[^{125}I]Iodonaphtylazide labeling selectively a cysteine residue in the F_0 of the ATP-synthase from *E. coli* is unsuitable for topographic studies of membrane proteins

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The ATP synthase from *E. coli* was reacted with the hydrophobic photolabel [^{125}I]iodonaphtylazide. Subunit *b* in the F_0-part was selectively labelled. Label was traced back to the single cysteine$_{21}$ in subunit *b*. Thus the reactive intermediate of INA generated by photolysis had a high preference for nucleophiles. Due to this high selectivity the detection of membrane spanning peptide segments by labelling with INA is not reliable.

Hydrophobic labeling *Nitrene* *Membrane protein* *Proton channel*

1. INTRODUCTION

In recent years hydrophobic photolabelling reagents have been developed [1–12]. In the dark these labels will distribute as an inactive form in the membrane. Illumination will generate a reactive group which is capable to react with a wide range of amino acid sidechains in contact with the lipid phase. The reactive groups are either nitrenes or carbenes. Two types of hydrophobic photolabels have been described:

(1) Lipids which bear a reactive group; data regarding modified amino acid side chains appeared in [10,13–15].
(2) Small diffusable hydrophobic reagents which due to their hydrophobicity will be distributed in the lipid phase.

So far, the specificity of these reagents at the level of modified amino acid residues, either nitrene precursor or carbene precursor, has not been investigated.

Here, the labelling of the ATP synthase from *E. coli* with the most commonly used reagent INA which was the first reagent used to modify integral membrane proteins from the hydrophobic core [6] will be described. As the complete amino acid sequence of the ATP synthase is known [16–20] it was possible to investigate labelling at the level of modified amino acid residues.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

Genotype of strain CM 2786 (CM 1470 (pBJC 706)): CM 1470 *E. coli* K12 F$^+$ *asn B32 thi-1 relA1 spo 11 atp 706* (del *atp I BEFHA*). *PBJC 706 atp* (BEFHAGDC)$^+$ on a dimer of pBR 322. Expression of the ATP synthase genes on the plasmid is mainly due to transcription from a promotor in pBR 322, resulting in ATP synthase levels 4–5-fold higher than in a wildtype strain.

2.2. *Growth and media*

Cells were grown on Vogel and Bonner minimal medium [22]. Carbon source was 1% glucose, ampicillin was added to 0.1 mg/ml. Cells were grown to late log phase in 200 l cultures. Preparation of membranes, ATP synthase (F_1F_0) and F_1-depleted

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membranes was performed as in [21,22]. The purity of F_1F_0 was >95%. The enzymatic activities were identical to those in [21].

2.3. *Labelling*

The label [^{125}I]iodonaphtylazide was synthesized at a specific radioactivity of 5 Ci/mmol as in [23]. To 2 mg F_1F_0 dissolved in 400 μl 50 mM Tris–HCl, 25 mM Aminoxid WS35, 1 mM $MgCl_2$, 0.2 mM EGTA, 100 mM KCl, 20% MeOH (pH 7.5), 0.1 mCi [^{125}I]INA in 10–20 μl ethanol were added in the dark at room temperature. Incubation time was 15 min. Membranes (2 mg protein) were adjusted to 6 mg protein/ml in 1 mM Tris–HCl, 0.2 mM EDTA, 5% glycerol (pH 7.5) and incubated with 0.1 mCi [^{125}I]INA for 15 min at room temperature. Photolysis was done in an Aminco fluorimeter equipped with a 250 W Xenon lamp at 360 nm using a band width of 40 nm in a 1 cm quartz cuvette for 4 min at room temperature. Samples were stirred with a small stirring rod.

2.4. *Separation of subunits and identification of modified residues*

F_1F_0 was precipitated by addition of 9 vol. acetone (–20°C) and incubation at –20°C for 1 h. The precipitate was collected by brief centrifugation and dissolved in 0.2 M Tris, 2% SDS. Subunits were separated by high-performance gel permeation chromatogaphy on a G 3000 SW column in 200 mM sodium phosphate buffer (pH 7.0) containing 0.1% SDS [15]. Purity was checked by SDS–polyacrylamide gel electrophoresis followed by autoradiography [22].

In some experiments subunit *b* was isolated from membranes by preparative SDS gel electrophoresis as in [22].

CNBr-cleavage, separation of peptides on Bio-Gel P30 in 80% formic acid and amino acid analysis and sequence analysis were performed as in [19,20,24].

3. RESULTS

3.1. *[^{125}I]INA labelled specifically subunit* b

The modification of F_0-subunits of the ATP synthase by [^{125}I]INA was studied under several different conditions. The results with purified ATP synthase (F_1F_0) dissolved in a buffer containing the non-denaturing detergent Aminoxid WS 35 are shown in fig.1A: Subunit *b* was selectively labelled. We used different preparations of F_1F_0 and different amounts of INA. In some cases the subunit β was labelled but the incorporation of label into the two other F_0 subunits *a* and *c* which are surely membrane integrated [15,22] could not be enhanced. The same pattern was observed after integration of F_1F_0 into proteoliposomes (not shown). In F_1-depleted membranes (fig.1B) subunit *b* was again selectively labelled and no labelling of subunit *c* could be detected. Even in the SDS denatured state there was no labelling of subunit *c* although it constitutes about 20% of the total protein mass (fig.1C).

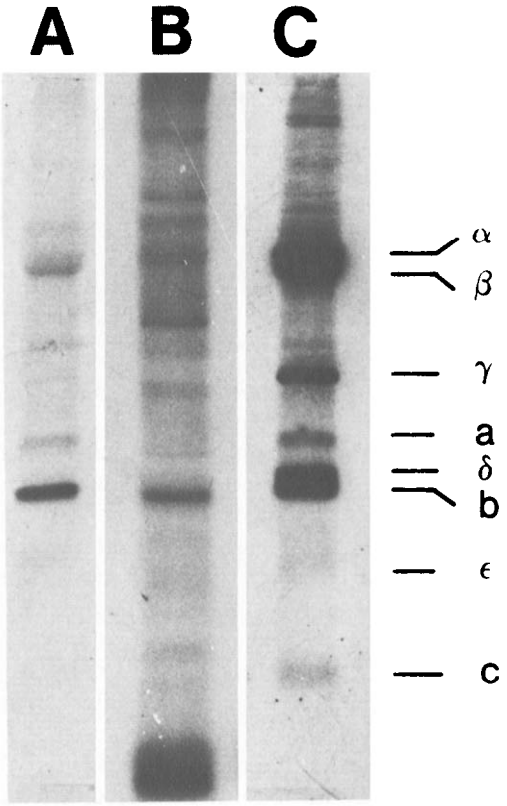

Fig.1. Distribution of [^{125}I]INA in F_1F_0 subunits. F_1F_0 or F_1-depleted membranes of the overproducing strain CM 2786 were incubated and reacted with [^{125}I]INA as in section 2. About 10 μg protein were subjected to SDS–polyacrylamide gelectrophoresis. The gel was stained with Coomassie blue, dried and exposed on Kodak X-O-Mat film for ~16 h at –70°C: (A) F_1F_0 in buffer with Aminoxid WS 35; (B) F_1-depleted membranes; (C) F_1F_0 in 2% SDS solution.

3.2. *Selective attachment of INA to a cysteine residue*

To investigate the nature of the high selectivity of labelling, subunit *b* was cleaved by CNBr at its methionine residues. This cleavage yields two small peptides (B2 and B3) located at the N-terminus and one large fragment B4. Radioactivity was exclusively found in peptide B2; i.e., residues 2–22 in the sequence of subunit *b* (fig.2). It should be noted that a large part of the label was detached from subunit *b* by CNBr-cleavage and appeared in the total column volume. The labelled amino acid(s) were identified by Edman degradation of the whole protein. Fig.3 shows that label was found exclusively at position 21 corresponding to the single cysteine in the entire F_0-part of the ATP synthase. The bound label was perfectly stable during Edman degradation in contrast to CNBr-treatment and more than 80% of the applied radioactivity was recovered up to step 21. Thus, in accordance with the results from CNBr fragmentation the label was exclusively bound at the N-terminus of subunit *b* at the cysteine residue 21.

4. DISCUSSION

F_0 of the ATP synthase from *E. coli* is an ideal subject to study the specificity of hydrophobic photolabels:

(i) The primary structures of its 3 components

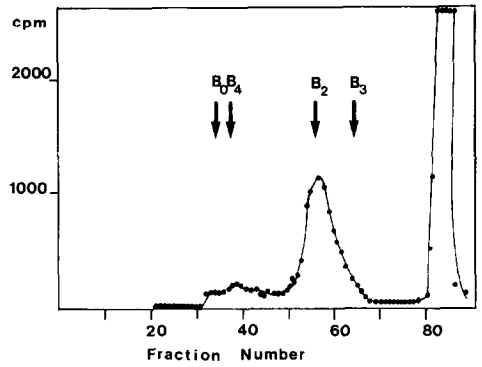

Fig.2. Separation of labelled CNBr-fragment from subunit *b*. Samples containing about 50 000 cpm were subjected to CNBr-cleavage. Peptides were separated in 80% formic acid on a Bio-Gel P 30 column (0.8 × 150 cm) [24]. The arrows indicate the elution volumes of the CNBr-fragments B 0 (uncleaved subunit *b* $M_r \sim 17\,000$) B 4 ($M_r \sim 15\,000$), B 2 ($M_r \sim 2300$), B 3 ($M_r \sim 900$).

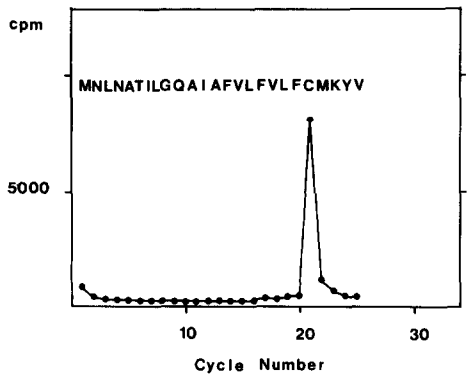

Fig.3. Identification of labelled residue(s) by Edman degradation. Labelled subunit *b* was immobilized on *p*-phenylendiisothiocyanate activated porous glass beads and subjected to Edman degradation [22]. PTH amino acids were identified by HPLC chromatography and analysed for ^{125}I radioactivity.

and their stoichiometry of a:b:c = 1:2:12 is known [16–18,25,26].

(ii) There are sufficient data on the membrane integration of the subunits *b* and *c* obtained by protease treatment experiments [22] and label experiments with photoreactive phospholipids [15]. Subunit *b* is only anchored in the membrane by a short segment (residue 1–26) at the N-terminus. Subunit *c* due to its hydrophobicity is soluble in organic solvents (e.g., chloroform/methanol) and is thus an integral membrane protein. It is deeply embedded in the membrane since protease added to membrane vesicles failed to cleave this subunit [22].

(iii) Protein sequence methods have been developed for subunits *b* and *c* [16,19,20,22]. It was thus possible to study the modification at the level of individual amino acids.

This study shows that the hydrophobic label [^{125}I]INA exhibits a high selectivity for nucleophiles; e.g., the thiol of a cysteine. Labelling of membrane integrated peptide segments will thus depend on the presence of such 'reactive' groups exposed at the lipid phase. Apparently, the reactivity of the generated nitrene was too low to cause effective insertion into C–H-bonds of the aliphatic side chains of Ala, Leu, Ile, and Val and into the side chains of Met or Phe. However, these risidues are the most abundant ones in hydrophobic mem-

brane spanning segments (cf. sequence of subunit *c*).

Selectivity problems may also result from long-lived products that are formed by intramolecular rearrangements of nitrenes. Such intermediates have been identified upon photolysis of some arylnitrenes [27,28]. Substituted arylazides [28] rapidly undergo an intramolecular rearrangement to the corresponding cycloazaheptatrienes which are strong electrophiles. Most likely, the cysteine residue in the subunit *b* provided a sink for such a reactive intermediate. Thus, labelling did not proceed via the nitrene but rather via an unintended byproduct upon photolysis of [^{125}I]INA.

If labelling should give reliable results on the exposure of peptide segments at the lipid boundary, then the chemically most inert aliphatic residues must be attacked even in the presence of a strong nucleophile such as a cysteine residue. In conclusion, [^{125}I]INA and more generally precursors of nitrenes seem not to fulfill this requirement as demonstrated by the non-labeling of the intrinsic membrane proteins *a* and *c* of the ATP synthase from *E. coli* which do not contain cysteine residues.

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