SHORT COMMUNICATION

Effects of Catabolite Repression and Inhibitors of Protein Synthesis on the Mitochondrial Carbodiimide Binding Site in *Saccharomyces cerevisiae*

By MICHAEL D. PARTIS,* ENRICO BERTOLI† AND DAVID E. GRIFFITHS

Department of Molecular Sciences, University of Warwick, Coventry CV4 7AL

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Mitochondria prepared from *Saccharomyces cerevisiae* grown on low concentrations of glucose bound less carbodiimide than mitochondria from *S. cerevisiae* grown on high concentrations of glucose. This decreased binding could be prevented by including cycloheximide in the growth medium. These phenomena are discussed in terms of a possible mechanism of carbodiimide inhibition of energy-linked reactions.

INTRODUCTION

Dicyclohexylcarbodiimide (DCCD) is a potent inhibitor of the mitochondrial ATP synthetase system (ATPase; EC 3.6.1.3) (Robertson *et al.*, 1968). Inhibition of the ATP synthetase may be correlated with covalent binding of [¹⁴C]DCCD to the inner membrane of beef heart mitochondria (Holloway *et al.*, 1966; Knight *et al.*, 1968). Mitochondria of *Saccharomyces cerevisiae* bind only a small fraction of an inhibitory concentration of [¹⁴C]DCCD (Partis *et al.*, 1975; Sebald *et al.*, 1978). It has been suggested that reaction with only one of six carbodiimide binding subunits of the yeast mitochondrial ATPase is sufficient to cause inhibition of the enzymic activity (Sebald *et al.*, 1978).

In the course of our investigation of the microenvironment of the carbodiimide binding protein, we found that binding of DCCD and the spin label analogue *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N'*-cyclohexylcarbodiimide (NCCD) to mitochondrial particles of *S. cerevisiae* could be enhanced by removing the catalytic subunits of the ATPase, or by growing the yeast under anaerobic conditions (Partis *et al.*, 1975). We now report that the binding of carbodiimides to *S. cerevisiae* mitochondria is increased during catabolite repression and that a cytoplasmically synthesized component is implicated in the decrease of binding during derepression.

METHODS

Abbreviations. DCCD, *N,N'*-Dicyclohexylcarbodiimide; NCCD, *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N'*-cyclohexylcarbodiimide.

Materials. NCCD was synthesized in this laboratory by Dr P. Iannou as described by Azzi *et al.* (1973). [¹⁴C]DCCD was provided by Professor R. B. Beechey.

Organism and growth conditions. *Saccharomyces cerevisiae* strain D22 (a, ad₂) was obtained from Dr D. Wilkie. Stock cultures were maintained on agar slopes containing (g l⁻¹ in distilled water): glucose, 20; yeast extract, 10; peptone, 5; agar, 20; the pH was 6.1. Cells were grown in 500 ml medium containing 1% (w/v)

* Present address: Biological Laboratory, University of Kent, Canterbury CT2 7NJ.

† Present address: Istituto di Biochimica, Università di Ancona, Italy.

Fig. 1. Binding of $[^{14}\text{C}]\text{DCCD}$ to mitochondria from glucose repressed and derepressed *S. cerevisiae*: (a) derepression in the absence of protein synthesis inhibitors; (b) derepression in the presence of 10^{-8} M-cycloheximide. $\circ$, Repressed; $\bullet$, harvested after 0.5 h derepression; $\triangle$, harvested after 1 h derepression; $\blacktriangle$, harvested after 3 h derepression; $\square$, harvested after 18 h derepression.

yeast extract, 0.5% (w/v) peptone, 0.01% (w/v) adenine sulphate, mineral salts (Wickerham, 1946) and 5% (w/v) glucose in 2 l baffled conical flasks shaken at 30 °C. Tributyl citrate (0.01%, v/v) was added as an antifoaming agent. A 2% (v/v) inoculum from a starter culture grown to late-exponential phase was used, and the cells were harvested, after 12 h growth, by centrifugation at 1200 g for 5 min. Derepression of these cells was carried out in medium containing 0.5% (w/v) glucose.

Isolation of mitochondria. Suspensions of washed cells in 0.25 M-sucrose containing 20 mM-Tris/HCl buffer (pH 7.5) and 1 mM-EDTA were broken by shaking with glass beads (0.45 to 0.50 mm) for 45 s in a cooled Braun homogenizer at 4000 oscillations min^{-1} . The homogenate was diluted with an equal volume of buffer. After two centrifugations at 3000 g to remove cell debris and nuclei, a mitochondrial pellet was obtained by centrifuging at 13000 g for 20 min. The crude mitochondrial pellet was resuspended in 0.25 M-sucrose, 20 mM Tris/HCl, 1 mM-EDTA (pH 7.5).

Analytical procedures. NCCD was incorporated into yeast mitochondria as described by Azzi *et al.* (1973). Electron spin resonance spectra were recorded at room temperature using a Decca Radar X1 spectrometer with a 7-in Newport Instruments magnet. Binding of $[^{14}\text{C}]\text{DCCD}$ was measured as described previously (Partis *et al.*, 1975). Protein was determined by the Lowry procedure. Inorganic phosphate and ATPase assays were as described by Griffiths & Houghton (1974).

RESULTS

The binding of $[^{14}\text{C}]\text{DCCD}$ to mitochondrial membranes from glucose repressed yeast and derepressed yeast is shown in Fig. 1(a). A decreased binding of the carbodiimide was expressed within 30 min of transfer to a medium low in glucose. This decrease in binding did not occur if cycloheximide was present during derepression, as shown in Fig. 1(b). No effect of derepression on the inhibitory potency of DCCD was observed. The concentration of DCCD causing 50% inhibition of ATPase in mitochondrial particles from glucose repressed yeast, derepressed yeast, and yeast derepressed in the presence of cycloheximide was $1.4 \text{ nmol (mg protein)}^{-1}$.

The freely tumbling motion of a nitroxide spin probe in a medium of low viscosity gives rise to a resonance spectrum of three sharp lines of nearly equal height. The electron spin resonance spectrum of NCCD bound to mitochondrial membranes from glucose-repressed yeast is shown in Fig. 2(a); the unequal broadening of the three lines is characteristic of immobilization of the probe. There was a decrease in the central peak height in the resonance spectra of NCCD bound to mitochondrial membranes from repressed yeast grown in low glucose medium. This decrease in peak height, corresponding to a lowered binding of carbodiimide, was almost complete at 30 min after transfer into low glucose medium. In addition, the resonance spectrum of NCCD in mitochondria from derepressing yeast was

Fig. 2. Electron spin resonance spectra of NCCD bound to mitochondria from glucose repressed and derepressed *S. cerevisiae*: (a) derepression in the absence of protein synthesis inhibitors; (b) derepression in the presence of 10^{-5} M-cycloheximide. The numbers indicate the time of harvesting (h) after transfer to low glucose medium.

not broadened to the same extent as that in mitochondria from repressed yeast. This reduction in broadening may be attributed either to an increase in the mobility of the bound NCCD, or to a decrease in the polarity of the microenvironment of the binding site.

The decrease in binding of NCCD was prevented by inclusion of cycloheximide in the low glucose medium (Fig. 2b), as found for the decreased binding of [14 C]DCCD (Fig. 1). Cycloheximide also partially prevented the reduction of broadening in the spectrum.

It is surprising that the binding of carbodiimides decreased during derepression with no concomitant decrease in inhibition. This would seem to imply a non-causal relationship between covalent carbodiimide binding and ATPase inhibition. However, a mechanism involving inhibition due to non-covalent interaction may be ruled out: the ATPase of DCCD-treated mitochondrial membranes is inhibited even when non-covalently bound DCCD has been removed by repeated washing (Holloway *et al.*, 1966). Moreover, the binding site for DCCD has been shown to be localized in the oligomycin-sensitive ATPase complex and to be bound to a glutamic acid residue in the proteolipid subunit 9 (Partis *et al.*, 1975; Wachter *et al.*, 1977). Covalent binding is thought to proceed through the formation of an unstable *O*-acyl urea and conversion to a stable *N*-acyl urea.

Broughall (1973) has suggested that the mechanism of action of DCCD in yeast mitochondria is similar to that found in erythrocyte membranes (Godin & Schrier, 1970) in which inhibition is initially due to carbodiimide binding as the *O*-acyl urea. Subsequent attack by an adjacent nucleophile causes loss of the DCCD as dicyclohexylurea, covalent binding of the nucleophile at the carbodiimide binding site and no loss of inhibition. The free inhibitor after incubation of DCCD with yeast mitochondria is predominantly dicyclohexylurea, indicating that such a reaction is taking place (Partis *et al.*, 1975). The results presented here are also consistent with this hypothesis. The presence of such a membrane nucleophile in mitochondria from derepressed yeast would lead to loss of bound DCCD as dicyclohexylurea, and reduced levels of the nucleophile in mitochondria from repressed yeast would cause enhanced binding of DCCD. In both cases, inhibition would follow binding, whether DCCD was permanently bound or not.

Inclusion of cycloheximide in the derepression medium prevents the decrease in carbodiimide binding, implying that the postulated nucleophile must be extramitochondrially synthesized. Identification of such a nucleophile as one of the components of the membrane component of the ATPase may be of value in the elucidation of the coupling mechanism in oxidative phosphorylation.

REFERENCES

- AZZI, A., BRAGADIN, M. A., TAMBURRO, A. M. & SANTATO, M. (1973). Site directed spin labelling of the mitochondrial membrane. *Journal of Biological Chemistry* **248**, 5520–5526.
- BROUGHALL, J. M. (1973). *The biochemistry of oligomycin resistance in mutants of the yeast Saccharomyces cerevisiae*. Ph.D. thesis, University of Warwick.
- GODIN, D. V. & SCHRIER, S. L. (1970). Mechanism of inactivation of erythrocyte membrane adenosine triphosphatase by carbodiimides. *Biochemistry* **9**, 4068–4077.
- GRIFFITHS, D. E. & HOUGHTON, R. L. (1974). Studies on energy linked reactions: modified mitochondrial ATPase of oligomycin resistant mutants of *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **46**, 157–167.
- HOLLOWAY, C. T., ROBERTON, A. M., KNIGHT, I. G. & BEECHY, R. B. (1966). The binding of [¹⁴C]-dicyclohexylcarbodiimide to mitochondrial fractions. *Biochemical Journal* **100**, 79p.
- KNIGHT, I. G., HOLLOWAY, C. T., ROBERTON, A. M. & BEECHY, R. B. (1968). The chemical nature of the site of action of dicyclohexylcarbodiimide in mitochondria. *Biochemical Journal* **109**, 27p.
- PARTIS, M. D., BERTOLI, E., MASCARELLO, S. & GRIFFITHS, D. E. (1975). The organisation of oligomycin sensitive ATPase. *Biochemical Society Transactions* **4**, 88–91.
- ROBERTON, A. M., HOLLOWAY, C. T., KNIGHT, I. G. & BEECHY, R. B. (1968). A comparison of the effects of *N,N'*-dicyclohexylcarbodiimide, oligomycin and aurovertin on energy linked reactions in mitochondria and submitochondrial particles. *Biochemical Journal* **108**, 445–456.
- SEBALD, W., SEBALD-ALTHAUS, M., LUKINS, H. B. & WACHTER, E. (1978). Biogenesis of the mitochondrial ATPase complex: the dual origins of subunits and genes. In *The Membrane ATPases* (in the Press). Edited by H. Peeters. Gent: European Press.
- WACHTER, E., SEBALD, W. & TZAGOLOFF, A. (1977). Altered amino acid sequence of the DCCD binding protein in the oli-1 resistant mutant D273-10b/A21 of *Saccharomyces cerevisiae*. In *Mitochondria 1977*, pp. 441–449. Edited by W. Bandlow, R. J. Schweyen, K. Wolf & F. Kaudewitz. Berlin: Walter de Gruyter.
- WICKERHAM, L. J. (1946). A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. *Journal of Bacteriology* **52**, 293–301.