inhibitor/mg of protein necessary for 50% inhibition of inhibitor-sensitive activity) for inhibition of ATPase activity by seven different inhibitors of energy conservation were observed. Activity was more sensitive to inhibition by oligomycin, efrapeptin, citreoviridin and quercetin when values were highest. 5. The results are discussed in relation to the phased organization of biosynthesis and degradation of cellular components known to occur during the cell cycle of this organism.

Extensive studies on the mitochondrial ATPase (EC 3.6.1.3) of yeast have revealed structural and functional similarities to the enzyme from mammalian sources (Kagawa & Racker, 1966; Schatz et al., 1967; Tzagoloff & Meagher, 1971). The enzyme comprises three functional components: \( F_0 \)-ATPase, the oligomycin-sensitivity-conferring peptide, and the membrane factor \( (F_0) \). The synthesis and assembly of the entire enzyme complex is under the dual control of mitochondrial and non-mitochondrial protein-synthesizing systems, and is coded for in part by nuclear DNA and in part by mitochondrial DNA (see Tzagoloff et al., 1979, for a review). A naturally occurring inhibitor protein of \( F_0 \)-ATPase has been isolated from many sources, and the presence or absence of this inhibitor in Schizosaccharomyces pombe is correlated with marked changes in sensitivities of ATPase to inhibitors of energy conservation (Lloyd & Edwards, 1976); when the natural inhibitor is present, sensitivities to inhibitors acting on \( F_0 \)-ATPase increase 2-fold, whereas sensitivities to inhibitors acting on the membrane factor show a greater increase. Removal of the inhibitor protein by gel filtration (Racker & Horstman, 1967) decreases the sensitivity of the mitochondrial ATPase of Acanthamoeba castellanii to inhibitors of energy conservation by between 1.5- and 14-fold (Edwards et al., 1982).

There have been few reports on the expression of ATPase activity during the cell cycle (Lloyd et al., 1982). In Escherichia coli ATPase activity reaches two maxima per cell cycle at 0.37 and 0.8 of the cycle, whereas sensitivity to Ruthenium Red is maximal at the middle of the cycle (Scott et al., 1980). In Alcaligenes eutrophus ATPase activity is maximal at 0.4 and 0.9 of the cycle, corresponding to mid-step rises in respiratory activities (Edwards & Jones, 1977) and minimal at 0.1 and 0.6 of the cycle (Edwards et al., 1978); inhibition by 4-chloro-7-nitrobenzofurazan was greatest at maximal ATPase activity, whereas maximal inhibition by \( NN' \)-dicyclohexylcarbodi-imide was when ATPase activity was lowest. Discontinuous accumulation of ATPase activity was also observed during the cell cycle of S. pombe (Edwards & Lloyd, 1977), and 11 inhibitors of energy conservation all showed cell-cycle-dependent variations in their \( I_{50} \) values (\( \mu g \) of inhibitor/\( \mu g \) of protein necessary for 50% inhibition of inhibitor-sensitive activity) (Lloyd & Edwards, 1977).

The cell cycle of A. castellanii is divided into phases of biosynthesis separated by degradative phases, whereby total RNA and total cellular protein accumulate discontinuously (Edwards & Lloyd, 1980). Thus, at 30\(^\circ\)C the generation time is 8 h (Edwards & Lloyd, 1978) and total protein oscil-
lates to give seven maxima per cell cycle (Edwards & Lloyd, 1980). The accumulation of catalase [a peroxisomal enzyme in this organism (Müller & Möller, 1969; Morgan et al., 1973)] is also oscillatory during the cell cycle, as determined by assays of both enzyme activity and enzyme protein by an immunological method (Edwards et al., 1981).

In the present study we have examined changes in ATPase activity during the cell cycle of *A. castellanii*, as well as changes in sensitivities to inhibitors of energy conservation, and show that the changes in ATPase activity are paralleled by changes in ATPase protein, as assayed by an immunological method.

**Experimental**

**Maintenance and growth of the organism**

*Acanthamoeba castellanii* was maintained and grown with shaking at 30°C exactly as described previously (Edwards et al., 1977). Cells were counted in a Fuchs–Rosenthal haemocytometer (Baird and Tatlock, Chadwell Heath, Essex, U.K.) after a suitable dilution in 50 mM-MgCl₂ (pH 7.4).

**Preparation of synchronous cultures**

Synchronous cultures were prepared by centrifugation of exponentially growing cultures, in growth medium at 800 rev./min (150 g; *r*ₑᵥ, 21 cm) for 2 min in the 6 x 1 litre head of an MSE Mistral centrifuge. Approx. 80% of the supernatant, containing the slowest-sedimenting cells of the culture (approx. 10%), was decanted and grown as a synchronous culture. Assessment of synchrony was as described previously (Edwards & Lloyd, 1980), by using the synchrony index of Blumenthal & Zahler (1962).

**Preparation of cell-free extracts**

Samples removed from synchronous cultures (50–100 ml) were centrifuged at 30000 rev./min (750 g; *r*ₑᵥ, 7.6 cm) for 2 min in the 6 x 250 ml head of an MSE 18 centrifuge. The pellet was re-suspended in 50 mM-Tris/H₂SO₄, pH 8.1, and a cell-free extract was produced by treatment with ultrasound (Branson Sonicator, 40 W for 10 s). This treatment resulted in 100% cell breakage and the suspension produced was used as a cell-free extract.

**Preparation of submitochondrial particles and ATPase-inhibitor protein**

Mitochondria were isolated from mid-exponential-phase cultures essentially as described previously (Edwards & Lloyd, 1978) in a disruption buffer containing 0.32 M-sucrose, 0.2 mM-EGTA and 10 mM-Tris/H₂SO₄, pH 8.1. The mitochondrial suspension in 50 mM-Tris/H₂SO₄ (pH 8.1) was treated with ultrasound for 10 s to produce submitochondrial particles. This suspension was used to assay the inhibitor protein after gel filtration, as described below.

The ATPase-inhibitor protein was prepared as described previously (Edwards et al., 1982) from cell-free extracts prepared from synchronous cultures.

**Other methods**

ATPase was assayed by the proton-release method (Edwards et al., 1982) in a stirred reaction mixture at 30°C containing 4 mM-ATP, 6 mM-MgSO₄ and 10 mM-Tris/H₂SO₄, pH 8.1. Activity was measured within 6 h of preparation of extracts.

The activity of the ATPase-inhibitor protein was measured as described previously (Edwards et al., 1982); one unit of activity is defined as the volume of inhibitor preparation required to inhibit ATPase activity of a preparation of inhibitor-depleted submitochondrial particles by 50%; the inhibitor activity was then expressed as units per mg of protein. The inhibitor was removed from submitochondrial particles by gel filtration through a Sephadex G-50 column (0.9 cm x 15 cm). Elution was with 50 mM-Tris/H₂SO₄ pH 8.1, and the particles were eluted in the void volume (3.6 ml).

Immunological determination of ATPase protein was by complement fixation by using serum prepared against F₁-ATPase as described previously (Edwards et al., 1982).

Protein was measured by the method of Lowry et al. (1951).

**Chemicals**

The sources of chemicals and inhibitors have been described previously (Edwards et al., 1982).

**Results**

**Changes in cell numbers, ATPase activity and F₁-inhibitor activity during the cell cycle**

A synchronous culture, containing 9% of the original exponential culture, was prepared as described in the Experimental section and followed for 9 h of growth. Cell numbers remained constant for the initial 5 h, then increased and doubled by just less than 8 h of growth (Fig. 1a). Throughout this period, ATPase activity per ml of culture doubled overall, but the pattern of accumulation was discontinuous (Fig. 1b), in that activity rose to seven maxima during the cell cycle. These oscillations in activity were also observed in measurements of specific activities (Fig. 1c) and the mean amplitude of these (peak–trough, % of minimum value) was 37%. Similar oscillations were observed in the activities of the F₁-inhibitor protein in these extracts (Fig. 1d); the mean amplitude (peak–trough, % of minimum value) was 74%. The timings of maxima and minima of enzyme activity and F₁-inhibitor activity closely
ATPase in *Amoeba* cell cycle

An exponentially growing culture (3.5 \times 10^6 cells/ml) was centrifuged at 800rev./min for 2 min as described in the Experimental section. The supernatant (containing 9% of the exponential culture) was decanted and grown as a synchronous culture. At frequent time intervals, samples were removed for measurement of (a) cell numbers, (b) ATPase activity/ml of culture, (c) ATPase activity/mg of protein and (d) F$_1$-inhibitor activity/mg of protein. The synchrony index, $F$ (Blumenthal & Zahler, 1962), was 0.68.

coincided. Similar changes in ATPase activity were observed in an experiment in which the disruption buffer contained the following proteinase inhibitors: 2 mM-phenylmethylaminesulphonyl fluoride; 5 mM-$p$-aminobenzamidine hydrochloride; 5 mM-$\epsilon$-amino-hexanoic acid (results not shown).

**Effect of inhibitors on ATPase activity during the cell cycle**

The effect of oligomycin on ATPase activity of extracts prepared at different stages of synchronous growth is shown in Fig. 2. ATPase activity over the initial 2.5h varied by 46%, and sensitivity to oligomycin also showed large changes and varied over a 4-fold range. Maximum sensitivity to oligomycin was observed when ATPase activity was highest, whereas minimum sensitivity was observed when activity was lowest.

![Fig. 1. Changes in cell numbers, ATPase activity and F$_1$-inhibitor-protein activity during synchronous growth of *A. castellanii*](image1)

![Fig. 2. Changes in ATPase activity and sensitivity to oligomycin during a portion of the cell cycle of *A. castellanii*](image2)

A synchronous culture (containing 15% of the exponential culture) was prepared as in the legend to Fig. 1. The initial cell population after centrifugation was $7 \times 10^6$ cells/ml, and growth was followed for 9h. The doubling time was 8h and the synchrony index ($F$) was 0.7. •, ATPase activity/mg of protein, and ○, $I_{50}$ values ($\mu$g of oligomycin/mg of protein required for 50% inhibition), over the initial 2.5h of synchronous growth.

In a similar experiment where ATPase activity varied by 96% (trough-peak, % of minimum value), large variations in sensitivities to efrapeptin, NN'-dicyclohexylcarbodi-imide and 4-chloro-7-nitrobenzofurazan were observed (Fig. 3). $I_{50}$ values for efrapeptin showed a reciprocal relationship to enzyme activity; thus sensitivity of ATPase to this inhibitor was maximal when activity was highest. Sensitivities to the other inhibitors tested in this experiment were not as clearly defined, however, except for the first minimum in activity, which was relatively insensitive to both NN'-dicyclohexylcarbodi-imide and 4-chloro-7-nitrobenzofurazan (Fig. 3b).

In another experiment, shown in Fig. 4, ATPase activity was measured and correlated with a totally independent assay for amount of ATPase protein by an immunological method. ATPase activity was high at the beginning of the experiment and again after 1h and 2h of synchronous growth (Fig. 4a); these changes in activity were closely paralleled by changes in amount of enzyme protein.
Fig. 3. Changes in ATPase activity and sensitivity to efrapeptin, 4-chloro-7-nitrobenzofurazan (Nbf-Cl) and NN'-dicyclohexylcarbodi-imide (DCCD) during synchronous growth of A. castellanii

A synchronous culture, initially containing $4.1 \times 10^5$ cells/ml (13% of the original exponential culture), was followed under conditions identical with those described in the legend to Fig. 2. The doubling time was 8.05 h and the synchrony index ($F$) was 0.71. (a) Specific activity; (b) $I_{50}$ values ($\mu$g of inhibitor/mg of protein necessary for 50% inhibition) for efrapeptin (●), DCCD (O) and Nbf-Cl (■).

Fig. 4. Changes in ATPase protein, ATPase activity and sensitivity to spegazzinine, citreoviridin and quercetin during synchronous growth of A. castellanii

Experimental conditions were identical with those described in the legend to Fig. 2. The synchronous culture initially containing $5 \times 10^5$ cells/ml (16% of the original exponential culture) doubled in cell numbers by 8.1 h; the synchrony index ($F$) was 0.68. (a) ATPase activity (munits/mg of protein) (●) and ATPase protein (immunological units/mg of protein) (O). (b) $I_{50}$ values ($\mu$g of inhibitor/mg of protein necessary for 50% inhibition) for spegazzinine (●), citreoviridin (O) and quercetin (■).
Sensitivity of ATPase activity to citreoviridin and quercetin were similar in that sensitivity was maximal at highest enzyme activity and was lowest at low activity (Fig. 4b). The sensitivity profile to spegazzinine was again less well defined, but lowest sensitivity to the inhibitor appeared to occur just after maximum activity. 

Table 1 summarizes the effects of inhibitors on ATPase activity during the initial 2.5 h of synchronous growth.

**Changes in ATPase activity, ATPase protein and F\textsubscript{1}-inhibitor during growth of control, asynchronous cultures**

To establish whether the variations in ATPase activity observed in Figs. 1–4 were genuinely associated with the cell cycle, or whether they resulted from artefacts arising from the method used to produce the synchronous cultures, the experiment in Fig. 5 was performed. An exponentially growing culture was centrifuged under conditions identical with those used to produce the synchronous cultures, but after centrifugation the pellet and supernatant were remixed and the entire contents were returned to the growth vessel. Cell numbers increased exponentially (doubling time 8 h), as did total cell protein and ATPase activity per ml of culture (Fig. 5a). The specific activities of ATPase did not vary significantly and averaged 20.3 munits/mg of protein. The coefficient of variation (v) for the data points shown in Fig. 5(b) (v = 100 \times \text{standard deviation/mean}) was 3.8%. A similar coefficient of variation for the \text{F}_{1}\text{-inhibitor-protein activity was 7%}. ATPase protein as determined by an immunological method also showed no fluctuations during this period.

**Discussion**

The results presented demonstrate that, in common with the majority of protein in this organism (Edwards & Lloyd, 1980), the accumulation of ATPase protein during the cell cycle is oscillatory. Since changes in ATPase activity closely parallel changes in enzyme protein, the possibility that these periodic fluctuations in activity of this enzyme are regulated by binding and release of the naturally occurring inhibitor protein can be eliminated. However, it cannot be discounted that the cell-cycle-dependent variations in sensitivities of inhibitors of energy conservation arise, at least in part, by alterations in the amount of bound inhibitor protein, since sensitivity to inhibitors can vary up to 14-fold, depending on the presence or absence of this natural inhibitor (Edwards et al., 1982).

Immunological determination of amount of enzyme protein during the cell cycle has very rarely been reported (Klevecz, 1969; Martin et al., 1969). This approach provides confirmation that enzyme activity represents amount of enzyme protein, and has confirmed that the accumulation of catalase protein in this organism is also discontinuous (Edwards et al., 1981). That similar changes were also observed when extracts were prepared in the presence of proteinase inhibitors eliminates the possibility that restricted proteolysis occurs in cell-free extracts. Thus the turnover of ATPase protein during the cell cycle of this organism is rapid under ideal growth conditions. The periodicity of these oscillations is about 1 h, and it should be emphasized that, as discussed previously (Edwards & Lloyd, 1980), the degree of synchrony in these cultures is underestimated, since DNA replication is completed in about 1 h (Edwards & Lloyd, 1978, 1980). As clearly shown in Fig. 5, these oscillations are genuinely associated with the cell cycle, since no oscillatory increase was observed in control (asynchronous) cultures that were subjected to experimental procedures identical with those employed to produce the synchronous cultures. Oscillatory enzyme formation, and the control processes that

<table>
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<tr>
<th>Inhibitor</th>
<th>Maximum inhibition (%)</th>
<th>Minimum inhibition (%)</th>
<th>I\textsubscript{50} range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligomycin</td>
<td>71</td>
<td>43</td>
<td>1.4–0.3</td>
</tr>
<tr>
<td>Spegazzinine</td>
<td>79</td>
<td>32</td>
<td>1.2–0.5</td>
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<tr>
<td>Citreoviridin</td>
<td>94</td>
<td>52</td>
<td>0.7–0.3</td>
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<tr>
<td>\text{NN'}-Dicyclohexylcarbodi-imide</td>
<td>84</td>
<td>39</td>
<td>1.75–0.6</td>
</tr>
<tr>
<td>4-Chloro-7-nitrobenzofurazan</td>
<td>68</td>
<td>30</td>
<td>0.8–0.2</td>
</tr>
<tr>
<td>Efrapeptin</td>
<td>68</td>
<td>21</td>
<td>0.04–0.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td>80</td>
<td>26</td>
<td>2.3–0.1</td>
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underlie this phenomenon, are discussed further in several reviews (Thurston, 1972; Halvorson, 1977; Lloyd et al., 1982).

A previous study of inhibitor sensitivity of ATPase activity during the cell cycle of S. pombe (Lloyd & Edwards, 1977) identified at least seven distinct inhibitor-binding sites on this enzyme. Clearly, in A. castellanii, no such model can be proposed. However, of those inhibitors tested, profiles for \( I_{50} \) values for oligomycin, efrapeptin, citreoviridin and quer cetin were similar to each other, in that maximum sensitivity was observed when activity was highest.

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References

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