A MODIFIED SUBUNIT OF MITOCHONDRIAL ATPase IN MUTANTS OF *SACCHAROMYCES CEREVISIAE* WITH DECREASED SENSITIVITY TO DICYCLOHEXYLCARBODIIMIDE

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1. Introduction

Biochemical genetic studies in this laboratory have led to the isolation of mutants of *Saccharomyces cerevisiae* with lowered sensitivity to oligomycin [1]. The mutations are located on one of three loci on mitochondrial DNA designated OLI, OLII and OLIII [2,3] and may be associated with cross-resistance to venturicidin [3]. It has been shown that the component conferring lowered sensitivity resides in the membrane subunits of the mitochondrial ATPase and not in the catalytic subunits [4].

Dicyclohexylcarbodiimide (DCCD) is a potent inhibitor of oxidative phosphorylation [5] and has been shown to bind to a low molecular weight proteolipid subunit of the ox heart mitochondrial ATPase [6,7]. Although DCCD inhibits yeast mitochondrial ATPase at low concentrations [8], the covalent binding of the inhibitor found in ox heart mitochondria cannot be demonstrated in aerobically grown yeast. Binding of carbodiimides to yeast promitochondria has been shown to be associated with subunit 9 of the oligomycin-sensitive ATPase [8]. We have investigated the physical properties of the carbodiimide binding protein of some oligomycin resistant mutants, and here present data indicating that the mutation at the OLIII locus causes an alteration of the smallest subunit of the oligomycin sensitive ATPase.

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2. Materials and methods

The parental strain of *S. cerevisiae* D22 (a, ad_2, ω^+, ρ^+) donated by Dr D. Wilkie, and the oligomycin-resistant strains D22A21, D22A19 and D22A61 have been described [1–3]. The growth on glucose medium [1] and isolation of mitochondria were as in [4], except that glucose repressed yeast was grown for 12 h on medium containing 5% glucose. Drug resistance was assayed by the drop-out technique [9]. Labelling of mitochondrial protein synthesis products was as in [10]. Protein was determined by the Lowry procedure [11], and inorganic phosphate and ATPase assays were as in [4]. NCCD was incorporated into yeast mitochondria as in [12]. Electron paramagnetic resonance (EPR) spectra were recorded at room temperature using a Decca Radar XI spectrometer with a 7 in. Newport Instrument Magnet.

Oligomycin sensitive ATPase was prepared as in [13]. Subunit 9 was prepared from submitochondrial particles as in [14], with an additional terminal step. Material from preparative layer chromatography (TLC) in chloroform–methanol–HCl–water [14] was further purified by chromatography on preparative layer silica plates in chloroform–methanol–17% NH_3 (2:2:1, by vol.). Subunit 9 has an R_F 0.72 in this system; purification was monitored with subunit

9 labelled with [^{3}H]leucine [10]. Isoelectric focussing was essentially as in [15], except that samples were first solubilised in 2% SDS and 9.5 M urea.

3. Results and discussion

Cross-resistance of OLI, ILII and OLIII mutants to DCCD at the whole cell level is shown in table 1. It can be seen that although mutations at all 3 loci may give rise to lowered sensitivity to DCCD, only mutation at OLIII produces resistance in all the mutants examined. The resistance to oligomycin in all 3 classes is not indicative of a common gene product; genetic evidence [3] shows that while OLI and OLIII are closely linked, OLII is located on a separate cistron. The cross-resistance of OLII mutants may be due to cooperative effects between subunits. The consistent resistance to DCCD found in OLIII mutants suggests that these mutants may be useful in identification of the hitherto unknown gene product with the carbodiimide binding site.

Figure 1 shows that the OLIII mutation is expressed as a lowered sensitivity of the mitochondrial ATPase to DCCD. Both D22A19 and D22A61 require increased concentrations of DCCD for inhibition of the membrane bound ATPase (I_{50} = 21.8 nmol/mg protein) compared with the parental strain (I_{50} = 1.5 nmol/mg protein). The same effect may be seen

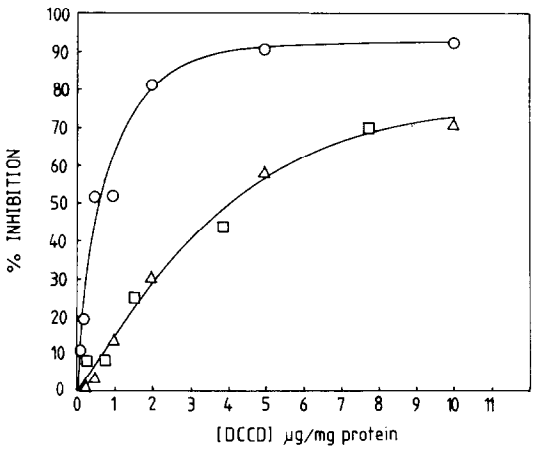

Fig.1. Inhibition of mitochondrial ATPase by DCCD. (○——○) D22 parental strain; (△——△) D2261; (□——□) D22A19.

in the detergent solubilised ATPase complex (I_{50} parental strain = 5.8 nmol/mg protein, I_{50} D22A19 = 33.0 nmol/mg protein). The ATPase activity of OLI and OLII mutants does not, in general, show increased tolerance to DCCD [4].

N-(tetramethylpiperidinyl-1-oxyl)-*N'*-(cyclohexyl)-carbodiimide (NCCD) is a spin label analogue of DCCD and an equally potent inhibitor of energy-linked reactions [11]. NCCD may be used as a sensitive monitor of the environment of the carbodiimide

Table 1
Resistance *in vivo* to inhibitors

Strain	Mitochondrial locus	Resistance to antibiotics in vivo (µg/mg)			
		DCCD	Oligomycin	Venturicidin	TET
D22	–	0.05	0.5	0.1	4
D22 A21	OLI	0.05	5	0.1	4
D22 A16	OLI	2	5	0.1	4
D22 A14	OLII	2	5	0.1	4
D22 A15	OLII	5	5	0.1	4
D22 C4	OLII	2	5	0.1	4
D22 A13	OLII	0.05	–	–	–
D22 B21	OLII	0.05	–	–	–
D22 A19	OLIII	2	5	2	4
D22 61	OLIII	0.5	5	2	4
D22 62	OLIII	2	5	2	4
D22 69	VI	2	0.5	–	20

The level of resistance is defined as the maximum concentration allowing normal growth of the strain

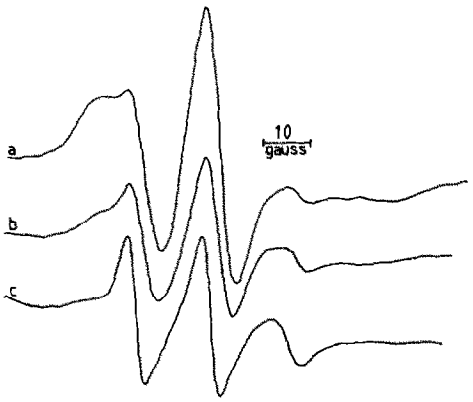

Fig.2. EPR of NCCD bound to mitochondrial membranes. (a) D22, parental strain; (b) D2261; (c) D22A19.

binding protein. The ESR spectrum of NCCD bound to mitochondrial membranes from glucose repressed parental and mutant strains is shown in fig.2. The spectrum of NCCD bound to mitochondrial membranes from the parental D22 strain shows the line broadening characteristic of an immobilised nitroxide spin label [11]. It is apparent that the central peak height of the spectrum of NCCD bound to the mitochondrial membranes of the OLIII mutants D2261 and D22A19 is reduced compared to that of the parental strain, indicating a reduced affinity for NCCD. Moreover, the line broadening of the nitroxide resonance spectrum is greater in the parental strain compared to the OLIII mutants, which may be due to either a greater degree of immobilisation of the carbodiimide or to a more polar microenvironment for the binding site in the parental strain.

In order to examine the carbodiimide binding protein further, the proteolipid was extracted and purified as in [14], and freed from remaining lipid by preparative TLC in chloroform/methanol/17% NH_3 (2:2:1, by vol.). The proteolipid protein, which was identified as a mitochondrially synthesised protein by co-chromatography with ^{3}H-labelled subunit 9, showed similar R_F values on TLC in two systems, whether prepared from parental or mutant strains. Isoelectric focussing in polyacrylamide gel containing urea demonstrated that the proteolipids from parental and mutant strains have pI values between 4.75 and 5.0. The OLII mutant D22A15 contains a proteolipid which is indistinguishable from that of the parental strain, but the pI of the proteolipid of OLI mutant D22A21 and the OLIII mutant D2261 is displaced towards the acid, indicating a modification of these peptides.

It has been shown [16] that the DCCD binding proteolipid from OLII mutants and the parental primarily in a low molecular weight form, while the proteolipids from OLII mutants and the parental strain exists as a 45 000 dalton aggregate which may be converted to the 8000 dalton form by treatment with base or organic solvents. The OLIII mutations possess the proteolipid in the aggregated 45 000 dalton form [17], are cross-resistant to venturicidin and DCCD [4], and are thus phenotypically distinct from the OLI mutation, although the two loci may be allelic [3].

The OLIII locus has been suggested [18] as the structural gene for subunit 6 of the mitochondrial ATPase, on the basis of decreased subunit 6 content in a temperature-sensitive OLIII mutant. However, an alteration of the electrophoretic mobility of subunit 9 in this mutant has been found [19] and the OLIII locus suggested to play some role in the specification of this subunit. The consistent cross-resistance to DCCD, the altered carbodiimide binding and the change in isoelectric point reported here for OLIII mutants are all consistent with this view.

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