Labeling of individual amino acid residues in the membrane-embedded F_0 part of the F_1 F_0 ATP synthase from *Neurospora crassa*
Influence of oligomycin and dicyclohexylcarbodiimide

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Three F_0 subunits and the F_1 subunit β of the ATP synthase from *Neurospora crassa* were labeled with the lipophilic photoactivatable reagent 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). In the proteolipid subunit which was the most heavily labeled polypeptide labeling was confined to five residues at the NH_2-terminus and five residues at the C-terminus of the protein. Labeling occurred at similar positions compared with the homologous protein (subunit *c*) in the ATP synthase from *Escherichia coli*, indicating a similar structure of the proteolipid subunits in their respective organisms.

The inhibitors oligomycin and dicyclohexylcarbodiimide did not change the pattern of accessible surface residues in the proteolipid, suggesting that neither inhibitor induces gross conformational changes. However, in the presence of oligomycin, the extent of labeling in some residues was reduced. Apparently, these residues provide part of the binding site for the inhibitor. After reaction with dicyclohexylcarbodiimide an additional labeled amino acid was found at position 65 corresponding to the invariant carbodiimide-binding glutamic acid. These results and previous observations indicate that the carboxyl side chain of Glu-65 is located at the protein-lipid interphase. The idea is discussed that proton translocation occurs at the interphase between different types if F_0 subunits. Dicyclohexylcarbodiimide or oligomycin might disturb this essential interaction between the F_0 subunits.

ATP synthases from various organisms are composes of two parts: F_1 and F_0. F_1 is peripheral and contains the binding sites for adenine nucleotides, F_0 is membrane-embedded and catalyzes H^+ conduction across the membrane. The availability of the complete primary structure of the eight subunits of the ATP synthase from *Escherichia coli*, as well as an increasing number of structural data, has lead to the proposal of models for the arrangement of the three F_0 subunits in the membrane [1−8].

In a recent publication the membrane-embedded segments of the F_0 subunits from *E. coli* have been analyzed using [^{125}I]TID. This lipophilic compound upon illumination generates a carbene which is reactive enough to insert even into aliphatic amino acid side chains [9, 10]. The positions of the attacked amino acids were determined by sequence analysis of the modified subunits *b* and *c*. The labeling patterns indicated that the reaction of amino acid side chains with the carbene is predominantly determined by the accessibility of a residue from the lipid phase and not by its chemical reactivity. Thus, this approach appeared to be suitable to map directly the surface of a membrane-spanning segment and to delineate from the lipid-accessible surface residues other conformational properties of the protein.

In the present study the mitochondrial ATP synthase from *Neurospora crassa* was analyzed by the same technique for two reasons. Firstly, the proteolipid subunit (subunit 9 or dicyclohexylcarbodiimide-binding protein) of the mitochondrial F_0 is homologous to the *E. coli* subunit *c*, but only 16 positions are occupied by identical residues in both proteins. Thus, it was interesting to see whether a comparable lipid-accessible surface area is determined. Secondly, the mitochondrial F_0 can be inhibited by oligomycin, and similar to the bacterial F_0, by the covalent binding of dicyclohexylcarbodiimide [11−13]. This prompted the question whether these inhibitors might change the accessible surface by either inducing a conformational change or by shielding part of the surface area.

EXPERIMENTAL PROCEDURES

Neurospora crassa wild type 74A was grown and mitochondria were prepared as described in [11]. Immunoprecipitation of the ATP synthase and polyacrylamide gel electrophoresis were done as in [14]. The proteolipid subunit was extracted from mitochondria with chloroform/methanol [11].

Photolabeling

Mitochondria (8 mg protein) were suspended in 4 ml 10 mM Tris/Cl pH 7.5. 0.8 mCi [^{125}I]TID in 80 μl ethanol (8 Ci/mmol, Amersham) were added at 0°C and the mixture was kept at 0°C for 15 min. Photolysis was done in a flat
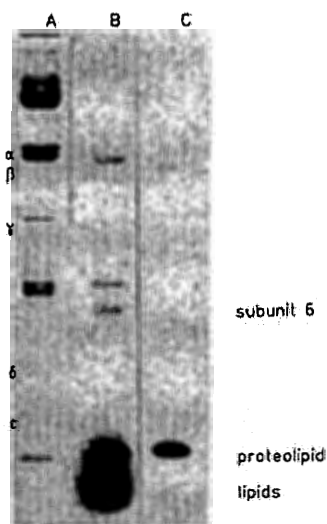
Fig. 1. *Immunoprecipitation of [^{125}I]TID-labeled ATP synthase from* Neurospora crassa *mitochondria*. (A) Coomassie staining of the immunoprecipitate after sodium dodecyl sulfate gel electrophoresis. (B) Autoradiography of A. (C) Autoradiography of the purified proteolipid subunit after sodium dedecyl sulfate gel electrophoresis

plastic trough covered with a glass plate with a 16-W ultraviolet lamp at 366 nm at 0 °C for 4 min. Aliquots of 200 µl were taken for immunoprecipitation of the ATP synthase. For the isolation of the proteolipid the remainder was centrifuged for 20 min at 40000 rpm to pellet the mitochondria. The pellet was then extracted with chloroform/methanol as described [11]. Lipids were removed by size-exclusion chromatography using a column (0.5 × 30 cm) packed with Sephadex LH 20 equilibrated in chloroform/methanol (2/1, v/v) containing 5% 1 M triethylammonium acetate pH 6.5; 100000 − 500000 cpm ^{125}I radioactivity were recovered at this step.

If indicated, oligomycin was added at 20 µg/mg protein. A preincubation was done for 5 min at 0 °C. Dicyclohexalcarbodiimide was added at 300 nmol/mg protein. Preincubation time was 4 h at 0 °C.

Sequencing and identification of labeled amino acid residues

Triethylamine (50 µl) were added to the proteolipid solution obtained after Sephadex LH 20 chromatography (2 ml). The protein was then incubated with 50 mg porous *p*-phenylendiisothiocyanate-activated glass beads for 4 h at 37°C. Residual isothiocyanate residues were inactivated by an incubation with 50 µl ethanolamine for 1 h at 37°C.

Sequencing was performed with a modified Sequemat 12 solid-phase sequenator using the following short program: 4 min methanol, 3 min buffer (1 M *N*-methylmorpholine/trifluoroacetate pH 8.1/pyridine, 2/3, v/v), 12 min buffer plus 10% phenylisothiocyanate in acetonitrile, 8 min methanol, 2 min dichloroethane, 5 min methanol, 15 min trifluoroacetic acid, 0.5 min methanol. Fractions were collected during the 15-min trifluoroacetic acid wash and the following methanol wash. Conversion to phenylthiohydantoins and thin-layer chromatography was done as described [15].

To estimate the repetitive yield, the amount of Pth-Ala was measured in the respective cycles by using high-performance liquid chromatography on a Spectra physics SP 8700 system. Phenylthiohydantoins were separated on a Zorbax CN 5-µm column (0.45 × 25 cm) by using a linear gradient from 12% B
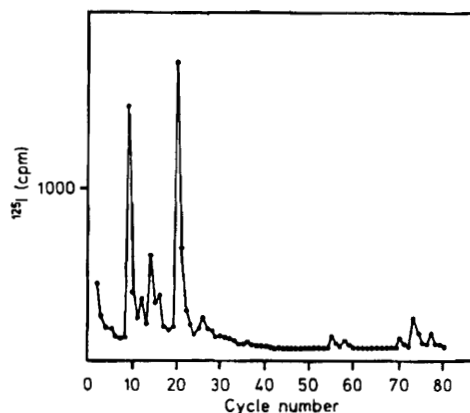

Fig. 2. *Edman degradation of the purified [^{125}I]labeled proteolipid subunit*. The proteolipid subunit was immobilized on phenylendiisothiocyanate-activated porous glass beads and sequenated in an automated solid-phase sequenator. Released samples were analyzed for ^{125}I radioactivity. The performance of the Edman degradation was monitored by thin-layer chromatography or high-performance liquid chromatography

in A to 78% B in A during 18 min, solvent A being 25 mM sodium acetate, 5% tetrahydrofuran pH 5.1, and solvent B being 60% acetonitrile in water. The flow rate was 1 ml/min and the temperature was 37°C.

Autoradiography of the thin-layer plates was done at −80°C using an intensifier screen. Radioactivity was determined by γ-counting.

RESULTS

Labeling of the ATP synthase subunits

After reaction with [^{125}I]TID the ATP synthase was immunoprecipitated from Triton-X-100-solubilized mitochondria by an antiserum raised against F$_1$. Fig. 1 shows the separation of the subunits after sodium dodecyl sulfate gel electrophoresis. Autoradiography revealed four labeled polypeptides: the proteolipid subunit (M_r 8000), a poorly stained subunit with M_r 20000 which is homologous to the F$_0$ subunit *a* from *E. coli* and a protein with M_r 24000 of unknown identity. The fourth band is the F$_1$ subunit *β*. All the other subunits of the ATP synthase were not modified. Throughout all our experiments with various photo-labels and various organisms we consistently observed labeling of subunit *β*. The reason for the labeling of this apparently peripheral protein is not clear. It seems possible that either it has some contact with the lipid bilayer or that [^{125}I]TID partitions into a hydrophobic pocket of this subunit.

More than 90% of the protein bound label is recovered in the proteolipid subunit which exists as an oligomer of at least six copies in the F$_0$ part [11].

Labeling of distinct amino acids residues in the proteolipid subunit

The labeled proteolipid subunit was isolated by chloroform/methanol extraction and lipids were removed by chromatography on Sephadex LH 20. Autoradiography after sodium dodecyl sulfate gel electrophoresis showed a homogeneous radioactive product migrating with M_r 8000 (Fig. 1). After attachment to porous glass beads, the protein was subjected to 80 cycles of Edman degradation. Previous sequence
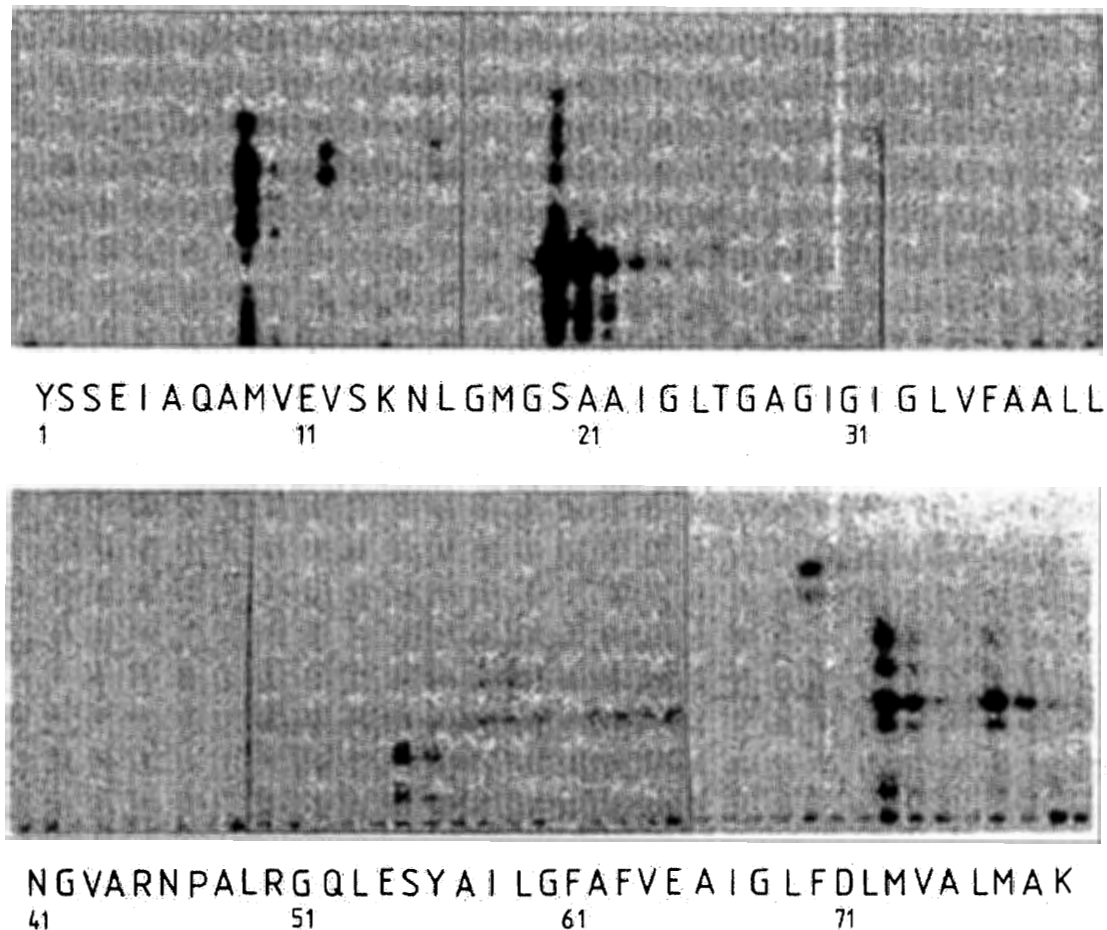
YSSEIAQAMVEVSKNLGMGSAAIGLTGAGIGIGLVFAALL
1   11   21   31

NGVARNPALRGQLESYAILGFAFVEAIGLFDLMVALMAK
41   51   61   71

Fig. 3. *Autoradiography of released [^{125}I]TID-labeled phenylthiohydantoins after thin-layer chromatography.* To compensate for the 5% loss during each cycle an increasing aliquot was spotted on the thin-layer plate (1/10 for residues 1 − 20, 2/10 for residues 21 − 40, 4/10 for residues 41 − 60, 8/10 for residues 61 − 80)

analyzes have shown that the protein is attached mainly via the lysyl residue at position 79, and that besides Phe-80 and Ala-81 the whole polypeptide chain can be sequenated in a single run. Fig. 2 shows the release of ^{125}I radioactivity during the individual cycles. Clearly discrete amino acids were labeled. The amount of the sequenced radioactivity was sufficient to allow a quantitative evaluation even at the final steps. The autoradiography of the phenylthiohydantoins after thin-layer chromatography revealed distinct characteristic patterns for the labeled amino acids, thus allowing the discrimination between truely labeled residues, carry over from the preceding step and artefactual release of radioactivity. Accordingly release of radioactivity during cycle 14 is not due to the labeling of the lysine residue at this position but is caused by a release of a small portion of the polypeptide chain bound via this lysine to the glass beads. Label was found to be associated with the following residues: Met-9, Val-12, Leu-16, Ser-20, Thr-26, Ser-55, Ile-58, Phe-70, Met-73 and Met-77. There was no labeling in the middle segment of residues 27 − 54. Fig. 4 depicts the amount of label in the individual amino acid residues after correction for a 5% loss during each cycle.

When oligomycin was added to the membranes prior to the photoactivation of [^{125}I]TID the labeling pattern did not change qualitatively. Apparently oligomycin did not induce large conformational changes in the oligomer of the proteolipid subunits. But labeling was reduced by more than 50% at the positions of Ser-55, Ile-58 and Phe-70 (Fig. 5). Obviously oligomycin shielded some residues from the labeling with [^{125}I]TID.

In another experiment the mitochondria were preincubated with dicyclohexylcarbodiimide at concentrations 10 − 20-fold higher than needed for maximal inhibition of the ATPase activity and sufficient to modify covalently the carboxyl group of Glu-65 in all proteolipids of the oligomer [11]. Again the same residues were found to be attacked by the carbene, as in the above experiment. However, [^{125}I]TID modified an additional amino acid residue at position 65 that corresponds to the invariant glutamic acid residue which binds the inhibitor dicyclohexylcarbodiimide covalently [13]. As previously discussed [10], the reaction products between carboxyl groups and the carbene are probably not stable during 65 cycles of Edman degradation and escape detection. Thus no information on the location of this important group could be obtained. The preloading of Glu-65 with dicyclohexylcarbodiimide lead to a reaction with the carbene, indicating that at least the bound inhibitor is accessible from the lipid phase.

DISCUSSION

The labeling of the ATP synthase subunits from mitochondria is largely similar to that previously observed for the *E. coli*

Fig. 4. *Histogram of the distribution of* ^{125}I *radioactivity among individual amino acid residues in the proteolipid subunit.* The figures (cpm) were corrected for decreasing yields during Edman degradation according to the determined repetitive yield of 95%

enzyme. Most of the bound label is found on the proteolipid subunit. This is expected, since as an oligomer of at least six copies it represents the major constituent of the F_0 [11]. Also the accessibility of the other F_0 subunits (a or b) is comparable in mitochondria and bacteria. A subunit homologous to subunit b in *E. coli* has not yet been identified in mitochondria. Since mitochondria F_0 contains several polypeptides of unknown identity, it is a distinct possibility that the labeled polypeptide of M_r 24000 corresponds to the *E. coli* subunit b which is also labeled by $[^{125}I]$TID. It should be mentioned that no labeling was found at the position of subunit 8 (aap_1) [16, 17] which, according to its amino acid sequence, should traverse the membrane once. Unexpected is the labeling of the F_1 subunit β, both in mitochondria and bacteria, which is certainly a peripheral protein. The hypothesis has been put forward that the β subunit might have contact with the lipid phase. But it is also feasable that this subunit contains a hydrophobic pocket which sequesters some of the added $[^{125}I]$TID. The labeling of the β subunit by dicyclohexylcarbodiimide as well as with other hydrophobic affinity labels would be in accordance with the latter possibility [1].

Amino acid sequence analysis of the carbene-modified proteolipid revealed that only discrete amino acid residues were labeled. The positions of the affected amino acids in the mitochondrial subunit are very similar to that in the homologous subunit c from *E. coli*. Labeled residues occur at identical positions (Val-20/Leu; Ile-58/Phe, Met-77/Tyr) or at juxtaposed positions (Met-9/Leu; Leu-16/Met-Tyr; Ser-20/ Val; Phe-70/Met). This substantiates the notion that the labeling is predominantly determined by the accessible surface and not by the chemical reactivity of the side chain. A different alignment of the N-terminal segments postulating a one-residue deletion in the *Neurospora* protein [4] would result in an even better coincidence of the carbene-labeled positions. Some positions are affected only in the mitochondrial or in the bacterial subunit [Thr-26/(Ile); Ser-55/(Thr); Met-73/(Gly); (Ile-23)/Leu; (Phe-61)/Met; (Phe-80)/Phe]. Phe-80 could not be analyzed in this study, since the sequencing stopped at position 79. The reason for the nonlabeling of the other residues remains uncertain at the present time. It is unreasonable to assume that these discrepancies result from major

Fig. 5. *Histogram of the distribution of* ^{125}I *radioactivity among individual amino acid residues in the proteolipid subunit.* Labeling was carried out after preincubation of the mitochondria with (A) 300 nmol dicyclohexylcarbodiimide (DCCD)/mg protein, (B) with 20 µg oligomycin/mg protein. The arrows indicate differences compared with the labeling pattern in the absence of inhibitors

F A F V E A I G L F D L M V A L M A K
61   71

Fig. 6. *Autoradiography of released* $[^{125}I]$*TID-labeled phenylthiohydantoins after thin-layer chromatography.* Labeling with $[^{125}I]$TID was carried out after preincubation with 300 nmol dicyclohexylcarbodiimide/mg protein. The arrow indicates the appearance of a new modified residue at position 65

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