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The Dicyclohexylcarbodiimide-Binding Protein of the Mitochondrial ATPase Complex from *Neurospora crassa* and *Saccharomyces cerevisiae*

Identification and Isolation

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Incubation of mitochondria from *Neurospora crassa* and *Saccharomyces cerevisiae* with the radioactive ATPase inhibitor [^{14}C]dicyclohexylcarbodiimide results in the irreversible and rather specific labelling of a low-molecular-weight polypeptide. This dicyclohexylcarbodiimide-binding protein is identical with the smallest subunit (M_r 8000) of the mitochondrial ATPase complex, and it occurs as oligomer, probably as hexamer, in the enzyme protein.

The dicyclohexylcarbodiimide-binding protein is extracted from whole mitochondria with neutral chloroform/methanol both in the free and in the inhibitor-modified form. In *Neurospora* and yeast, this extraction is highly selective and the protein is obtained in homogeneous form when the mitochondria have been prewashed with certain organic solvents. The bound dicyclohexylcarbodiimide label is enriched in the purified protein up to 50-fold compared to whole mitochondria.

Based on the amino acid analysis, the dicyclohexylcarbodiimide-binding protein from *Neurospora* and yeast consists of at least 81 and 76 residues, respectively. The content of hydrophobic residues is extremely high. Histidine and tryptophan are absent. The N-terminal amino acid is tyrosine in *Neurospora* and formylmethionine in yeast.

The mitochondrial ATPase complex has been resolved in the soluble ATPase, F_1, which provides the catalytic sites for the adenine nucleotides, and in the membrane-integrated part, F_0, which is insolved in the transport of protons across the mitochondrial inner membrane (for review see [1–3]). Inhibitors as oligomycin [4,5] and dicyclohexylcarbodiimide [6] have been found to act on the membrane factor, F_0 [7,8] and to block the enzymatic activity of the whole complex as well as the proton permeability of F_1-depleted membranes [9–11].

Dicyclohexylcarbodiimide reacts irreversibly and quite specifically with a polypeptide of low molecular weight as has been shown first with beef heart mitochondria in 1971 [12]. This dicyclohexylcarbodiimide-binding protein has been classified as a proteolipid [13] since it is extracted by chloroform/methanol from whole mitochondria [12] as well as from oligomycin-sensitive ATPase [14]. In the meantime dicyclohexylcarbodiimide-binding proteins were isolated from *Escherichia coli* plasma membrane [15,16], lettuce chloroplasts [17] and rat liver mitochondria [18]. For the *E. coli* protein a molecular weight of about 8000 and an extremely hydrophobic amino acid composition was reported [15].

During studies on the biogenesis of the mitochondrial ATPase complex from yeast [19,20] and *Neurospora crassa* [21–23], a proteolipid subunit was observed with a molecular weight of about 8000 and a very hydrophobic amino acid composition. Thus, it was a distinct possibility that this subunit is identical in both micro-organisms and that it corresponds to the dicyclohexylcarbodiimide-binding proteins mentioned above. A more convincing proof for such an identity appeared to be required, however, since in yeast this subunit is synthesized by the mitochondrial protein-synthesizing system [20] whereas the possibly corresponding subunit of the *Neurospora* ATPase is synthesized outside the mitochondria on cytoplasmic ribosomes [23]. This suggested for the first time that a homologeous polypeptide may be synthesized at different sites of the cell in different organisms [24].

Enzyme. ATPase or ATP phosphohydrolase (EC 3.6.1.3).

The present communication describes experiments indicating indeed that the 8000-M_r subunit of the mitochondrial ATPase complex from both *Neurospora* and yeast reacts specifically and irreversibly with the inhibitor dicyclohexylcarbodiimide. Different lines of evidence indicate that this subunit occurs in the ATPase complex as oligomer, probably as hexamer. A simple procedure for purification of the protein from the two micro-organisms is presented. The present results provide the basis for a further analysis of the dicyclohexylcarbodiimide-binding protein in *Neurospora* and yeast. The amino acid sequences, the identification of the dicyclohexylcarbodiimide-binding residue and the identification of amino acid substitutions in oligomycin-resistant mutants will be communicated later. Preliminary reports on these latter topics already have been published [25–27].

MATERIALS AND METHODS

Growth and Labelling of Cells

Neurospora crassa, wild type SL74A (Fungal Genetic Stock Center, Arcata, Calif., U.S.A.), was grown under aeration in Vogel's minimal medium plus 2% sucrose at 25 °C into late log phase [28]. 10 to 15 g wet weight of hyphae were obtained in 1 l culture medium after a growth period of 18 to 20 h. Cellular proteins were homogeneously labelled with [^{3}H]leucine (New England Nuclear Co., Boston, Mass., U.S.A.) (55 Ci/mmol) by growing cells of a 15-h culture for one doubling time in the presence of the radioactive amino acid [29].

Commercial baker's yeast, *Saccharomyces cerevisiae*, [30] (National Yeast Products, New York, N.Y., U.S.A.) and strain D-1827 leu$^-$ auxotrophic for leucine (Genetic Institute, University Munich) were used for isolation of mitochondria and analysis of the dicyclohexylcarbodiimide-binding protein. For labelling of cellular proteins with [^{3}H]leucine, strain D-1827 leu$^-$ was grown in 1 l of YPD medium (1% yeast extract, 2% peptone, 3% glucose) at 30 °C into stationary phase (1 to 2×10^8 cells/ml). Cells were then diluted fivefold with fresh YPD medium and grown for another 2 to 3 h. After a wash with minimal medium (0.67% yeast nitrogen base) plus 3% glycerol, cells were resuspended in 0.2 l of this medium and starved for 15 min. Then [^{3}H]leucine was added and 30 min later a chase of unlabelled leucine (2 mM) for 3 h.

Isolation of Mitochondria and ATPase Complex

Neurospora mitochondria were prepared by differential centrifugation after disruption of the hyphae by a grind mill [28]. Yeast cells were mechanically broken with glass beads in a Bronwill MSK cell homogenizer [31]. For large scale preparation of yeast mitochondria, cells were frozen in liquid nitrogen and then disrupted in a Waring blendor [32]. Submitochondrial particles from beef heart were obtained from the total mitochondrial fraction isolated according to [34].

The ATPase complex of *Neurospora* and yeast was isolated by immunoprecipitation with antiserum to F_1 ATPase, essentially as described [35]. Antisera to F_1 ATPase and the 8000-M_r subunit were obtained from rabbits. F_1 protein (5 mg per ml 10 mM Tris-acetate, 1 mM EDTA, 2 mM ATP, pH 7.5) and 8000-M_r subunit (2 mg per ml 2% sodium dodecylsulfate) were emulsified with two volumes of complete Freund's adjuvant. The animals were injected subcutaneously into the back, first with 0.6 ml and four weeks later with 1.2 ml. The sera were collected two weeks after the second injection. Oligomycin-sensitive ATPase was prepared from beef heart mitochondria according to Tzagoloff et al. [36].

Labelling with [^{14}C]Dicyclohexylcarbodiimide

Mitochondria were suspended in 10 mM Tris-acetate, pH 7.5, at a protein concentration of 5 to 10 mg per ml at 0 °C. [^{14}C]Dicyclohexylcarbodiimide was added as an ethanolic solution up to 50 µl per ml mitochondrial suspension. When not otherwise indicated, about 10 nmol dicyclohexylcarbodiimide was added per mg protein. The incubation period was 4 h throughout the experiments.

For determination of bound [^{14}C]dicyclohexylcarbodiimide label, the incubation mixture was poured into 9 volumes of methanol, and the precipitated proteins were washed four times with chloroform/methanol/diethylether (2/1/12). In some experiments, an aliquot of the incubation mixture was diluted 10-fold with 10 mM Tris-acetate, pH 7.5, and the membranes were sedimented at 226 000 × *g* for 30 min. The sediment was washed five times with chloroform/methanol/ether (2/1/12). Higher specific dicyclohexylcarbodiimide contents (nmol/mg protein) were obtained in the latter case since unlabelled proteins are removed by the centrifugation step. The dried proteins were dissolved in 2% sodium dodecylsulfate. Aliquots were used for determination of protein and radioactivity. Before gel electrophoresis, mercaptoethanol and Tris base were added to a final concentration of 5% and 0.1 M, respectively.

Bound dicyclohexylcarbodiimide also was determined after washing the mitochondria with phospholipids [7]. Sedimented mitochondria were resuspended in 10 mM Tris-acetate, pH 7.5, and then diluted with 10 volumes of a buffer containing 2% Asolectin, 25%

sucrose and 10 mM Tris-acetate, pH 7.5. The mixture was centrifuged for 60 min at 226000 × *g*. This wash was repeated five times. A sample of the mitochondria resuspended in the 10 mM Tris buffer was withdrawn for determination of radioactivity and ATPase activity.

Isolation of the Dicyclohexylcarbodiimide-Binding Protein

Neurospora mitochondria were sedimented by a 30-min centrifugation at 226000 × *g* after a 10-fold dilution of the suspension with 10 mM Tris-acetate, pH 7.5. The sediment was homogenized with at least 20 volumes of chloroform/methanol (2/1). Then four volumes of ice-cold diethylether were added. The mixture was stirred for 10 min at 0 °C and then centrifuged for 5 min at 27000 × *g*. The sediment was again homogenized in a small volume of chloroform/methanol (2/1), and four volumes of ice-cold ether were added. After centrifugation, the washing with chloroform/methanol/ether was repeated a third time. After this prewash the mitochondrial protein was homogenized with 2 to 3 volumes of chloroform/methanol (2/1) and stirred for 10 min at room temperature. The mixture was centrifuged for 10 min at 8000 × *g*. The supernatant was saved, and the sediment was extracted three additional times with a small volume of chloroform/methanol. The combined extracts were reduced to near dryness in a flash evaporator at 30 °C. The residue was taken up in a small volume of chloroform/methanol, and the protein was precipitated with four volumes of ether. The protein was dissolved in chloroform/methanol and precipitated with ether until it was completely soluble in chloroform/methanol. Insoluble material was removed each time by centrifugation for 10 min at 8000 × *g*.

The dicyclohexylcarbodiimide-binding protein of yeast was isolated by the same procedure, but instead of the first prewash with chloroform/methanol/ether, a concentrated mitochondrial suspension (10 mM Tris-acetate, pH 7.5) was poured into nine volumes of ice-cold methanol [20].

Analytical Procedures

ATPase activity was determined at 37 °C and pH 8.5 with 10 mM ATP as substrate [33]. The formation of inorganic phosphate was found to be linear for at least 10 min when 0.1 or less ATPase units (μmol P_i/min) per ml were present during the assay. F_1 ATPase was released from the membrane by shaking a mitochondrial suspension for 5 min with chloroform [35]. The activity of F_1 ATPase was measured in the upper water phase obtained after a 5-min centrifugation at 12000 × *g*, and the activity was related to the protein present in the original mitochondrial suspension.

Protein was determined by the Lowry method [37] using bovine serum albumin as standard.

^3H and ^{14}C radioactivities were measured in a Packard Tri-Carb liquid scintillationspectrometer. All samples were first dissolved in 0.5 ml dodecylsulfate buffer (1% dodecylsulfate, 0.1 M Tris-acetate, pH 8), and then 15 ml of the scintillation mixture (600 ml toluene, 400 ml ethyleneglycol monomethyl ether, 6 g Packard Permablend III) were added. Counting efficiencies were 45.7% for ^{14}C and 24.5% for ^3H radioactivity.

Dodecylsulfate-gel electrophoresis was performed in horizontal gel slabs (12.5% or 15% acrylamide plus 1/30 bisacrylamide) which had been polymerized and stored in 0.5% dodecylsulfate, 0.1 M Tris-acetate, pH 8 [38]. Gels were stained over night with 0.25% Coomassie blue R-250 in acetic acid/methanol/water (1/5/5), destained with the same solvent and stored in 10% acetic acid [39]. For determination of radioactivity, the unstained gels were cut into 1-mm slices, and the slices were then eluted with 0.5 ml dodecylsulfate buffer at 50 °C over night.

The determination of the apparent molecular weight of the smallest ATPase subunit from *Neurospora* [21] and yeast [19] by dodecylsulfate-gel electrophoresis already has been described. Recently, molecular weights of 8300 and 7790 were obtained by amino acid sequence analysis of the proteolipid from *Neurospora* and yeast, respectively [27]. The apparent molecular weights of the dicyclohexylcarbodiimide-binding protein fractions from beef heart were calculated from their electrophoretic mobility on dodecylsulfate gels using the subunits of the *Neurospora* ATPase complex as standards [21].

Before amino acid analysis the dicyclohexylcarbodiimide-binding protein was submitted to a chromatography on Bio-Gel P-30, minus 400 mesh, in the presence of 80% formic acid. Aliquots of the protein were then hydrolysed for 24, 48, 72 and 120 h in 5.7 M HCl at 105 °C. Cysteine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation [40]. The amount of the individual residues was measured with a Durrum 500 amino acid analyser.

N-terminal amino acids were determined by the dansylation method [41]. A N-terminal formyl group was removed by a 4-h incubation of the protein in 0.5 M methanolic HCl at room temperature [42].

Materials

[^{14}C]Dicyclohexylcarbodiimide was synthesized from [^{14}C]urea (New England Nuclear Co., Boston, Mass., U.S.A.) (20 Ci/mol) via dicyclohexylurea [43, 44].

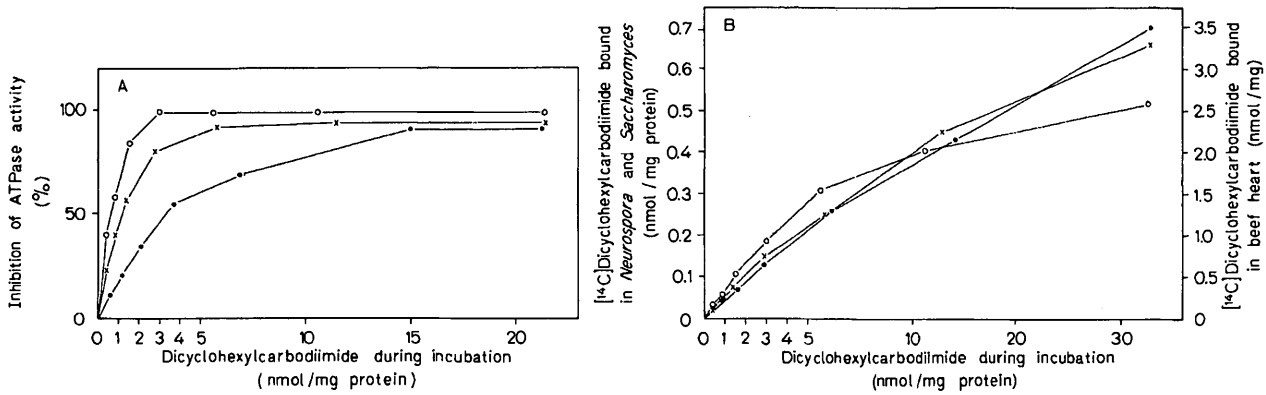


Fig. 1. *Inhibition of mitochondrial ATPase and recovery of bound label at various concentrations of [^{14}C]dicyclohexylcarbodiimide.* (A) Inhibition of mitochondrial ATPase from *Neurospora crassa* (●——●), *Saccharomyces cerevisiae* (×——×) and beef heart (○——○) at various concentrations of dicyclohexylcarbodiimide. Mitochondria or submitochondrial particles (beef heart) suspended (5 mg per ml) in 10 mM Tris-acetate, pH 7.5, were incubated for 4 h at 0 °C with the indicated amounts of dicyclohexylcarbodiimide. Aliquots were withdrawn for determination of ATPase activity. (B) Recovery of bound [^{14}C]dicyclohexylcarbodiimide label in mitochondrial protein from *Neurospora crassa* (●——●), *Saccharomyces cerevisiae* (×——×) and beef heart (○——○) at various concentrations of [^{14}C]dicyclohexylcarbodiimide. Mitochondria or submitochondrial particles (beef heart) were incubated with [^{14}C]dicyclohexylcarbodiimide as described above. Aliquots of 0.1 ml were diluted 10-fold with 10 mM Tris-acetate, pH 7.5, and the mitochondria were sedimented for 30 min at 226000 × *g*. The mitochondria were washed with organic solvents (see Methods) in order to remove unbound [^{14}C]dicyclohexylcarbodiimide, and were then dissolved in dodecylsulfate buffer. Aliquots were used for determination of protein and ^{14}C radioactivity

RESULTS

Analysis of the Action of Dicyclohexylcarbodiimide at the Level of Whole Mitochondrial Membranes

The inhibition of the ATPase activity of mitochondria from *Neurospora* and yeast at various dicyclohexylcarbodiimide concentrations is shown in Fig. 1 A. The dose dependence of the inhibition is different in the mitochondria from these two microorganisms, and higher dicyclohexylcarbodiimide concentrations are necessary than for inhibition of the beef-heart enzyme. After incubation with dicyclohexylcarbodiimide for 4 h at 0 °C, half-maximal inhibition is found at 3–4, 1–1.5 and 0.4–0.5 nmol dicyclohexylcarbodiimide per mg protein for the *Neurospora*, yeast and beef-heart ATPase, respectively. The concentrations necessary for maximal inhibition are 10–15, 4–6 and 2–3 nmol per mg protein, respectively. The values measured for the beef heart ATPase are similar to those already reported in the literature [7, 9].

The binding of dicyclohexylcarbodiimide was analysed by means of the ^{14}C-labelled compound which had been synthesized from [^{14}C]urea at a reasonably high specific radioactivity (20 Ci/mol). After incubation of mitochondria with various inhibitor concentrations, bound [^{14}C]dicyclohexylcarbodiimide was determined by extensively washing the mitochondria with organic solvents which do not extract the proteolipid (see Methods). A low percentage (5% and less) of the added ^{14}C radioactivity is found to be bound to whole mitochondrial protein from *Neurospora* and yeast (Fig. 1 B). At corresponding inhibitor concentrations, the binding of dicyclohexylcarbodiimide is up to seven-times higher in beef-heart membranes. In all three types of mitochondria the labelling increases more and more with higher dicyclohexylcarbodiimide concentrations even when maximal inhibition of ATPase activity has been achieved. But also at lower dicyclohexylcarbodiimide concentrations no clear correlation is observed between inhibition of ATPase activity and binding of ^{14}C label.

Bound [^{14}C]dicyclohexylcarbodiimide also could be recovered after repeated washes of the mitochondria with phospholipids (Table 1). After five washes an amount of residual ^{14}C label was found in the membrane which was only slightly higher (about 30%) than after the wash with organic solvent. But nevertheless, the ATPase activity remained maximally inhibited. The ATPase activity could be reactivated, however, by incubation of the mitochondria with chloroform. This treatment is known to release the F_1 ATPase from the membrane [35, 45]. This result indicates that the inhibitor acts on the membrane factor of the ATPase complex and not on the F_1 component, as already has been shown for the beef-heart ATPase [7, 8]. It shows further that the bound dicyclohexylcarbodiimide is the only ^{14}C-labelled reaction product which can be responsible for the inhibitory effect. The result does not exclude the possibility, however, that dicyclohexylcarbodiimide leads to the formation of an inhibitory product, e.g. by a condensation reaction [54, 55], which is not detected by bound ^{14}C radioactivity (see Discussion).

When *Neurospora* and yeast mitochondria were incubated with up to 10 nmol dicyclohexylcarbo-

Table 1. *Irreversible inhibition of mitochondrial ATPase from* Neurospora crassa *and* Saccharomyces cerevisiae *by dicyclohexylcarbodiimide as demonstrated by extensive washing with phospholipids*
Mitochondria from *Neurospora* and *Saccharomyces* were incubated for 4 h at 0 °C with [^{14}C]dicyclohexylcarbodiimide, $(cHxN)_2C$, 11.5 and 8.4 nmol per mg mitochondrial protein, respectively. The mitochondria were washed with phospholipid medium as detailed under Methods. ATPase activity was determined for mitochondria suspended in 10 mM Tris buffer, pH 7.5, as well as for aliquots treated with chloroform. [^{14}C]Dicyclohexylcarbodiimide radioactivity, $(cHxN)_2C$, was measured in the original suspensions and in aliquots washed in addition with organic solvents (see Methods)

Fractions	ATPase activity		[^{14}C]Radioactivity	
	mitochondria	mitochondria treated with chloroform	mitochondria	mitochondria washed with organic solvents
	$\mu mol\ P_i \times min^{-1} \times mg\ protein^{-1}$		nmol $(cHxN)_2C$/mg protein	
Neurospora crassa				
Mitochondria	2.2	2.2	–	–
Mitochondria incubated with $(cHxN)_2C$	0.3	2.3	11.5	0.6
Mitochondria after five washes with phospholipids	0.5	3.3	0.67	0.5
Saccharomyces cerevisiae				
Mitochondria	10.3	8.9	–	–
Mitochondria incubated with $(cHxN)_2C$	0.5	9.1	8.4	0.42
Mitochondria after five washes with phospholipids	0.6	9.3	0.45	0.3

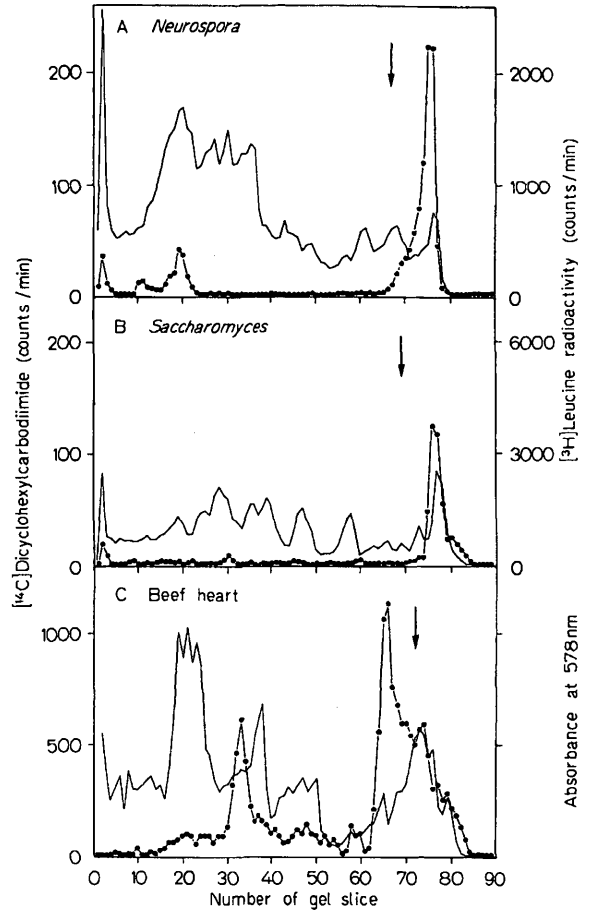


Fig. 2. *Recovery of bound [^{14}C]dicyclohexylcarbodiimide label in mitochondrial protein after dodecylsulfate-gel electrophoresis.* The mitochondria from (A) *Neurospora crassa* and (B) *Saccharomyces cerevisiae* containing a homogeneous [^{3}H]leucine label were incubated with [^{14}C]dicyclohexylcarbodiimide as described in the legend of Table 3. Submitochondrial particles from (C) beef heart were reacted with 2.5 nmol [^{14}C]dicyclohexylcarbodiimide per mg protein. The closed circles (●——●) represent the [^{14}C]dicyclohexylcarbodiimide radioactivity. The proteins from *Neurospora* and *Saccharomyces* were monitored by their [^{3}H]leucine radioactivity (continuous line). The distribution of the beef heart protein was determined by densitometry of the stained proteins which had been separated in parallel on the same gel slab. The arrow indicates the migration distance of cytochrome *c*

diimide per mg protein, the bound ^{14}C label is recovered at 80 to 100% in a fraction from gel electrophoresis corresponding to a polypeptide of about 8000 molecular weight. This applies to the dicyclohexylcarbodiimide label resistant to the wash with organic solvents (Fig. 2A and B) as well as to the label resistant to the wash with phospholipids (data not shown). At higher inhibitor concentrations during incubation, the labelling of the 8000-M_r polypeptide increases (see Fig. 5), and other labelled products appear at higher molecular weight range. In beef-heart membranes several labelled products already appear at much lower inhibitor concentrations and below the range where maximum inhibition of ATPase activity is obtained. At 2.5 nmol dicyclohexylcarbodiimide per mg protein during incubation, labelling is most pronounced in a polypeptide of 14000-M_r (Fig. 2C). Other labelled products occur at positions corresponding to polypeptides of molecular weights of 8000 and 45000. These multiple fractions may represent aggregates of one labelled species, since only one labelled polypeptide is observed when the beef-heart membranes are separated by gel electrophoresis under acidic conditions, as published elsewhere [46].

Association of the Dicyclohexylcarbodiimide-Binding Protein with the ATPase Complex

The ATPase complex of *Neurospora* and yeast was isolated from whole mitochondria by immunoprecipi-

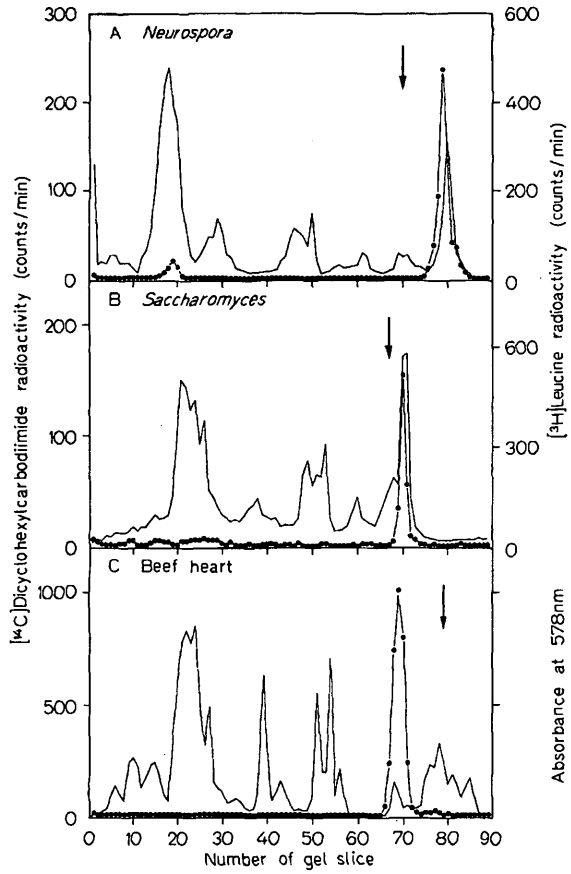


Fig. 3. *Recovery of bound [^{14}C]dicyclohexylcarbodiimide label after dodecylsulfate-gel electrophoresis of the ATPase complex from (A)* Neurospora crassa, *(B)* Saccharomyces cerevisiae *and (C) beef heart. Neurospora* mitochondria labelled with [^{3}H]leucine (273000 counts × min^{-1} × mg protein^{-1}) were incubated with 11 nmol [^{14}C]dicyclohexylcarbodiimide per mg, and contained 0.39 nmol bound per mg. Yeast mitochondria labelled with [^{3}H]leucine (857000 counts × min^{-1} × mg protein^{-1}) were reacted with 8.5 nmol [^{14}C]dicyclohexylcarbodiimide, and contained 0.27 nmol bound per mg. Both mitochondria were dissolved with Triton X-100 and the ATPase complex was immunoprecipitated with antiserum to the F_1 ATPase. Submitochondrial particles from beef heart were incubated with 2.5 nmol [^{14}C]dicyclohexylcarbodiimide per mg protein, and 1 nmol was bound per mg. The oligomycin-sensitive ATPase was purified from these particles according to [36]. (●—●) [^{14}C]Dicyclohexylcarbodiimide radioactivity; (——) [^{3}H]leucine radioactivity (*Neurospora* and yeast) or Coomassie blue stain (beef heart). The arrow indicates the migration distance of cytochrome *c*

tation with antiserum to F_1 ATPase [19,21]. The mitochondria were obtained from cells grown in the presence of [^{3}H]leucine and they were then reacted with [^{14}C]dicyclohexylcarbodiimide. After dodecylsulfate-gel electrophoresis of the immunoprecipitated ATPase protein, the ^{14}C label is found to comigrate with the smallest ATPase subunit of about 8000 M_r (Fig. 3A and B). The dicyclohexylcarbodiimide label and the protein label do not exactly coincide. This result will be further discussed below (see also Fig. 5).

Table 2. *Recovery of bound [^{14}C]dicyclohexylcarbodiimide label in the ATPase complex from* Neurospora crassa *immunoprecipitated at different concentrations of Triton X-100*
Mitochondria labelled homogeneously with [^{3}H]leucine (520000 counts × min^{-1} × mg^{-1}) were incubated for 4 h at 0 °C with [^{14}C]-dicyclohexylcarbodiimide, $(cHxN)_2C$, (11.5 nmol per mg protein). Then 10 mM Tris-acetate, pH 7.5, and 10% Triton X-100 was added to yield a protein concentration of 2 mg per ml and the concentrations of the detergent as indicated in the table. After mixing, the samples were immediately centrifuged for 5 min at 12000 × *g*. 0.2 ml of the supernatant were incubated with an equal volume of antiserum to F_1 ATPase for 4 h at 0 °C. The immunoprecipitates were washed three times with 10 mM Tris-acetate, pH 7.5, and dissolved in dodecylsulfate buffer. The amount of precipitated ATPase protein was determined by the [^{3}H]leucine label. The amount of $(cHxN)_2C$ bound to the starting mitochondria (0.4 nmol per mg protein) was taken as 100%

Concentration of Triton X-100	ATPase protein	ATPase-bound [^{14}C]$(cHxN)_2C$ label	Specific content of ATPase
%	% of total		nmol $(cHxN)_2C$/ mg protein
0.1	18.4	66	1.49
0.2	24	95	1.65
0.5	19.5	95	2.04
0.8	15.9	63	1.66
1	16	59	1.54
2	14.8	43	1.2
5	15.4	34	0.91

Because of the double-labelling technique, the inhibitor to protein ratio can be calculated for the 8000-M_r subunit. The ^{14}C/^{3}H ratios of 0.8 and 0.18 calculated for the whole peak correspond to 18 and 13 nmol inhibitor bound per mg protein in *Neurospora* and yeast, respectively. The beef heart enzyme was isolated from [^{14}C]dicyclohexylcarbodiimide labelled submitochondrial particles as oligomycin-sensitive ATPase [36]. Dodecylsulfate-gel electrophoresis reveals that only one dicyclohexylcarboddimide-modified protein of about 14000 M_r is present (Fig. 3C) as already has been reported by Stekhoven et al. [14]. Thus, the dicyclohexylcarbodiimide-binding protein from beef heart shows a different electrophoretic behaviour on dodecylsulfate gels compared to the *Neurospora* and yeast protein. It has to be noticed, however, that the ^{14}C label can not be correlated with a polypeptide band in the densitometric trace. As published elsewhere [46], the electrophoretic mobility of the beef-heart protein and especially of the inhibitor-modified form is unusual. Protein chemistry studies indicate that this protein has also a molecular weight of about 8000 [46].

Neurospora mitochondria (2 mg/ml) are completely solubilized at concentrations of Triton X-100 higher than 0.5% [21,22]. This allows a quantitative immunoprecipitation of the ATPase complex. Since antibodies

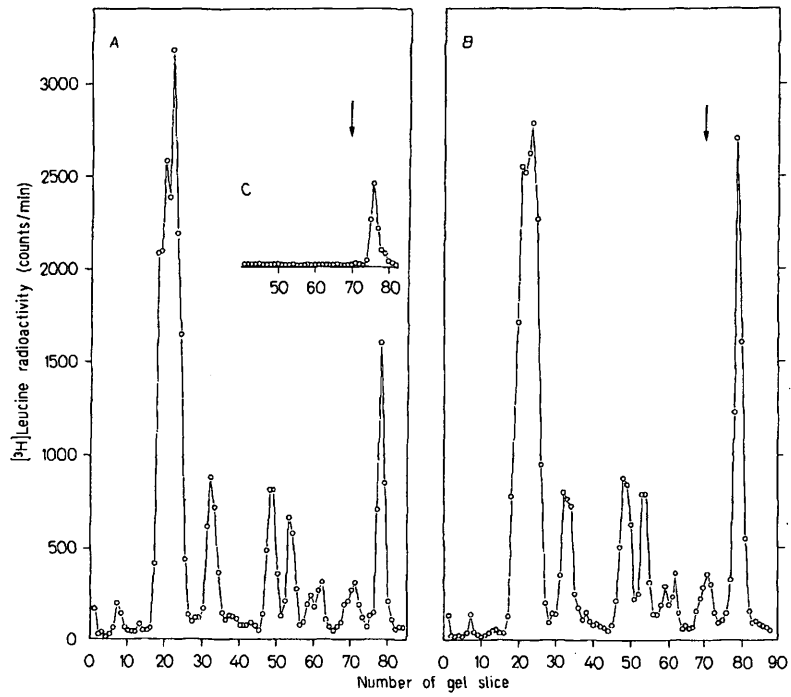
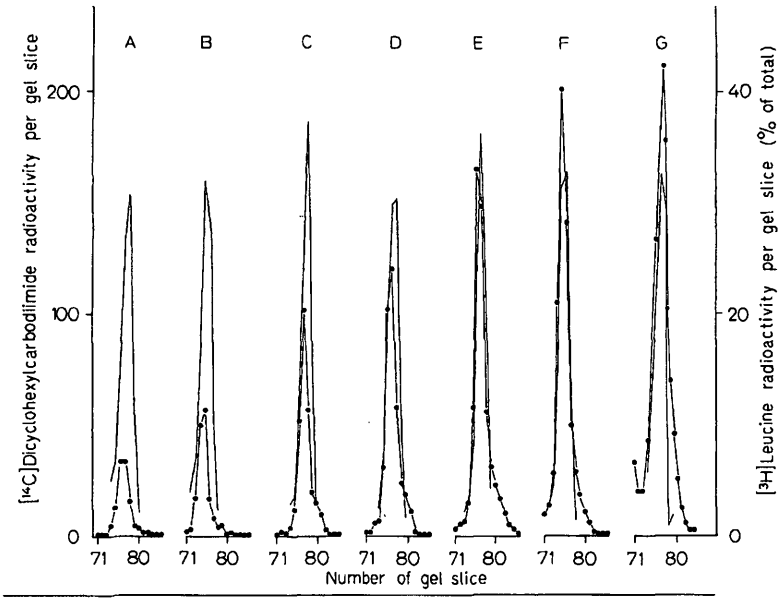


Fig. 4. *Partial loss of the 8000-*M_r *subunit after solubilisation of the ATPase complex with 1% Triton X-100. Neurospora* mitochondria labelled homogeneously with [^{3}H]leucine (1.2×10^6 counts $\times$ min^{-1} $\times$ mg^{-1}) were dissolved with 1% Triton X-100 at a protein concentration of 2 mg per ml. (A) One aliquot was incubated with antiserum to F_1 ATPase. (B) A second sample of the dissolved mitochondria was treated with antiserum to the 8000-M_r subunit together with antiserum to F_1 ATPase. The immunoprecipitates were sedimented by centrifugation and analysed by dodecylsulfate-gel electrophoresis. (C) The insert shows the immunoprecipitate which was obtained when the supernatant from the anti-F_1 precipitate was further treated with antiserum to the 8000-M_r subunit. The ^{3}H radioactivities of gel slices 1 to 50 were zero and are not shown in the figure. The [^{3}H]leucine protein label (O——O) is drawn in the same scale for all three immunoprecipitates. The arrow indicates the migration distance of cytochrome *c*

directed to only the F_1 component have been used, the possibility has to be considered that the dicyclohexylcarbodiimide-binding subunit is partially dissociated from the complex in the presence of the detergent and therefore not completely recovered in the immunoprecipitate. Table 2 shows the effect of different Triton X-100 concentrations on the yield of ^{14}C label as well as on the yield of the immunoprecipitated ATPase protein which is monitored by a homogeneous [^{3}H]leucine label. A high specific dicyclohexylcarbodiimide content (2 nmol/mg protein) and a high yield of ^{14}C label (95%) is obtained at 0.5% Triton X-100. Under this condition 19.5% of total mitochondrial protein is recovered, but the ATPase protein shows some minor contaminants [22]. At lower detergent concentration the mitochondria are incompletely dissolved and the immunoprecipitate is heavily contaminated [22]. When the Triton concentration is increased from 0.8 to 5%, the yield of ^{14}C label and also the specific inhibitor content of the immunoprecipitated ATPase complex drastically declines. This result leads to the conclusion that the dicyclohexylcarbodiimide-binding subunit is released from the *Neurospora* ATPase complex at higher detergent concentrations. The protein cleaved-off at 1% Triton X-100 is detected when the immunoprecipitation is performed with antiserum to the 8000-M_r subunit. Fig. 4C demonstrates that part of this polypeptide is recovered in free form when the supernatant from the anti-F_1 immunoprecipitate is incubated further with antiserum directed to this subunit only. Furthermore, a substantially higher amount of the 8000-M_r subunit (17.8% of the total [^{3}H]leucine label) is obtained when the solubilized mitochondria are treated with antisera to both F_1 and the 8000-M_r polypeptide (Fig. 4B). The 95% recovery of the ^{14}C label observed at a concentration of 0.5% Triton X-100 indicates that *in situ* the whole dicyclocarbodiimide-binding protein is associated with the ATPase complex.

The 8000-M_r subunit comprises a considerable amount of the total ATPase protein as deduced from the distribution of the homogeneous [^{3}H]leucine label after dodecylsulfate-gel electrophoresis. When the complex has been dissolved in the presence of 1% Triton X-100, the 8000-M_r subunit contains 13.1 $\pm$ 2.6% of the [^{3}H]leucine label of an anti-F_1 immunoprecipitate [21] and 17.8% of the [^{3}H]leucine label of an immunoprecipitate obtained with antisera to both F_1 ATPase and the 8000-M_r protein (Fig. 4B). The amount of this subunit, however, is overestimated by



fraction	[14C]Dicyclohexylcarbodiimide in sample						
	A	B	C	D	E	F	G
	nmol/mg Lowry protein						
incubation mixture	15	30	60	115	240	310	460
mitochondria	0.46	0.72	1.43	2.2	3.4	3.8	4.7
8000-M_r subunit	23	34	54	75	99	118	145

Fig. 5. *Labelling with [^{14}C]dicyclohexylcarbodiimide of the 8000-*M_r *subunit of the ATPase complex from* Neurospora crassa *at various dicyclohexylcarbodiimide concentrations. Neurospora* mitochondria labelled homogeneously with [^{3}H]leucine (273000 counts × min^{-1} × mg protein^{-1}) were incubated with the amounts of [^{14}C]dicyclohexylcarbodiimide indicated in the table beneath. In an aliquot of samples A to G, the label bound to whole mitochondrial protein was determined. The ATPase complex was immunoprecipitated by antibodies to F_1 ATPase from each sample and then separated by dodecylsulfate-gel electrophoresis. Only the gel-electrophoretic fractions corresponding to the 8000-M_r subunit (gel slices 71 to 85) were analysed for [^{3}H]leucine (——) and [^{14}C]dicyclohexylcarbodiimide radioactivity (●——●). The total [^{3}H]leucine radioactivity of the 8000 M_r fraction, which varied between 370 and 700 counts/min, was taken as 100%, and the ^{3}H and ^{14}C radioactivities present in the individual gel slices were related to these normalized values. The [^{3}H] leucine radioactivity of the 8000-M_r polypeptide was used to calculate the amount of Lowry protein, assuming that the specific ^{3}H radioactivity (counts × min^{-1} × mg Lowry protein^{-1}) of this polypeptide is 1.6-times higher than that of whole mitochondrial protein (see Table 4)

the [^{3}H]leucine label due to the extremely high leucine content (1.33 μmol/mg) (see Table 4). The leucine content of the F_1 protein from *Neurospora* is 1.6-times lower (0.83 μmol/mg) [47]. As a first approximation the F_1 protein may be assumed to be representative for the leucine content of the whole complex. Then it can be calculated [38] that the 8000-M_r subunit comprises 8.2% and 11%, respectively, of the ATPase protein immunoprecipitated with the antisera described above. These values correspond to a molecular weight of 42000 and 55000, respectively, in an ATPase complex of about 500000 M_r [1]. Accordingly, 5 to 7 molecules of the 8000-M_r polypeptide are present in the ATPase complex.

As has been shown in Fig. 3 A and B, the ^{14}C label is slightly displaced compared to the protein label of the 8000-M_r subunit during gel electrophoresis. Furthermore, the dicyclohexylcarbodiimide content of less than 20 nmol per mg subunit protein appears to be very low considering that the ATPase activity is strongly inhibited under this condition. In the previous paragraph first evidence has been presented that this subunit is present as oligomer, probably as hexamer. Thus, it appears possible that only one or a few of the monomers carry a dicyclohexylcarbodiimide label whereas the others are unmodified. This could account for the low ^{14}C label. The displacement during gel electrophoresis between the ^{14}C label and the protein then also could be explained. Since the dicyclohexylcarbodiimide-modified polypeptide may exhibit a slightly reduced electrophoretic mobility. Therefore, it was investigated whether the whole subunit protein can be saturated with dicyclohexylcarbodiimide at higher inhibitor concentrations. Mitochondria of *Neurospora crassa* were incubated with 15 to 460 nmol [^{14}C]dicyclohexylcarbodiimide per mg protein (Fig. 5). From each sample the ATPase complex was isolated by immunoprecipitation and then separated on dodecylsulfate gels. Only the electrophoretic fractions containing the inhibitor-binding subunit

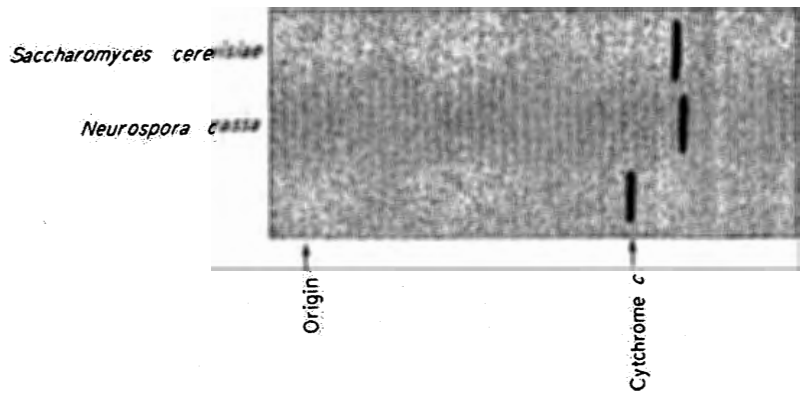


Fig. 6. *Dodecylsulfate-gel electrophoresis of the purified proteolipid from* Neurospora crassa *and* Saccharomyces cerevisiae. The purified proteolipids were applied at a concentration of 1 mg per ml dodecylsulfate buffer. Cytochrome *c* was applied at a fivefold lower concentration. All proteins were analysed in parallel on the same gel slab and stained with Coomassie blue R-250

were analysed, as shown in Fig. 5. Mitochondria containing a homogeneous [^{3}H]leucine label were used. Thus, the bound ^{14}C label could be immediately related to the protein present. At the low inhibitor concentration of 15 nmol per mg mitochondrial protein during incubation, 23 nmol dicyclohexylcarbodiimide are calculated to be bound per mg subunit protein. The ^{14}C label is found to be displaced by one or two fractions compared to the protein label. The labelling increases up to sixfold and the displacement becomes less pronounced when higher inhibitor concentrations have been used. At the highest concentration of 460 nmol per mg mitochondrial protein, the ^{14}C label coincides with the subunit protein, and 145 nmol dicyclohexylcarbodiimide are calculated to be bound per mg subunit protein. This amount of bound inhibitor nearly corresponds to a theoretical specific dicyclhexylcarbodiimide content of 125 nmol per mg protein, which is to expect after a 1:1 reaction of a 8000-M_r polypeptide. This high specific inhibitor content does not necessarily indicate that each of the 8000-M_r subunits has been modified at the same site. The coincidence of ^{14}C label and protein strongly suggests, however, that each of the subunits contains bound dicyclohexylcarbodiimide. Furthermore, it has to be noticed that even at the extreme inhibitor concentration the labelling of the 8000-M_r subunit is 30-fold higher than that of whole mitochondrial protein. This indicates that a preferential labelling of this polypeptide is retained. Thus, a modification of each of the subunits at the same site is likely. As also shown in Fig. 5, the labelling of the dicyclohexylcarbodiimide-reactive subunit increases continuously up to the highest inhibitor concentrations. Thus, when binding sites are calculated from the amount of dicyclohexylcarbodiimide bound at maximum inhibition of ATPase activity, accidental and misleading values may be obtained [15, 16].

Isolation and Properties of the Dicyclocarbodiimide-Binding Protein

The dicyclohexylcarbodiimide-binding protein of *Neurospora* and yeast can be extracted with chloroform/methanol from whole mitochondria, similarly to the beef-heart protein [12]. In both micro-organisms the extraction is rather specific, and the protein is obtained in pure form (Fig. 6), but only when the mitochondria have been extensively prewashed with organic solvents. The procedure described under Methods is similar to the method used by Sierra and Tzagoloff [20] for the isolation of the 8000-M_r subunit of the yeast ATPase complex (subunit 9, proteolipid). The thin-layer chromatography on silica gel applied by these authors as final purification step can be ommited, when the mitochondria are washed two times with chloroform/methanol/ether subsequent to the treatment with 90% methanol. The *Neurospora* protein becomes insoluble in chloroform/methanol when pretreated with 90% methanol. Therefore, these mitochondria were washed three times with chloroform/methanol/ether only. The overall yield of the dicyclohexylcarbodiimide-binding protein is about 5 mg per g of total mitochondrial protein in both organisms (Table 3). Unfortunately, this simple procedure can not be applied for the isolation of the beef heart protein. After the prewash, only a low percentage of the protein is extracted with chloroform/methanol, and the extract contains several other proteolipids. The dicyclohexylcarbodiimide-binding protein from this organism could be recently isolated using carboxymethyl-cellulose chromatography as major purification step [46].

When the *Neurospora* and yeast protein is purified from [^{14}C]dicyclohexylcarbodiimide-treated mitochondria, the yield appears to be the same for the free and the inhibitor-modified species. This may be

Table 3. *Protein yield and enrichment of bound [^{14}C]dicyclohexylcarbodiimide label during purification of the proteolipid from* Neurospora crassa *and* Saccharomyces cerevisiae
Mitochondria labelled with [^{3}H]leucine from *Neurospora crassa* (400000 counts × min^{-1} × mg^{-1}) and *Saccharomyces cerevisiae* (857000 counts × min^{-1} × mg^{-1}) were incubated with [^{14}C] $(cHxN)_2C$ (8.8 and 8.5 nmol per mg protein, respectively). The purification steps are detailed under Methods. Analysis of the described [^{14}C]-$(cHxN)_2C$ labelled mitochondria by dodecylsulfate gel electrophoresis is shown in Fig. 2A and B. n.d., not determined

Fraction	Protein (Lowry)	[^{3}H]Leucine label	Bound [^{14}C]-$(cHxN)_2C$
	mg	counts × min^{-1}	nmol/mg protein
Neurospora crassa			
Mitochondria	255	102×10^6	0.28
Sedimented mitochondria	181	72×10^6	0.4
Residue after chloroform/methanol extraction	175	70×10^6	0.22
Total chloroform/methanol extract	n.d.	1.77×10^6	n.d.
Purified proteolipid	1.5	1.02×10^6	11.8
Saccharomyces cerevisiae			
Mitochondria	60	51.4×10^6	0.27
Purified proteolipid	0.32	0.54×10^6	13.4

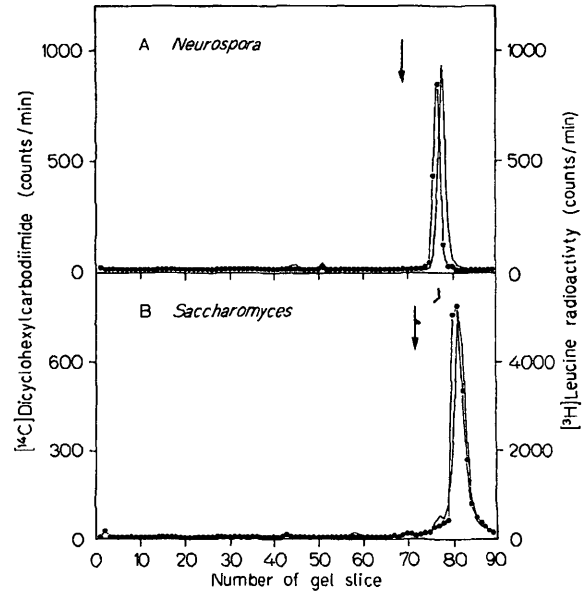


Fig. 7. *Distribution of [^{14}C]dicyclohexylcarbodiimide label and [^{3}H]-leucine radioactivity in the purified dicyclohexylcarbodiimide-modified proteolipid from (A)* Neurospora crassa *and (B)* Saccharomyces cerevisiae *after dodecylsulfate-gel electrophoresis.* The proteolipids were isolated from the same mitochondria as the ATPase complexes described in the legend to Fig. 3. When the total ^{14}C label of the peak fractions is related to the total [^{3}H]leucine radioactivity, 18.8 and 13.4 nmol bound dicyclohexylcarbodiimide per mg Lowry protein are calculated for the *Neurospora* and yeast protein, respectively. The arrow indicates the migration distance of cytochrome *c*

deduced from the finding that the inhibitor content is very similar in the purified protein (Fig. 7A and B) and in the 8000-M_r subunit displayed by dodecylsulfate-gel electrophoresis of the ATPase complex isolated from the same mitochondria (Fig. 3A and B). The specific dicyclohexylcarbodiimide content (nmol/mg protein) is enriched up to 50-fold in the isolated protein compared to whole mitochondrial protein. The lower enrichment observed in some experiments may be explained by the occurrence of additional dicyclohexylcarbodiimide-modified polypeptides in the membrane. A 50-fold enrichment would indicate that the dicyclohexylcarbodiimide-binding protein amounts to 2% of the total mitochondrial protein. This value is in reasonable agreement with the content derived from the amount of immunoprecipitated ATPase protein (see Table 2) and the amount of 8000-M_r subunit present in the ATPase complex (see Fig. 4B). Thus, this result gives further support to the notion that the dicyclohexylcarbodiimide-binding protein is present in the ATPase complex as oligomer, probably as hexamer.

The homogeneity of the purified dicyclohexylcarbodiimide-binding protein is indicated by the following observations. Firstly, the protein migrates as single band during gel electrophoresis in the presence of dodecylsulfate (Fig. 6 and 7) or in the presence of phenol/formic acid [23]. Secondly, only one single N-terminal amino is detected by the dansylation method (see below). Thirdly, the amino acid sequence [27] is completely accounted for by the amino acid composition shown in Table 4.

Based on the amino acid analysis the *Neurospora* and yeast protein have a slightly different size in containing 81 and 76 residues, respectively (Table 4). These values are in good agreement with the apparent molecular weights determined by dodecylsulfate-gel electrophoresis. It may be mentioned, however, that in the described gel electrophoretic system (see Methods) the yeast protein exhibits as slightly lower electrophoretic mobility than the *Neurospora* protein (Fig. 6). The N-terminal residue is tyrosine in *Neurospora* and formylmethionine in yeast. The occurrence of formylmethionine may be related to the finding that the yeast protein is synthesized by the mitochondrial protein-synthesizing system [19, 20], which is known to start with this amino acid [48]. In contrast, the *Neurospora* protein is synthesized on extramitochondrial ribosomes [21, 23]. Both proteins contain an unusually low amount of polar amino acid residues. No tryptophan and no histidine is present, and only a few lysines and arginines. This low percentage of basic residues may explain the poor staining properties of these proteins after gel electrophoresis. Com-

Table 4. *Amino acid composition of the purified dicyclohexylcarbodiimide-binding proteolipid from* Neurospora crassa *and* Saccharomyces cerevisiae

The best integral number of residue is shown in parentheses

Amino acid	Amount in proteolipid from			
	Neurospora crassa		*Saccharomyces cerevisiae*	
	mol/mol			
Aspartic acid	3.91	(4)	3.19	(3)
Threonine	2.11	(2)	3.11	(3)
Serine	4.75	(5)	5.03	(5)
Glutamic acid	4.93	(5)	2.03	(2)
Proline	1.21	(1)	1.78	(2)
Glycine	10.7	(11)	10.1	(10)
Alanine	13.9	(14)	9.77	(10)
Cysteine	–	(0)	1.15	(1)
Valine	6.01	(6)	5.71	(6)
Methionine	3.74	(4)	3.20	(3)
Isoleucine	5.89	(6)	8.66	(9)
Leucine	10.9	(11)	12.1	(12)
Tyrosine	1.29	(2)	0.91	(1)
Phenylalanine	5.68	(6)	5.89	(6)
Tryptophan	–	(0)	–	(0)
Lysine	2.01	(2)	2.00	(2)
Histidine	–	(0)	–	(0)
Arginine	1.97	(2)	0.97	(1)
N-terminal amino acid	tyrosine		formyl-methionine	
Total residues	81		76	
Polarity[a]	24.7		21.1	

[a] Calculated according to [49].

pared to cytochrome *c*, the staining intensities per mg Lowry protein are five to six times lower (Fig. 6).

DISCUSSION

In the present experiments, the action of the ATPase inhibitor dicyclohexylcarbodiimide was studied on the level of whole mitochondria, the isolated ATPase complex and the purified 8000-M_r proteolipid. As a result of these studies it is shown that the hydrophobic carbodiimide binds with a remarkable selectivity to the smallest subunit (M_r 8000) of the mitochondrial ATPase complex from *Neurospora crassa* and *Saccharomyces cerevisiae*. In both micro-organisms this 8000-M_r subunit has been analysed already during earlier biogenetic studies [19–21, 23, 24]. Their identity, however, was not finally established. The specific binding of dicyclohexylcarbodiimide as demonstrated in the present experiment indicates that this subunit represents in both micro-organisms the dicyclohexylcarbodiimide-binding protein as it has been identified in beef heart mitochondria [12, 14, 46] and *E. coli* plasma membrane [15, 16]. Recently, by sequence analysis it could be shown that the dicyclohexylcarbodiimide-binding protein from *Neurospora* and yeast exhibit extensive homology. In the two polypeptides 40 positions are occupied by identical residues [26, 27].

The purification of the dicyclohexylcarbodiimide-protein is particularly easy in *Neurospora* and yeast, since it can be extracted by neutral chloroform/methanol from whole mitochondria in pure form and in reasonable yield. This specific extraction, however, was obtained only when the mitochondria had been extensively prewashed with certain organic solvents. Several other proteins were found in the extract in addition to the dicyclohexylcarbodiimide-binding protein when the mitochondria were treated immediately with chloroform/methanol. The prewash removes the water and the bulk of the phospholipids, and it denatures probably most of the mitochondrial proteins. This appears to be responsible for the fact that the other potential proteolipids are not longer extracted. Unfortunately, this simple method could not be adapted to other mitochondria (beef heart, *Locusta migratoria*, *Schizosaccharomyces pompe*, *Aspergillus nidulans*), or to spinach chloroplasts and *E. coli* plasma membranes. With these membranes several proteins are extracted even after the prewash, and in some cases the dicyclohexylcarbodiimide-binding protein is obtained only in poor yield (W. Sebald, unpublished observations). The dicyclohexylcarbodiimide-binding protein from *E. coli* [15, 16] and beef heart [46] has been purified from complex proteolipid mixtures by chromatography in the presence of chloroform/methanol on DEAE-cellulose and LH-60 or on CM-cellulose, respectively.

The dicyclohexylcarbodiimide-binding protein from *Neurospora* and yeast exhibits an amino acid composition which is comparable to that of the corresponding proteins from *E. coli* [15] and beef heart [46]. The content of hydrophobic residues is extremely high even when compared to that of other intrinsic membrane proteins (see e.g. [24, 38, 49–51]). Most abundant are the amino acids glycine, alanine and leucine. The polarity calculated according to Capaldi and Vanderkooi [49] is below 25%. As already noted by Fillingame [15], a similarly low polarity is found with glycophorin [51] and bacteriorhodopsin [52] in those segments of the polypeptide chain which are supposed to span the phospholipid belayer of the membrane. In all four dicyclohexylcarbodiimide-binding proteins tryptophan and histidine is absent. The lack of cysteine and serine or the lack of cysteine, however, is a special feature of the protein from *E. coli* or *Neurospora crassa*, respectively.

The dicyclohexylcarbodiimide-binding protein occurs as oligomer in the ATPase complex as indicated by three independent lines of evidence. At the moment, the best estimate leads to a molar ratio of 6 to 7 mole-

cules per ATPase. This estimate is based on the following observations. Firstly, in *Neurospora*, the dicyclohexylcarbodiimide-binding protein is completely separated from the other ATPase subunits by dodecylsulfate-gel electrophoresis. Its amount, as a percentage of total ATPase protein, thus can be calculated from the distribution of a homogeneous [^{3}H]leucine label based on the leucine contents (μmol per mg protein) of the whole complex and the subunit, respectively [38]. Secondly, the content of the protein in whole mitochondria (nmol per mg mitochondrial protein) may be derived from the enrichment up to 50-fold of the specific inhibitor content in the isolated proteolipid (nmol dicyclohexylcarbodiimide bound per mg protein) compared to that of whole mitochondria. A value of 2.5 nmol dicyclohexylcarbodiimide-binding protein per mg mitochondrial protein is thereby obtained which is 6 to 7 time higher than the molar amount of ATPase present in the mitochondria. From quantitative immunoprecipitation data values of 0.35 to 0.4 nmol ATPase per mg mitochondria are obtained. Thirdly, in yeast, the 8000-M_r subunit of the ATPase complex has been found to occur in a high-molecular-weight form of 45000 when whole mitochondria were dissolved in dodecylsulfate buffer [19,20]. This 8000-M_r subunit is now identified as dicyclohexylcarbodiimide-binding protein, and the 45000-M_r species probably represents a native hexamer, which in yeast appears to be stable even in the presence of dodecylsulfate. Preliminary evidence already has been reported, that the dicyclohexylcarbodiimide-binding protein of *E. coli* [15,16] and lettuce chloroplasts [17] and also a 5400-M_r subunit of the ATPase complex from the thermophilic bacterium PS-3 [53] occur as oligomers.

In mitochondria from *Neurospora* and yeast, dicyclohexylcarbodiimide binds with a striking specificity to the 8000-M_r subunit of the ATPase complex. Furthermore, as shown recently the bound inhibitor is found exclusively at one position, a glutamyl residue [27], of the polypeptide chain. This specificity is unexpected at the first view, since carbodiimides are known to be highly reactive compounds [54,55]. It has to be considered, however, that within the mitochondria a potentially reactive group has to meet some requirements in order to become labelled with [^{14}C]dicyclohexylcarbodiimide. Firstly, the hydrophobic carbodiimide probably concentrates when added to the mitochondrial suspension immediately in the phospholipid bilayer. Thus, only groups located in a hydrophobic environment will react. Secondly, dicyclohexylcarbodiimide is a rigid and rather bulky molecule. Thus, its attachment may require a certain geometry of the binding site. This could explain the occurrence of dicyclohexylcarbodiimide-resistant mutants in *E. coli* [56,57], as well as the higher reactivity of the 8000-M_r subunit in beef heart compared to *Neurospora* or yeast. Thirdly, the bound ^{14}C label is stable under a variety of conditions including concentrated organic acids. Therefore, the reaction product most likely is a *N*-acylurea which originates from the unstable *O*-acylisourea by intramolecular rearrangement [54,55]. This reaction has to be favoured compared to other possible reactions which do not lead to a stable bound ^{14}C label [54,55]. Fourthly, at present, no information is available on the labelling kinetics of the 8000-M_r subunit by dicyclohexylcarbodiimide. It is tempting to speculate, however, that the inhibitor-reactive site of the ATPase subunit is labelled faster than other potentially reactive groups due to a high local H^+ activity. The formation of an *O*-acylisourea is known to be catalysed by protons [54].

The identification of a dicyclohexylcarbodiimide-reactive ATPase site leads to the question whether or not the bound product is responsible for the inhibition of the enzymatic activity of the ATPase complex. The present experiments with *Neurospora* and yeast indicate that no other dicyclohexylcarbodiimide-labelled products can be involved, since inhibition of ATPase activity as well as the labelling of only the 8000-M_r subunit persists after removal of excess inhibitor by a wash with phospholipids. This result, however, does not exclude the possibility that dicyclohexylcarbodiimide leads to the formation of another inhibitory product, e.g. by a condensation reaction, which no longer contains a carbodiimide-label. In *Neurospora* and yeast mitochondria only 3–4% of the applied dicyclohexylcarbodiimide radioactivity is recovered as bound ^{14}C label. In beef heart mitochondrial membranes the situation is more favourable. After an incubation time of 4 h about 40% are recovered as bound dicyclohexylcarbodiimide (Fig. 1 B) [46]. From reports in the literature [9,12] it can be concluded that the added ^{14}C label is nearly completely bound when submitochondrial particles from beef heart are incubated for 16–20 h with 0.6–1 nmol/mg. This result strongly suggests that at least in this membrane unlabelled reaction products are not formed to a major extent. A direct correlation between the binding of [^{14}C]dicyclohexylcarbodiimide and the inhibition of the ATPase activity could be established in *E. coli* [57], since the 8000-M_r proteolipid was not labelled by [^{14}C]dicyclohexylcarbodiimide in membranes of a mutant resistant to the effects of the inhibitor. Thus, it is reasonable to conclude that also in *Neurospora* and yeast the inhibition of the ATPase activity is due to the modification of that site of the 8000-M_r subunit which is identified by the bound dicyclohexylcarbodiimide label. The low amount of 15–20 nmol inhibitor bound per mg subunit protein at nearly maximum inhibition then indicates that the modification of one of the probably six sites of the subunit oligomer is sufficient to block the enzymatic activity of the complex.

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