Labeling of Subunit b of the ATP Synthase from Escherichia coli with a Photoreactive Phospholipid Analogue*

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Purified ATP synthase (F1:F0) from Escherichia coli K12 was labeled with the hydrophobic photoreactive label 1-palmitoyl-2-(2-azido-4-nitro)benzoyl sn-glycero-3-[^3H]phosphocholine in reconstituted proteolipo-
somes. The F0-subunit b was predominantly labeled. A very low amount of label was detected on the other F0-
subunits a and c. The label in subunit b could be traced back by proteolytic digestion to the NH2-terminal fragment 1 to 53 which contains the stretch of hydrophobic amino acid residues 1 to 32. By sequencing the intact protein, the distribution of label among the amino acids in this segment was determined. Cysteine 21 was predominantly labeled. Other labeled amino acids occurred at the NH2-terminal (Asn-2) and at position 26 (tryptophan). Due to the restricted mobility of the label in the lipid bilayer, these residues are suggested to be located in or close to the polar head of the lipid bilayer. These results will be compared with predictions for the arrangement of the polypeptide b derived from the hydrophobicity profile.

ATP synthases generally are composed of a hydrophilic component F1 and a membrane-integrated part F0 (1-3). The ATP sites are located in the F1-part, whereas the F0-part catalyzes the proton conduction across the membrane. In all organisms so far analyzed, F1 is composed of the five subunits α, β, γ, δ, and ε (1-3). But only for Escherichia coli the subunit composition of the F0 has been unequivocally determined. Three genes could be assigned to the three protein bands present in the highly purified preparation of F0 (4-7). The DNA sequence of the genes has been determined and thus the amino acid sequence is known (8-10). F0 consists of subunit a with a Mr = 30,287 which according to its low polarity seems to be an intrinsic membrane protein, subunit b with a Mr = 17,212, an amphiphilic protein, and then subunit c with a Mr = 8,288 which is also referred to as the proteolipid or the dicyclohexy carbodiimide-binding protein (11,12). This integral membrane protein has been identified in numerous organisms and the amino acid sequences of these proteins from nine species have been reported (13,14). A stoichiometry for α:β:γ:δ:ε of 1:2:10 has been suggested (15).

For the elucidation of the proton pathway across the membrane, the arrangement and the folding of the polypeptide chains in the membrane have to be established. At the present time, no data on the molecular organization are available.

Hydrophobic photolabeling reagents have been developed in the recent years and their versatility and specificity have been well documented (16-19). These labels distributed in the lipid phase on illumination give rise to a reactive group, which reacts with amino acid side chains in contact with the lipid phase. Among the various reagents, phospholipid derivatives containing a photoreactive (19) nitrophenylazide group attached at the polar head group of the phospholipid are of particular interest. This label most likely will only modify amino acid residues which are in proximity of the polar phase of the lipid bilayer and thus will give first information about the accessibility of a subunit from the lipid bilayer. So far, this reagent has been successfully applied for cytochrome c oxidases and the ATP synthase from beef heart (20,21). For subunit α of cytochrome c oxidase, the label could be traced back to single amino acid residues, marking the beginning of one hydrophobic stretch (20). Here we wish to report the labeling of the E. coli ATP synthase, where subunit b was almost exclusively labeled. Two short segments of labeled amino acids located in the NH2-terminal region of the protein were identified. Possible arrangements of this hydrophobic segment of the polypeptide subunit b in the lipid bilayer will be discussed.

MATERIALS AND METHODS

ATP Synthase from E. coli K12—This was prepared as described by Friedl et al. (22). Enzymatic activities were identical as described (ATPase activity, 23 units/mg, ATP-driven H+ translocation, 2,000 units F1/mg). The purity of the ATP synthase complex estimated by scanning the Coomassie blue-stained gel was greater than 95% (cf. Fig. 1). The label (1-palmitoyl,2-(2-azido-4-nitro)benzoyl sn-glycero-3-[3H]phosphocholine was synthesized as described at a specific radioactivity of 3.1 Ci/mmol (19).

Reconstitution of the ATP Synthase—This was done according to Friedl et al. (22) using a lipid to protein ratio of 1000:1 (w/w) for the analytical scale. For the preparative scale, a ratio of 250:1 was chosen and 0.1 mCi of 3H radioactivity was associated with the phospholipid analogue and 2 mg of ATP synthase were used.

Photolysis—The lipid protein complexes were then illuminated at 0 °C with a 100-watt mineral lamp (Ultra-Violet Products, San Ca-
briel, CA) for 20 min, with a glass filter to cut down the radiation lower than 300 nm. After illumination, the protein was recovered by centrifugation through 10% sucrose, 10 mM sodium phosphate buffer, pH 7.2.

Electrophoresis—Electrophoresis was performed on a 0.8-cm long linear gradient i4 to 19% polyacrylamide gel with a modification of the Laemmli procedure (23), 15% sucrose was incubated in a 1-cm-
long 5% polyacrylamide stacking gel, and the running gel contained a 0 to 20% linear sucrose gradient.

Separation of the ATP Synthase Subunits—ATP synthase subunits were separated by high performance liquid chromatography on a G 300 SW column (60 x 60 cm) (Toyo Soda, Tokyo) equilibrated...

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in 20 mM sodium phosphate buffer, pH 7.0, containing 0.1% SDS.\textsuperscript{1} Samples of 300 \(\mu\)l containing 1 mg of protein were injected. The flow rate was 0.7 ml/min. Fractions of 0.17 ml were collected. Proteins were detected at 280 nm.

Proteolytic Cleavage by Staphylococcus aureus Protease V8 (Miles)—This was performed in 200 mM sodium phosphate buffer, pH 7.8 containing 0.1% SDS. To the isolated labeled \(\beta\)-subunit (50,000 cpm), 300 \(\mu\)g of unlabeled protein was added. Digestion was carried out for 16 h at 30 \(^\circ\)C with 50 \(\mu\)g of protease. Then, another 50-\(\mu\)g aliquot was added and the incubation was continued for 8 h at 30 \(^\circ\)C. The mixture was then lyophilized.

The dried sample was dissolved in 0.9 ml of 80% formic acid. Peptides were separated on a P-30 column (0.8 x 150 cm) equilibrated in 80% formic acid (24). Flow rate was 1 ml/h. Fractions of 0.5 ml were collected. Aliquots were taken for thin layer chromatography, polyacrylamide gel electrophoresis, determination of radioactivity, and amino acid analysis (Biotronic LC 2000) (24).

Sequencing Methods—For the immobilization on the solid support, an equal amount of 0.53 M dimethyldialanine buffer, pH 9.0, containing 66% pyridine was added to the solution of the purified labeled \(\beta\)-subunit. Seventy mg of phenylendisiothiocyanate-activated porous glass beads (230 \(\mu\)m) were then added and incubated for 16 h at room temperature. The glass was washed with water, methanol, and trifluoroacetic acid. Deformylation was carried out in 1 M HCl dissolved in dry methanol for 2 h at room temperature. The glass was washed with methanol, diethyl ether and then dried. Sequencing and conversion was performed as described (24). The degradation was checked by analyzing the phenylhydantoin derivative of the amino acids by high performance liquid chromatography on a Lichrosorb 10\(\mu\)m column (5 \(\mu\)m, Merck) according to Ref. 25. For determination of radioactivity, the sample was dissolved in 50 \(\mu\)l of trifluoroacetic acid. Aliquots of 30 \(\mu\)l were counted (24).

RESULTS

Fig. 1 shows the distribution of label in the individual subunit of the ATP synthase. Subunit \(\beta\) was predominantly labeled. The position of the labeled subunit \(\beta\) was slightly shifted because of the increased molecular weight due to the covalently bound phospholipid analogue. As expected, no labeling was noticed in the \(\alpha\)-subunits which are supposed not to penetrate the lipid bilayer. Surprisingly, only faint, if any, labeling was found in the other \(\alpha\)-subunits \(a\) and \(c\), which represent the major protein mass of the \(\alpha\)-moiety. The yield of label in subunit \(\beta\) was 0.015 moles of lipid/mol of subunit \(\beta\).

For the identification of the modified amino acids, the yield of labeling was increased by choosing a lower lipid to protein ratio (250:1, w/w). This resulted in the following distribution of radioactivity in the individual subunits: \(b\), 83%; \(\beta\), 10.3%; \(a\), 4.0%; \(c\), 2.7%. About 0.06 mol of bound lipid per mol of subunit \(b\) was recovered. Subunit \(b\) was purified by gel permeation chromatography on a G-3000 SW high performance column (Fig. 2). Unlike in the gel electrophoresis, aggregates of the subunits are found in the void volume. This may account in part for the lower recovery of radioactivity in the subunit \(b\). Furthermore, the labeling of subunit \(b\) was decreased to about 0.04 mol of lipid per mol of subunit \(b\) during high performance liquid chromatography. The purity of subunit \(b\) was greater than 90% as checked by polyacrylamide gel electrophoresis in the presence of SDS.

To trace back the label to fragments of the polypeptide chain, CNBr cleavage was tried followed by separation of the peptides on Bio-Gel P-30 in 80% formic acid (data not shown). No radioactivity was recovered at positions corresponding to the molecular weight of peptides. Instead, all the radioactivity was found in the column volume corresponding to a molecular weight of less than 200. Apparently, the label was destroyed or detached from the respective amino acids during CNBr treatment.

Cleavage with Staphylococcus aureus protease V8 was predicted to result in numerous small fragments and one large fragment containing the hydrophobic NH\(_2\)-terminus. This fragment can be easily recognized during P-30 chromatography. Fig. 3 shows the separation of the peptides. Again, a large portion of the label was found in the column volume, but two radioactivity peaks could be recognized. The first peak was uncleaved subunit \(b\). The second one with a \(M_r\) = 5000 showed UV absorbance at 280 nm which was expected for a peptide from the NH\(_2\)-terminus because only the small segment of residue 1 to 26 contained 1 tyrosine and 1 tryptophan; the rest of subunit \(b\) is devoid of aromatic amino acids. No other peak in UV absorbance or radioactivity was observed down to a molecular weight of 500. The recovery of label was about 0.003 mol of lipid per mol of peptides. The amino acid analysis of the pooled fraction (shaded area) is consistent with a mixture of the peptides 1 to 48 and 1 to 53 (Table I). This is in good agreement with the molecular weight determined by the P-30 chromatography. These experiments indicated that the covalently bound label predominantly resides in the NH\(_2\)-terminal part of the subunit \(b\).

The distribution of label among the individual amino acids was determined by sequencing the NH\(_2\)-terminal region of the
**FIG. 3.** Separation of peptide from subunit b generated by cleavage with *Staphylococcus aureus* V8 protease on P-30. For details, see "Materials and Methods." Aliquots of 200 μl were counted. The arrows indicate molecular weight standards: subunit b, 17,300; subunit c, 8,300; peptide B6 from subunit c, 4,200; peptide B8 from subunit c, 1,200; peptide B3 from subunit c, 0,600. The solid line indicates absorbance at 280 nm, the dotted line "H radioactivity. Fractions from the shaded area were pooled and further analyzed.

**TABLE 1**

Amino acid composition of the large fragment generated by *Staphylococcus aureus* V8 protease treatment

The values in parentheses are taken from the known amino acid sequence for the peptide 1 to 53.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagine</td>
<td>3.7 (4)</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Serine</td>
<td>1.1 (1)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4.8 (5)</td>
</tr>
<tr>
<td>Proline</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.8 (6)</td>
</tr>
<tr>
<td>Cysteine*</td>
<td>0.7 (1)</td>
</tr>
<tr>
<td>Valine</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Methionine</td>
<td>n.d. (3)</td>
</tr>
<tr>
<td>Isoleucine*</td>
<td>1.9 (4)</td>
</tr>
<tr>
<td>Leucine*</td>
<td>3.1 (6)</td>
</tr>
<tr>
<td>Tyrosine*</td>
<td>0.2 (1)</td>
</tr>
<tr>
<td>Phenylalanine*</td>
<td>3.8 (3)</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.2 (3)</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.9 (2)</td>
</tr>
</tbody>
</table>

* Determined as Cys-SO₃⁻.

A low recovery of hydrophobic amino acid residue occurring in a cluster is typical, especially for the short hydrolysis time of 16 h in this experiment.

intact subunit b in two independent sequenator runs applying each time 30,000 cpm (Fig. 4). Significant amounts of radioactivity were recovered in the first steps. Because of the gradual decrease of radioactivity, this might reflect a leakage. There are two possible mechanisms for such a leakage: 1) Leakage of the protein which was not covalently attached to the glass. Despite washing the glass with trifluoroacetic acid, still a small fraction remains adsorbed to the glass and is released in the first cycle. This was noticed throughout all our experiments with various labels. Thus, no statement about the labeling of the first residue can be done. In all experiments using various labels (24 and Footnote 2), in the second step radioactivity due to adsorbed protein was found to be negligible. It is thus unlikely that the radioactivity at step 2 in this experiment is caused by the elution of noncovalently bound protein. 2) Gradual destruction of the label. The lipid analogue itself was found to be stable at the condition used in the

**DISCUSSION**

The finding of a cysteine residue probably close to the polar head group of the bilayer explains several initially surprising features.

1. The high preference for the subunit b over the other *F₀* subunits. Apparently, nitrenes exhibit a selectivity for nucleophiles. This favors the labeling of the strong nucleophilic thiol of the cysteine. In a recent publication, Ross et al. (26) described the labeling of the transmembrane domain of glycophorin A with phospholipid analogues containing carbene...
precursors (aryldiazirine). They found that Glu-70 was the predominant site of cross-linking with the phospholipid derivatives. Brunner and Richards (27) found a glutamic acid and a tryptophan as the major sites for the binding of carbene- and nitrene-generating phospholipid analogues in contact with gramicidin. Thus, even though these reagents may react with all amino acids, some residues might receive the bulk of the label. Segments devoid of these residues might be poorly labeled even though they are in contact with the lipid phase. As a consequence, only positive assignment on the accessibility of a certain subunit from the lipid phase can be done.

2. Most likely, during the reaction with the -SH, an S-N bond will be formed which is suspected to be rather unstable, especially to CNBr cleavage during which a sulfonium cation is formed. In this intermediate, the S-N bond is further weakened and most likely cleaved.

Two short segments in the NH2-terminal hydrophobic part of the subunit b appeared to be attacked by the label. Since the mobility of the reactive nitrene is restricted by the fixed location of the phospholipid analogue in the bilayer, these segments are likely to be located in or close to the polar head region of the lipid bilayer. This implies that the NH2-terminus is completely embedded in the bilayer.

REFERENCES

Labeling of subunit b of the ATP synthase from Escherichia coli with a photoreactive phospholipid analogue.
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