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Identification of Amino Acid Substitutions in the Dicyclohexylcarbodiimide-Binding Subunit of the Mitochondrial ATPase Complex from Oligomycin-Resistant Mutants of *Saccharomyces cerevisiae*

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1. Oligomycin-resistant mutants of *Saccharomyces cerevisiae* strain D273-10B/A1 have been assigned to three previously defined mitochondrial genetic loci, *oli1*, *oli2* and *oli3*. Standard crosses of *oli1*-resistant mutants yielded a low percentage of sensitive recombinants indicating that the mutations were in closely linked but separate alleles.

2. The oligomycin-resistant mutants were used to study the amino acid sequence of the dicyclohexylcarbodiimide-binding subunit of the mitochondrial ATPase complex. Two different amino acid substitutions have been identified in the dicyclohexylcarbodiimide-binding protein of *oli1*-resistant mutants: leucine/phenylalanine at residue 53 or cysteine/serine at residue 65. The protein of the single *oli3*-resistant strain examined had a leucine/valine exchange in residue 57. No differences were noted in the dicyclohexylcarbodiimide-binding protein of the *oli2*-resistant mutant when the amino acid composition of cyanogenbromide fragments as well as a partial sequence were compared with the wild type. These data indicate that the *oli1* and *oli3* locus are in the structural gene of the dicyclohexylcarbodiimide-binding protein and they suggest that the *oli2* locus is in some other still unidentified mitochondrial gene.

3. Amino acid sequence data have also been obtained for the dicyclohexylcarbodiimide-binding protein of a revertant of an ATPase-deficient *pho2* mutant. The protein of the revertant strain was ascertained to have a serine/leucine exchange at residue 42. The localization of the *pho2* locus in the structural gene of the dicyclohexylcarbodiimide-binding protein is in agreement with previous evidence that mutations in the *pho2* and *oli1* loci are genetically linked.

4. The frequencies of recombination among the *oli1* and *oli3* resistant mutants have been related to the distances of the amino acids affected by the mutations. It is concluded that mutations in adjacent amino acids recombine with a frequency of 0.01 %.

As a result of recent studies in several laboratories [1–5] it has become evident that the mitochondrial genome of *Saccharomyces cerevisiae* codes for some if not all the proteins that are synthesized on mitochondrial ribosomes. This set of proteins includes three subunits of cytochrome oxidase [6,7], four subunits of the oligomycin-sensitive ATPase [8] and cytochrome *b* [9]. The identification of presumptive mitochondrial gene products has been based largely on correlative studies of mit$^-$ mutations which results in a loss or an alteration of the molecular size of specific mitochondrial translation products (mit$^-$ mutants have point mutations or small deletions in mitochondrial DNA that result in respiratory deficiency. Mutations in *oxi* loci cause a deficiency of cytochrome oxidase, *cob* (*box*) loci of cytochrome *b* and *pho* loci of the ATPase.) For example numerous mutations in the *cob* (or *box*) loci have been found to cause a reduction in the molecular weight of cytochrome *b* [2–4]. Similarly, mutations in *oxi1* and *oxi3* can cause the appearance of altered forms of subunits 2 and 1 of cytochrome oxidase, respectively [5]. In the case of the ATPase, the evidence so far has also been of an indirect nature. Certain oligomycin-resistant mutants in the *oli1* locus exhibit a modified dicyclohexylcarbodiimide-binding subunit which fails to form the

Table 1. *Names and genotypes of strains*

Name	Genotypes		References
	nuclear	mitochondrial	
D273-10B/A1	α, *met*	ϱ^+, ω^+	[10]
D273-10B/A21	α, *met*	ϱ^+, ω^+, O^R_{625}, P^R_{626}, E^R_{624}	[10]
D273-10B/A31	α, *met*	ϱ^+, ω^+, O^R_{101}	(this study)
D273-10B/A32	α, *met*	ϱ^+, ω^+, O^R_{102}	(this study)
D273-10B/A33	α, *met*	ϱ^+, ω^+, O^R_{103}	(this study)
D273-10B/A48	α, *met*	ϱ^+, ω^+, O^R_{118}	(this study)
D273-10B/A68	α, *met*	ϱ^+, ω^+, O^R_{119}	(this study)
CB-11	a, *ade1*	ϱ^+, ω^+	from Dr R. Needleman
CD21-1A	a, *ade1*	ϱ^+, ω^+,	(D273-10B/A21 × CB11)
CD31-3A	a, *ade1*	ϱ^+, ω^+, O^R_{101}	(D273-10B/A31 × CB11)
CD32-9A	a, *ade1*	ϱ^+, ω^+, O^R_{102}	(D273-10B/A32 × CB11)
CD33-7A	a, *ade1*	ϱ^+, ω^+, O^R_{103}	(D273-10B/A33 × CB11)
D22	a, *ade1*	ϱ^+, ω^+	[12]
D22/A15	a, *ade2*	ϱ^+, ω^+, O^R_{144}	[12]
D22/A16	a, *ade2*	ϱ^+, ω^+, O^R_{145}	[12]
D22/V61	a, *ade2*	ϱ^+, ω^+, V^R_{61}	[13]
M339-45/R17	α, *met*	ϱ^+, ω^+, mit^-_{945}	[14]

hexamer usually present in wild-type oligomycin-sensitive strains [10]. (The dicyclohexylcarbodimide-binding protein has also been referred to as the proteolipid or subunit 9 of the ATPase complex.)

The determination of the complete amino acid sequence of the wild-type dicyclohexylcarbodiimide-binding protein (unpublished) has made it feasible to extend the structural analyses to bther strains carrying mutations in the *oli1*, *oli2* and *oli3* loci and thereby directly test whether these mitochondrial genetic markers are associated with the structural gene of this ATPase subunit. In this communication we present evidence that mutations which can be genetically ascribed to the *oli1* and *oli3* loci lead to single amino acid substitutions in the dicyclohexylcarbodiimide-binding protein and are therefore included within the structural gene. Similar data have been obtained for a revertant of a mutant in the *pho2* locus. The identification of three genetic markers with a structural gene of yeast mitochondrial DNA should be useful for future studies on the isolation and sequencing of the gene.

MATERIALS AND METHODS

Isolation of Oligomycin-Resistant Strains

Some of the oligomycin-resistant strains were obtained from the wild-type sensitive haploid D273-10B/A1. A freshly grown culture of D273-10B/A1 (10^6-10^7 cells) was plated on R medium (1% yeast extract, 2% peptone, 3% glycerol, 2% agar, 5 μg rutamycin/ml). Spontaneous resistant mutants were collected after 4–5 days of incubation of the plates at 30 °C. The resistant strains were purified and the mutations ascertained to be mitochondrial by mitotic and meiotic segregation tests. In both tests, the mutants were crossed to the oligomycin-sensitive haploid *Saccharomyces carlsbergensis* CB11. After 20–30 generations of growth on WO medium (Wickerham's medium without amino acids, supplemented with 2% glucose), the prototrophically selected diploids were spread for single colonies on WO plates and replicated on rutamycin medium. Mutants yielding an equal distribution of rutamycin-sensitive and rutamycin-resistant segregants were further checked to be mitochondrially inherited by tetrad dissection of a single resistant diploid colony. In all cases 4:0 segregation ratios of the meiotic spore progeny were obtained. Some of the resistant strains were constructed by mating the original mutants to *S. carlsbergensis* CB11 and isolating an 'a'-mating-type oligomycin-resistant spore after tetrad dissection. Other standard oligomycin-resistant strains were kindly provided by Drs D. Griffiths and B. Lancashire. Table 1 lists the genotypes and sources of the various strains used.

Recombination of Oligomycin-Resistant Alleles

Oligomycin-resistant haploid strains in the opposite mating type and with complementary auxotrophies were manually mated on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar). After overnight incubation at 30 °C, the mating patch was replicated on WO medium and incubated for an additional two days. The prototrophically selected diploids were spread for single colonies on WO medium and after 2–3 days of growth replicated on YEPG medium (1% yeast extract, 2% ethanol, 3% glycerol, 2% agar) and R medium. Colonies that grew on the glycerol but not the rutamycin plates were scored as oligomycin-sensitive recombinants. The recombination frequency was equated to the percentage of oligomycin-sensitive colonies in the total glycerol-positive population.

Preparation of Submitochondrial Particles and of the Dicyclohexylcarbodiimide-Binding Protein

Overnight cultures of the wild-type and mutant strains (10 ml) were inoculated into 10 l of liquid YPD medium in a 14-l fermenter cup (New Brunswick Microferm). The culture was grown to stationary phase by vigorous aeration at 30 °C for 17 h. The cells were disrupted with glass beads in a Bronwill homogenizer and mitochondria prepared by previously published procedures [15]. The mitochondria were suspended in 0.25 sucrose, 10 mM Tris-acetate pH 7.5 and sonically irradiated for 30 s in a Raytheon 10-kHz Sonic Oscillator. The submitochondrial particles were collected by centrifugation at 79000 × *g* for

60 min, suspended in 10 mM Tris-acetate pH 7.5 at a protein concentration of 20–25 mg/ml and used directly for the isolation of the proteolipid. Under these conditions the yield of submitochondrial particles was 0.8–1 g of protein. When larger amounts of proteolipid were required, cells were grown in 100 l of YPD medium and mitochondria were prepared by the liquid nitrogen method of cell disruption [16].

The procedure for the purification of the dicyclohexylcarbodiimide-binding protein originally described by Sierra and Tzagoloff [17] was modified. The following protocol was used, starting from 1 g of submitochondrial particles. The suspension of particles (about 20 mg/ml) was added to 9 vol. of ice-cold methanol. Following 10 min of stirring, the mixture was centrifuged at $2000 \times g$ for 10 min, and the pellet was homogenized with 50 ml of chloroform/methanol (2/1) and the mixture centrifuged at $10000 \times g$ for 10 min in stainless steel bottles. The supernatant was saved and the pellet extracted two additional times with the same volumes of chloroform/methanol (2/1). The combined extracts were reduced to near dryness in a flash evaporator and the residue dissolved in 10 ml of chloroform/methanol (2/1). The proteolipid was precepitated with 5 vol. of cold ether and centrifuged at $10000 \times g$ for 10 min. The ether precipitation was usually repeated three additional times. We have noted that following ether precipitation some material failed to be dissolved in chloroform/methanol and was removed by centrifugation. The yield of proteolipid from 1 g of particles was about 2 mg which was sufficient for the amino acid analysis and sequencing of the 17-residue cyanogen bromide fragment.

Preparation and Separation of Cyanogen Bromide Fragments

The purified dicyclohexylcarbodiimide-binding protein was dissolved in 0.4 ml of a 1 M solution of cyanogen bromide in 98% formic acid. Following dissolution of the protein 0.1 ml H_2O was added and the cleavage reaction was carried out overnight at room temperature. The mixture was dried in a flash evaporator, the residue dissolved in 0.5 ml of 80% formic acid. The three fragments generated by cyanogen bromide were separated on a Bio-Gel P-30 column developed with 80% formic acid. The details of this procedure are described in a forthcoming paper.

Amino Acid Sequence Determinations

All sequencing was done on the 17-residue fragment coupled through the C-terminal homoserine lactone to 3-aminopropyl glass. The details of the solid-phase sequencing are described in a forthcoming paper.

Table 2. *Phenotypes of strains used for the analysis of the dicyclohexylcarbodiimide-binding protein*
Strain M339-45/R17 was isolated as a spontaneous revertant of the mit$^-$ mutant M339-45 which was determined to have a mutation in the *pho2* locus. Since the parent (D273-10B/A1) is a wild-type antibiotic-sensitive strain, the oligomycin resistance of the revertant is the result of the second correcting mutation [14]

Strain	Affected locus	Phenotype
D273-10B/A1	none	sensitive
D273-10B/A21	*oli1*	oligomycin resistant
D273-10B/A31	*oli1*	oligomycin resistant
D273-10B/A32	*oli1*	oligomycin resistant
D273-10B/A33	*oli1*	oligomycin resistant
D273-10B/A48	*oli2*	oligomycin resistant
D273-10B/A68	*oli3*	oligomycin and venturicidin rasistant
M339-45/R17	*pho2*	oligomycin resistant

Amino Acid Analyses

The fragments were hydrolyzed in 6 M HCl for 24 h at 105 °C. Amino acid analyses were performed in a Durrum amino acid analyser (model 500).

RESULTS

Oligomycin-Resistant Strains Used for Structural Analysis of the Dicyclohexylcarbodiimide-Binding Protein

Since the amino acid sequence of the dicyclohexylcarbodiimide-binding protein of the oligomycin-sensitive haploid D273-10B/A1 had been determined [11], all the proteins examined in this study were obtained from spontaneous oligomycin-resistant mutants of this strain. The mutants chosen represented the four mitochondrial genetic loci, *oli1*, *oli2*, *oli3* and *pho2*, based on their phenotypes (Table 2) and recombination data with standard oligomycin-resistant strains isolated in other laboratories (Table 4).

Recombination Frequencies of Oligomycin-Resistant Mutants in Intra-Locus and Inter-Locus Crosses

Even though the strains used for the measurement of recombination frequencies, were not isogenic either in their nuclear or mitochondrial DNA, the segregation of the resistance markers indicated that the input of mitochondrial DNA [18] in crosses involving CB11, D22 and D273-10B/A1 were not significantly different. This is shown in Table 3 where crosses of the wild-type CB11 and D22 to independent oligomycin-resistant isolates of D273-10B/A1 are seen to yield an approximately equal proportion of sensitive and resistant diploid segregents.

The mutated loci in the resistant strains derived from D273-10B/A1 were studied by quantitative re-

Table 3. *Segregation of oligomycin resistance in crosses involving D22, CB11 and D273*

Cross	Colonies scored	Number of oligomycin resistant colonies	Sensitives
			%
CB11 × D273-10B/A21	543	263	48.4
D22 × D273-10B/A21	144	61	42.4
D22 × D273-10B/A31	202	115	56.9
D22 × D273-10B/A32	194	94	48.5
D22 × D273-10B/A33	191	104	54.5

Table 4. *Intra-locus and inter-locus crosses*

Crosses	Colonies scored	Number of sensitive colonies	Fraction recombined
			%
oli1^R × *oli1*^{R}			
D273-10B/A21 × D22/A16	7265	0	0
D273-10B/A21 × CD31-3A	4979	3	0.06
D273-10B/A21 × CD32-9A	5814	8	0.14
D273-10B/A21 × CD33-7A	7231	9	0.12
D22/A16 × D273-10B/A31	6100	3	0.05
D22/A16 × D273-10B/A32	9045	6	0.07
D22/A16 × D273-10B/A33	5814	6	0.1
D273-10B/A31 × CD32-9A	8687	1[a]	0.01
D273-10B/A31 × CD33-7A	7880	0	0
D273-10B/A32 × CD33-7A	10635	0	0
oli2^R × *oli2*^{R}			
D273-10B/A48 × D22/A15	832	0	0
oli3^R × *oli3*^{R}			
D273-10B/A68 × D22/V61	2199	1	0.05
oli2^R × *oli1*^{R}			
D22/A15 × D273-10B/A21	175	16	9.1
D22/A15 × D273-10B/A31	164	21	12.8
D22/A15 × D273-10B/A32	211	11	5
D22/A15 × D273-10B/A33	217	19	8.7
D273-10B/A48 × D22/A16	138	7	5.1
oli3^R × *oli1*^{R}			
D273-10B/A68 × CD21-1A	2847	1	0.035

[a] Since the mutations in D273-10B/A31 and D273-10B/A33 have been shown in this study to cause the same amino acid substitution we cannot account for the single sensitive colony. A possible explanation is that the A31 or A33 allele may have a low frequency of reversion which produced a sensitive segregant.

combinational analysis in crosses to the standard testers; D22/A16 (*oli1*), D22/A15 (*oli2*) and D22/V61 (*oli3*). The results of the crosses are summarized in Table 4. No sensitive recombinants were found among diploids scored in the cross of D22/A16 to D273-10B/A21. This mutant also did not exhibit any cross-resistance to venturicidin and was therefore assigned to the *oli1* locus. In view of the rather large number of mitotic segregants scored (more than 7000), the results suggest that the mutation in D273-10B/A21 and D22/A16 are either in the same or very closely linked alleles. The three other strains (D273-10B/A31, D273-10B/A32 and D273-10B/A33) were also characterized as *oli1*-resistant mutants. These strains were not cross-resistant to venturicidin but produced a low percentage of sensitive recombinants when crossed to D22/A16 or D273-10B/A21 (0.06–0.14%). Crosses of the latter three mutants in pairwise combinations, failed to yield recombinants with the exception of one sensitive colony that was scored in the cross of D273-10B/A31 to CD33-9A (this is a constructed strain containing the mutated allele of D273-10B/A33). Based on these recombination data, the mutations in D273-10B/A31, A32 and A33 are either in the same or closely linked alleles. All three mutants, however, are genetically distinct from D22/A16 and D273-10B/A21.

Among the spontaneous resistant clones isolated from D273-10B/A1, several were found to be cross-resistant to venturicidin. This phenotype is characteristically associated with mutations in the *oli3* locus and one of the strains (D273-10B/A68) was chosen for further study. In the cross of D273-10B/A68 to D22/V61, a standard *oli3* resistant mutant [13], only one sensitive recombinatant was observed in over 2000 diploids scored. The mutated allele in D273-10B/A68 was therefore tentatively assigned to the *oli3* locus. It is of interest that in our studies, the frequency of recombination between *oli1* and *oli3* as exemplified by the cross of D273-10B/A68 to CD21-1A is considerably lower than reported by other laboratories. Using other *oli1* and *oli3* resistant strains, Lancashire et al. [13] and Trembath et al. [19] have measured recombination frequencies as high as 1.5% between the two loci.

One of the resistant mutants, D273-10B/A48 was found to have a mutation in *oli2*. This strain was not cross-resistant to venturicidin and did not yield sensitive recombinants when crossed to the *oli2* tester D22/A15. The assignment of the mutated allele in A48 to the *oli2* locus has been confirmed by crosses to other *oli2* resistant strains (Dr W. Lancashire, private communication).

Although we have not carried out an extensive analysis of inter-locus crosses, data based on rather small numbers of colonies scored indicate only weak linkage of the *oli1*-resistant alleles of D273-10B/A21, A31 and A33 with the *oli2* marker of D22/A15 (Table 4).

Sequence Analyses of the Dicyclohexylcarbodiimide-Binding Proteolipid Isolated from oli1, oli2 *and* oli3 *Resistant Mutants*

In order to identify possible changes in the primary structure of the dicyclohexylcarbodiimide-binding pro-

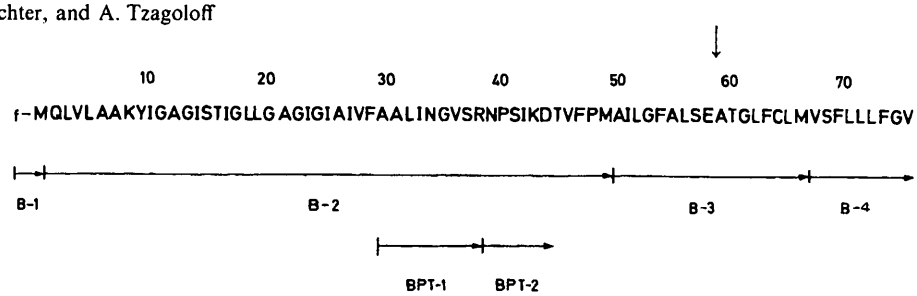

Fig. 1. *Amino acid sequence of the dicyclohexylcarbodiimide-binding protein of the ATPase-complex of* Saccharomyces cerevisiae, *strain D273-10B.* The fragments B-1 to B-4 were obtained after cleavage with cyanogen bromide. The fragments BPT-1 and BPT-2 are tryptic peptides prepared from a pepsin digest of fragment of B-2. The amino acid residues are represented by a one-letter code according to the convention of [20]. The inhibitor dicyclohexylcarbodiimide is covalently bound to the glutamyl residue 59 marked by an arrow [21]

Table 5. *Amino acid composition of cyanogen bromide fragment B-2 of the dicyclohexylcarbodiimide-binding protein from oligomycin-resistant strains*

Amino acid	Number of residues in strain						
	wild type	D273-10B A21	D273-10B A31	D273-10B A32	D273-10B A33	D273-10B A68	D273-10B A48
	mol/mol						
Aspartic acid	3.11	3.08	3.07	3.0	3.03	3.01	3.08
Threonine	1.97	2.07	2.09	1.9	2.08	2.13	2.08
Serine	2.92	2.86	3.16	2.8	3.08	3.13	2.96
Glutamic acid	0.99	1.17	1.10	1.0	1.18	0.97	1.15
Proline	1.95	2.25	2.02	1.9	2.21	2.13	2.11
Glycine	7.35	7.15	7.35	6.9	6.88	7.06	7.05
Alanine	6.85	6.33	6.67	6.5	6.58	6.33	6.38
Valine	3.06	3.33	3.69	3.5	3.62	3.35	3.38
Isoleucine	6.2	6.22	6.53	6.6	6.43	6.28	6.73
Leucine	5.24	5.09	5.18	4.9	4.86	4.87	5.04
Phenylalanine	1.84	1.79	1.97	2.3	1.78	1.56	1.72
Tyrosine	0.87	1.04	1.04	1.2	1.07	0.98	0.99
Lysine	1.96	1.97	1.82	1.7	1.79	2.02	1.93
Arginine	1.11	1.11	0.98	0.9	0.97	1.09	1.06
Homoserine	+	+	+	+	+	+	+

tein of the oligomycin-resistant mutants, the structural analyses were limited to the cyanogen bromide cleavage products which could be easily obtained following chromatography of the total digest on BioGel P30. Since amino acid analyses of the cyanogen bromide fragments were sufficiently sensitive to detect single amino acid substitutions, amino acid compositions were initially determined for each of the three cyanogen bromide fragments. In the case of the proteins isolated from the oligomycin-resistant strains, the compositions of fragments B-2 and B-4 (cf. Fig. 1) were in each case found to be indistinguishable from that of the wild-type within the limits of experimental error (Table 5), and they were not further sequenced. Fragment B-3 containing the sequence from residues 51–67, however, showed clear compositional differences from the corresponding wild-type peptide for the four *oli1* and one *oli3* mutants examined (Table 6). The fragment of the D273-10B/A21 protein had one less leucine and an additional phenylalanine. The B-3 fragments of the other three *oli1* resistant strains, D273-10B/A31, A32 and A33, each were missing the single cysteine, measured as cysteic acid, and had an extra serine. The composition of fragment B-3 from the *oli3* resistant mutant D273-10B/A68 indicated the loss of one leucine and the appearance of an extra valine which is absent in the wild-type peptide. No differences were noted in the composition of the B-3 fragment of the *oli2* resistant mutant D273-10B/A48.

The amino acid changes seen in the composition of the cyanogen bromide fragments were confirmed by solid-phase sequencing. The sequence data also permitted an exact identification of the residue position at which the amino acid substitution occurred. This is summarized in Fig. 2 for both the *oli1* and *oli3* resistant mutants. In D273-10B/A21 a phenylalanine is substituted for leucine at position 53 of the sequence. Mutants D273-10B/A31, A32 and A33 have identical substitutions of a serine for cysteine at position 65. The *oli3* mutation in D273-10B/A68 occurs at the intervening residue 57, where a leucine is replaced by a valine. The sequence of the fragment

Table 6. *Amino acid composition of cyanogen bromide peptide B-3 of the dicyclohexylcarbodiimide-binding protein from wild-type and mutant strains*
The integral numbers were obtained by sequence analysis. The values in parenthesis were determined by amino acid analysis

Amino acid	Number of residues in strain							
	D273-10B A1	D273-10B A21	D273-10B A31	D273-10B A32	D273-10B A33	D273-10B A68	D273-10B A48	M339-45 R17
	mol/mol							
Cysteic acid	1 (0.65)	1 (0.45)	**0** (–)	**0** (–)	**0** (–)	1 (0.62)	1 (0.68)	1 (0.44)
Threonine	1 (0.95)	1 (0.96)	1 (1.00)	1 (1.00)	1 (0.99)	1 (1.02)	1 (0.95)	1 (0.98)
Serine	1 (1.14)	1 (1.11)	**2 (1.98)**	**2 (1.90)**	**2 (1.89)**	1 (1.09)	1 (1.26)	1 (1.08)
Glutamic acid	1 (0.98)	1 (0.99)	1 (1.04)	1 (1.10)	1 (1.01)	1 (0.88)	1 (0.91)	1 (0.98)
Glycine	2 (2.04)	2 (1.98)	2 (2.00)	2 (1.99)	2 (1.99)	2 (2.06)	2 (2.20)	2 (2.03)
Alanine	3 (2.76)	3 (2.77)	3 (3.05)	3 (3.00)	3 (2.94)	3 (3.17)	3 (2.86)	3 (2.76)
Valine	0 (–)	0 (–)	0 (–)	0 (–)	0 (–)	**1 (1.17)**	0 (–)	0 (–)
Isoleucine	1 (1.16)	1 (0.98)	1 (0.95)	1 (0.92)	1 (1.03)	1 (0.93)	1 (1.27)	1 (1.00)
Leucine	4 (3.84)	**3 (3.14)**	4 (3.94)	4 (4.10)	4 (4.31)	**3 (3.01)**	4 (3.65)	4 (3.64)
Phenylalanine	2 (2.04)	**3 (3.02)**	2 (2.25)	2 (1.98)	2 (1.53)	2 (2.40)	2 (2.09)	2 (2.11)
Homoserine	1 (+)	1 (+)	1 (+)	1 (+)	1 (+)	1 (+)	1 (+)	1 (+)

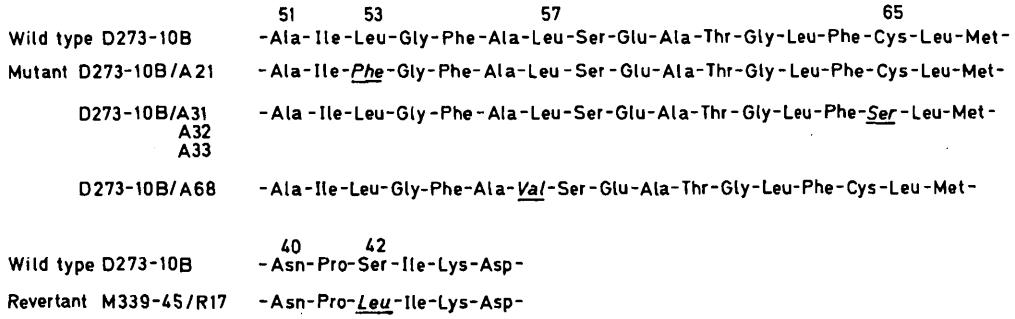

Fig. 2. *Altered amino acid sequences of the dicyclohexylcarbodiimide-binding protein from* oli1 *and* oli3 *resistant mutants and the mit⁻ revertant M339-45*

from *oli2*-resistant mutant D273-10B/A48, which was determined as a control, was found to be unaltered.

Analysis of the Dicyclohexylcarbodiimide-Binding Protein in a Revertant of a pho2 *Mutant*

Previous studies have shown that mutations in the *pho2* locus cause a deficiency of the oligomycin-sensitive ATPase [14]. Since mutations in this locus are closely linked to the *oli1* resistance marker and result in a molecularly altered form of the dicyclohexylcarbodiimide-binding protein, it was proposed that *pho2* mutational sites are also in the structural gene of this protein [14]. To verify this conclusion we have analyzed the amino acid composition and sequence of the protein isolated from a revertant of the *pho2* mutant M339-45. This revertant (M339-45/R17) is oligomycin resistant and grows on glycerol with a generation time longer than the wild-type. It is therefore probable that M339-45/R17 contains a second correcting mutation (supressor mutation). Genetic tests on the revertant have shown that the correcting mutation is cytoplasmically inherited and is probably intragenic since it was not readily genetically dissectable from the original mit⁻ mutation [14].

Amino acid analyses of the dicyclohexylcarbodiimide-binding protein of M339-45/R17 indicate that fragments B-3 (Table 6) and B-4 are indistinguishable from the wild-type. This was confirmed by sequencing the two fragments. The cyanogen bromide fragment B-2 containing residues 2–50, however, was found to differ from the corresponding wild-type peptide with respect to the number of serine and leucine residues (Table 7). A partial sequence of the B-2 fragment indicated that residues 2–35 are identical with the wild type. After cleavage of fragment B-2 with pepsin, a peptide containing residues 31–45 was isolated. This peptic fragment was digested with trypsin and the fragments BPT-1 and BPT-2 were obtained [11] (see Fig. 1). Fragment BPT-1 showed the same amino acid composition as the corresponding wild-type peptide, whereas fragment BPT-2 lacked serine and had an additional leucine (Table 7). Sequencing of this fragment revealed that the serine in position 42 is substituted by a leucine (Fig. 2). This serine/leucine exchange probably resulted from two separate muta-

Table 7. *Amino acid composition of fragments of the dicyclohexylcarbodiimide-binding protein from the* pho2 *revertant strain M339-45/R17*
The cyanogen bromide fragment B-2 was treated with pepsin. A peptide fragment containing residues 31–45 was further cleaved with trypsin yielding fragments BPT-1 and BPT-2. The procedures will be described in a forthcoming paper. The origin of the fragments is shown in Fig. 1

Amino acid	Number of residue in fragments					
	B-2		BPT-1		BPT-2	
	wild type	M339-45/R17	wild type	M339-45/R17	wild type	M339-45/R17
	mol/mol					
Aspartic acid	3.11	3.44	1.10	1.33	2.00	1.87
Threonine	1.97	2.07				
Serine	2.92	**1.25**	1.00	0.42	1.04	**0.00**
Glutamic acid	0.99	1.22				
Proline	1.95	2.10			0.78	0.98
Glycine	7.35	7.30	1.10	1.17		
Alanine	6.85	6.90	2.00	1.88		
Valine	3.06	3.20	1.04	0.96		
Isoleucine	6.20	6.50	1.05	1.06	1.04	1.13
Leucine	5.24	**6.00**	0.99	1.02	0.00	**0.96**
Phenylalanine	1.84	2.25				
Tyrosine	0.87	0.90				
Lysine	1.96	1.93			0.84	1.01
Arginine	1.11	0.98	0.98	0.91		
Homoserine	+	+				

tions, since the correcting mutation of the revertant appears to be located in the same gene as the original mit$^-$ mutation (see above) and since no other alterations could be detected in the revertant proteolipid.

DISCUSSION

Prior genetic and biochemical studies suggested that the *oli1* and *pho2* genetic markers of the yeast mitochondrial genome are located in the structural gene of the dicyclohexylcarbodiimide-binding protein [10, 14]. This was confirmed for the *oli1* marker by amino acid sequence analysis of the protein isolated from wild type and the *oli1*-resistant mutant D273-10B/A21 [22]. In the present study we have sequenced the dicyclohexylcarbodiimide-binding protein from several different *oli1* as well as *oli2* and *oli3* resistant mutants and from a revertant of a *pho2* mutant.

With the exception of the *oli2* resistant strain whose protein showed no compositional differences to the wild type, all the other proteins examined were found to have altered primary structures. The amino acid sequence data conclusively localize the *oli1*, *oli3* and *pho2* loci in the structural gene of the dicyclohexylcarbodiimide-binding protein of the yeast ATPase complex. The amino acid substitutions in the proteins of the *oli1* and *oli3* resistant strains can be explained by single base changes in the mitochondrial DNA. In the case of the *pho2* revertant, it is necessary to invoke two separate mutations in the same codon, the first causing the ATPase deficiency in the mit$^-$ mutant M339-45 and the second correcting the original mutation.

The identification of an *oli3* mutation in the structural gene of the dicyclohexylcarbodiimide-binding protein is somewhat unexpected. *oli3*-resistant mutants are characterized by cross-resistance to venturicidin [13]. The D273-10B/A68 strain was ascertained to be cross-resistant to venturicidin and to have a mutation tightly linked to another standard *oli3* mutation (D22/V61). The fact that D273-10B/A68 gave less than 0.1% sensitive recombinants when crossed to an *oli1*-resistant mutant is inconsistent with previous reports that the recombination frequencies between *oli1* and *oli3* range from 0.5–1.5% [13, 19]. There are several possible explanations for this discrepancy. (a) The D273-10B strain may in general have lower recombination frequencies than other yeast strains. (b) The structural gene of the dicyclohexylcarbodiimide-binding protein may have an insertion between the *oli1* and *oli3* loci in some yeast strains. (c) There may be another gene in yeast mitochondrial DNA which contains allele(s) that confer oligomycin and venturicidin resistance. The latter possibility gains some support from the studies of Groot-Obbink et al. [23] who have found an altered form of subunit 6 of the ATPase complex in a temperature-sensitive *oli3*-resistant mutant.

The identification of the amino acid substitutions in the polypeptide chains of the mutant proteins allow the recombination frequencies to be correlated with the distances between the altered amino acids. Crosses of the A31, A32 or A33 mutants to the A21 strain

produced 0.06–0.14% wild-type recombinants. When the results of the three crosses are pooled an average recombination frequency of 0.11% is obtained between the two mutations. The A21 strain has an amino acid substitution at position 53 which is 12 residues away from the amino acid exchange in A31, A32 and A33. Thus, the average recombination frequency per amino acid residue is approximately 0.01%. A similar value is obtained from the cross of A21 to A68 where the mutations are four amino acids apart. The percentage of sensitive recombinants scored in the latter cross was 0.05%. Assuming co-linearity of the structural gene and the absence of polar effects, the maximal recombination frequency for mutation in the most distant amino acids of this 76-residue polypeptide is 0.8%.

The dicyclohexylcarbodiimide-binding protein is a subunit of the membrane factor of the ATPase-complex of mitochondria [24–26], chloroplasts [27] and bacteria [28–30]. Although the precise function of this protein in the catalytic mechanism of the ATPase is not known, there is considerable evidence suggesting that it acts as a protonophore and that it provides binding sites for certain inhibitors of the enzyme. Dicyclohexylcarbodiimide, a potent inhibitor of oxidative phosphorylation and of the ATPase has been shown to bind covalently to the glutamyl residue at position 59 of the yeast protein [21]. There is also evidence that oligomycin reacts with an amino group on the protein to form a Schiff base. Criddle et al. [31] have reported that the dicyclohexylcarbodiimide-binding protein of yeast is labeled with [^{3}H]borohydride in the presence but not absence of oligomycin. Furthermore, the protonophoric activity of the protein was inhibited by oligomycin when it was isolated from wild type but not oligomycin-resistant mutants [32].

The isopolar substitutions leucine/phenylalanine (residue 69), leucine/valine (residue 42) identified in this study, each gives rise to the oligomycin-resistance phenotype. The functional properties of the ATPase complex do not appear to be affected by the mutations since the cell mass yield of the mutants is similar to that of the wild type. The mit$^-$ revertant, M339-45/R17, however, has a slightly longer generation time. Resistance of the mutant ATPase to oligomycin is most simply explained by an alteration of the binding site resulting in a lowered affinity for the inhibitor. The amino acid exchanges detected so far in the oligomycin-resistant strains span residues 42–65 of the polypeptide chain. Oligomycin-resistant mutants of *Neurospora crassa* have also been shown to have amino acid substitutions in the same region of the protein [33]. It is also of interest that some of the mutations occur at positions which are near to the glutamyl residue involved in dicyclohexylcarbodiimide-binding. These data suggest that the amino acid side chains of the residues affected in the mutant proteins may be located at the surface of the ATPase membrane factor (protein-lipid interphase) and that they are part of the binding site for oligomycin.

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