Laser-activated carbene labels the same residues in the proteolipid subunit of the ATP synthase in energized and nonenergized chloroplasts and mitochondria

H. Weber, W. Junge*, J. Hoppe and W. Sebald

Dept of Cytogenetics, Gesellschaft für Biotechnologische Forschung, 3300 Braunschweig and **Fachbereich Biologie/Chemie, Universität Osnabrück, 4500 Osnabrück, FRG*

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The membrane-embedded proteolipid of the F_0 part of the ATP synthase in mitochondria (su 9) and chloroplasts (CF_0III) was labeled with a carbene generated from the photoactivatable hydrophobic reagent [^{125}I]-TID by a single UV-laser pulse. Amino acid sequence analysis of the isolated proteolipid revealed that the carbene modified distinct residues which apparently are accessible from the lipid phase. No differences were detected in the labeling pattern upon energization of the membrane. Labeling of mitochondrial proteolipid in the presence of oligomycin led to a reduced modification of several side chains which map directly a surface involved in oligomycin binding.

ATP synthase *Proteolipid* *Laser-photoaffinity labeling* *Oligomycin*

1. INTRODUCTION

Recent experiments analyzed the structure of the proteolipids of *Neurospora crassa* and *Escherichia coli* ATP synthase by using a lipid-soluble carbene [1,2]. Distinct amino acid residues were labeled which represent a surface on the proteolipid oligomer accessible from the lipid phase. Furthermore possible binding sites for the inhibitors DCCD and oligomycin were detected [2]. These results demonstrated that the carbene generated from [^{125}I]TID provides a valuable tool for analyzing membrane protein topology [2,3].

For the F_1 part of the ATP synthase, large conformational changes have been observed upon membrane energization (review [4]). On the other hand, there is a lack of direct information for the F_0 part. This prompted the present experiments analyzing possible changes in the carbene-accessible surface of the proteolipid in dependence of the functional state of the enzyme and the binding of the inhibitor oligomycin.

The previous work did not consider the functional state of the ATP synthase. The carbene was generated by a 5 min exposure with UV light in simple buffer solution at 0°C. In the present study conditions were maintained allowing normal phosphorylation rates in mitochondria and thylakoids. The reactive carbene was generated by a pulse from a frequency-doubled ruby laser (50 ns duration). Since the lifetime of the carbene is presumed to be below 1 μs [3], a time-resolved analysis of the ATP synthase in different functional states appeared to be feasible.

Experiments were performed with suspensions of *Pisum sativum* chloroplasts and with suspensions of mitochondria isolated from *N. crassa*. The proteolipid was isolated, sequenced and analyzed for radioactive labeling. The results confirmed the labeling pattern of su 9 determined during earlier

Abbreviations: [^{125}I]TID, 3-(trifluoromethyl-3-(*m*-[^{125}I]phenyl)diazirine; DCCD, dicyclohexylcarbodiimide

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studies with one minor exception. It was noteworthy that energization of the membrane did not affect the labeling pattern, both in chloroplasts and in mitochondria. The present studies revealed a more extended binding site of oligomycin in comparison to previous experiments [2].

2. MATERIALS AND METHODS

2.1. *Preparation*

N. crassa was cultured and mitochondria were prepared as described in [5]. They were frozen and stored in liquid nitrogen in buffer solution.

Thylakoids (class II chloroplasts) from *P. sativum* were prepared using the method in [6]. They were used within 2 h of preparation of a concentrated suspension.

2.2. *Energization of membranes*

Whole mitochondria (2 mg protein/ml) were energized at 30°C with succinate or NADH in phosphorylation buffer 30 s before carbene activation. Oxidative phosphorylation was determined by [^{32}P]phosphate incorporation using the method in [7] and respiration rates measured using an oxygen electrode.

Thylakoids (0.7 mg protein/ml) were energized at room temperature with strong red light (2 s, 100 mW/cm^2) in a buffer described in [8]. Photophosphorylation was measured using the luciferin-luciferase assay [8]. Phenazine methosulfate was used as cofactor for cyclic electron transport.

2.3. *Carbene labeling and identification of labeled amino acids*

150 μl aliquots of a total volume of 5 ml containing 1 mCi [^{125}I]TID were filled into cone-shaped Eppendorf tubes. They were exposed to a single laser pulse of 350 mJ at wavelength 347 nm and of 50 ns duration.

Purification of su 9, subsequent sequence analysis and separation of amino acids by thin-layer chromatography were described in [2].

Purification of CF_0III was reported in [9]. 40% of radioactivity present in the purified CF_0III could be coupled to *p*-phenylenediisothiocyanate-activated glass mainly via Lys-48. After deformylation [1] sequence analysis was performed as reported for *N. crassa* su 9 [2].

3. RESULTS AND DISCUSSION

3.1. *Labeling of surface residues with a lipid-soluble carbene in isolated mitochondria from* N. crassa

Labeling of *N. crassa* mitochondria was performed under three different conditions. (i) 30 s after energization with succinate which resulted in an oxidative phosphorylation rate of 0.3–0.4 μmol ATP/mg protein per min. The rate was linear for 40–50 s. [^{125}I]TID present in the membrane did not influence phosphorylation. O_2 uptake was about 100 nmol O_2/mg protein per min with a respiratory quotient of 1.8 (fig.1). (ii) In phosphorylation buffer without ADP and succinate where no O_2 uptake or oxidative phosphorylation could be measured. (iii) In the presence of oligomycin which decreased the phosphorylation rate in energized mitochondria by 98%.

Purified labeled su 9 was sequenced in a single run up to Lys-79. The amount of radioactivity was sufficient to allow quantitative evaluation even at the final steps. Carbene label was found to be attached to Met-9, Val-12, Leu-16, Ser-20, Thr-26, Ser-55, Tyr-56, Ile-58, Phe-70, Met-73 and Met-77. Oligomycin present in the mitochondrial membrane resulted in a decreased [^{125}I]TID accessibility

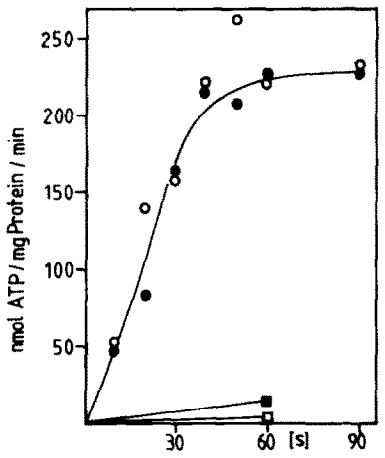

Fig.1. ATP synthesis during oxidation of succinate in mitochondria of *N. crassa* (○). Influence of: 30 nmol TID/mg protein (●); 30 μg oligomycin/mg protein (■) 60 μg/mg protein (□).

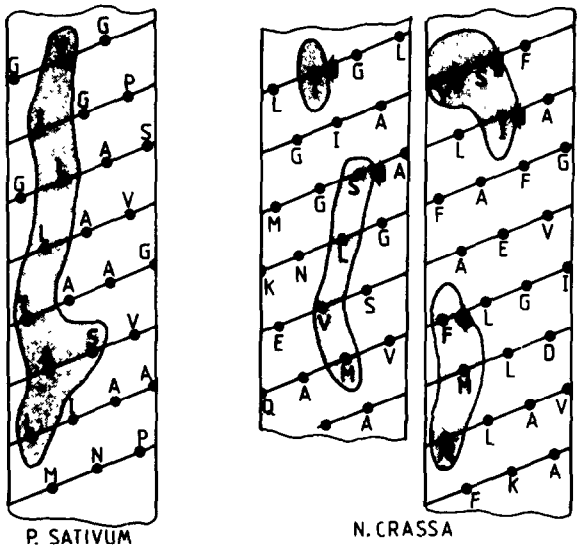

Fig.2. A planar representation of the two putative α-helices at the C-terminus and at the N-terminus is shown for *N. crassa* su 9. For *P. sativum* only the N-terminus is shown. Marked areas indicate contact to the lipid phase as revealed by labeling with [^{125}I]TID. Oligomycin in the membrane during the labeling procedure resulted in a more than 50% reduction in detected radioactivity in positions marked by arrows.

of Ser-20, Thr-26, Ser-55, Tyr-56, Ile-58 and Phe-70 (fig.2).

The following conclusions can be made about the carbene labeling of su 9. (i) The carbene-attacked residues are oriented to one side of a putative α-helix and represent a surface on the protein accessible from the lipid phase (fig.2). (ii) Energization did not change the carbene-accessible surface. (iii) Oligomycin caused a decrease in carbene label of certain residues. This indicates that oligomycin masks a surface involved in oligomycin binding.

Glu-65 which according to independent evidence is thought to be oriented to the lipid phase [2,10] was not labeled. Probably the reaction product between carboxyl groups and the carbene was gradually cleaved during the 65 cycles of Edman degradation and thus escaped detection [11].

Additional labeling of Tyr-56, in comparison to previous experiments [2], is thought to be an effect of higher temperature (30°C instead of 0°C). Possibly the membrane-embedded su 9 oligomer is less rigid at 30°C, so that the space-filling side group of Tyr-56 could partially overlap with Ser-55 and also receive label.

Compared to the earlier experiment the binding site of oligomycin also extends to the segment at the N-terminus (fig.3). This could be a similar effect of temperature.

For mitochondrial proteolipid of *Saccharomyces cerevisiae* oligomycin resistance resulted from an amino acid substitution Gly-23–Ala-23 [12]. This residue corresponds to Gly-29 in *N. crassa* su 9, located just one α-helix turn away from carbene-labeled Thr-26 which was influenced by oligomycin.

3.2. *Labeling of surface residues in isolated thylakoids from* P. sativum

Labeling of *P. sativum* thylakoids was performed at room temperature under two different conditions. (i) 2 s after energization with red light and ADP in phosphorylation buffer which resulted in photophosphorylation of 1–2 μmol ATP/mg protein per min (10–20 μmol ATP/mg chlorophyll per min). This rate was not influenced by addition of [^{125}I]TID, either before or after exposure to a single UV-laser flash. (ii) In buffer solution without red light and ADP. No phosphorylation was observed.

The labeled and purified CF_0III could be analyzed up to Lys-48. Label was attached to Leu-4,

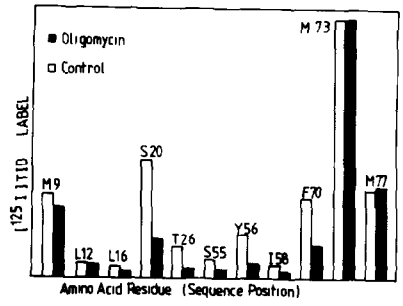

Fig.3. Histogram of the distribution of [^{125}I]TID label among individual residues in *N. crassa* su 9. Labeling was performed in the absence and presence of oligomycin (60 μg/mg protein). Amounts of radioactivity in the respective residues are corrected based upon a determined repetitive yield of 95% per cycle during Edman degradation.

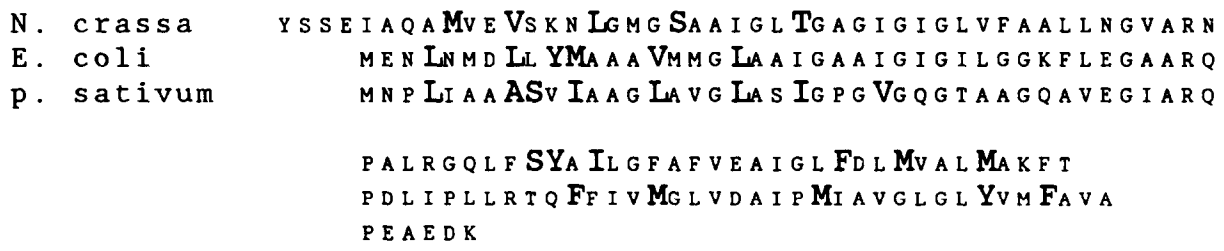

Fig.4. Sequences of the proteolipid subunits of *N. crassa*, *E. coli* [1] and *P. sativum* (shown until position 48). Carbene-labeled residues are indicated by bold letters.

Ala-8, Ser-9, Ile-11, Leu-15, Leu-19, Ile-22 and Val-26 (figs 2,4). Energization of the membrane did not change the labeling pattern.

The pigments present in thylakoids absorbed most of the laser pulse energy. Only 15% of the radioactivity was recovered in isolated CF_0III compared to experiments performed with su 9 of *N. crassa*.

4. CONCLUSIONS

Our experiments demonstrate that carbene labeling is also possible after nanosecond activation. Thus, in principle time-resolved measurements on dynamics of membrane proteins should be possible. Although even minor differences in labeling due to oligomycin could be analyzed in the case of su 9 we did not find a change in the labeling pattern upon energization of membranes. It can be estimated that any additional labeled residue should be detected if it represents at least 10% of the radioactivity present in the analyzed amino acids. Therefore it might be concluded that the proteolipid oligomer forms a rigid and compact core in the F_0 part whose conformation is not greatly altered by inhibitor binding and during ATP synthesis.

Recently the binding of DCCD and oligomycin to F_0 has been reported to reduce dramatically the binding of [γ-^{32}P]ATP in high-affinity catalytic sites, possibly due to conformational interactions between F_0 and F_1 [13]. The results of the functional and conformational studies could be reconciled if one visualizes that proton translocation in F_0 involves a rotational movement of the proteolipid oligomer against another F_0 subunit. Such a model is discussed in some detail in [2,14–16].

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