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Identifying subunits of ATP synthase $TF_0 \cdot F_1$ in contact with phospholipid head groups

α-Subunits are labelled selectively by a new photoreactive phospholipid designed for hydrophilic photolabelling

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A new phospholipid photolabel was introduced by modifying 1,2-dipalmitoylphosphatidylethanolamine with 4-azidosalicylate to 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-(4-azido-2-hydroxybenzoyl)ethanolamine (ASA-PE), which could be radioiodinated easily to ^{125}I-ASA-PE. The ATP synthase $TF_0 \cdot F_1$ of the thermophilic bacterium PS3 was reconstituted with soybean phospholipids forming proteoliposomes with high ATP-$^{32}P_i$ exchange activity. These proteoliposomes were incubated with ^{125}I-ASA-PE to provide its selective incorporation into the outside of the phospholipid bilayer. Upon illumination with ultraviolet light α-subunits of TF_1 were predominantly labelled.

ATP synthase; Photoreactive phospholipid; Hydrophilic photolabeling; α-Subunit; Membrane surface; (Thermophilic bacterium P53)

1. INTRODUCTION

The ATP synthase of thermophilic bacterium PS3 is composed of an integral membrane protein complex TF_0 and a peripheral protein complex TF_1. It consists of five different subunits α, β, γ, δ, ϵ and its subunit stoichiometry is $\alpha_3\beta_3\gamma\delta\epsilon$ [1]. TF_0 is probably composed of three subunits, subunit 4 (M_r 19000), subunit 6 (M_r 13500) and subunit 8 (M_r 5400). In order to study the arrangement of the membrane part F_0 of various ATP synthases within the phospholipid bilayer, radioactive 2-lysophosphatidylcholine was recently modified by the photoactive 2-azido-4-nitrobenzoic acid (directly or bound to a lipophilic ligand) [2,3]. These probes were thought to react with the hydrophobic sector of integral membrane proteins. It was found that these nitrene-generating phospholipid analogues photolabelled predominantly subunit b in *E. coli* ATP synthase and nearly all subunits of F_0 ($F_0$1–$F_0$6, $F_0$8, $F_0$10) in the mitochondrial enzyme [4].

We introduce here a new class of radioactive, nitrene-generating phospholipid analogues, in which the amino group in the polar head group of phosphatidylethanolamine was first reacted with 4-azido-2-hydroxybenzoic acid to the related amide. This compound could be easily radioiodinated in the last step of the synthesis.

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Abbreviations: ASA-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-(4-azido-2-hydroxybenzoyl)ethanolamine; ^{125}I-ASA-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-(4-azido-3-[^{125}I]iodo-2-hydroxybenzoyl)ethanolamine; NBD-PE, *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine

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This new probe was useful in identifying subunits of the thermophilic ATP synthase $TF_0 \cdot F_1$ in contact with phospholipid head groups on the proteoliposome surface.

2. MATERIALS AND METHODS

ATP synthase $TF_0 \cdot F_1$ was prepared from thermophilic bacteria PS3 according to Kagawa and Sone [1].

Activities were measured after addition of purified asolectin (ATPase activity: 13.7–15.2 U/mg protein at 56°C). Asolectin was purchased from Associated Concentrates, NY and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine from Sigma. 4-Azido-2-hydroxybenzoyl-*N*-hydroxysuccinimide ester was synthesized according to Ji and Ji [4]. ASE-PE was obtained by the reaction of the phosphatidylethanolamine with the *N*-hydroxysuccinimide ester. This product could be radioiodinated easily to ^{125}I-ASA-PE (spect. act. 200 Ci/mol) [5]. All operations involving the photoreactive compounds were performed under red safety light.

$TF_0 \cdot F_1$ was reconstituted into preformed liposomes by incubation according to Racker [6], with a modification introduced for $CF_0 \cdot F_1$ by Samoray and Hauska (personal communication). 50 μg of lyophilized $TF_0 \cdot F_1$ were dissolved in 100 μl of 0.5 M Tricine-NaOH, pH 8.0, plus 550 μl distilled water and incubated for 5 min at 37°C. 125 μl of a vesicle solution (for which 40 mg asolectin with 1 ml of 50 mM Tricine-NaOH, pH 8.0, were sonified in an ice bath under nitrogen until clear) were added immediately. The incorporation of $TF_0 \cdot F_1$ into the vesicles was established after 10 min at 37°C. The ATP-$^{32}P_i$ exchange activity was determined as in [1]. 0.5 mCi of ^{125}I-ASA-PE dissolved in chloroform were dried with a stream of nitrogen and the solution of $TF_0 \cdot F_1$ vesicles was added to it for an incubation time of 20 min at 37°C.

Photolabelling was performed by irradiation with a 125 W ultraviolet lamp (HPK 125 W/L, Philips) for 10 min at 0°C. A special glass filter was used to absorb the ultraviolet light below 300 nm. The protein was solubilized by addition of the detergent Chaps to a final concentration of 2%. It was centrifuged through a 10% sucrose gradient containing 50 mM Tricine-NaOH, 0.1% Chaps, pH 8.0, at 200000 × *g* for 2 h. This step separated unbound radioactive phospholipid and asolectin from the photolabelled protein. Horizontal SDS electrophoresis in ultrathin (0.5 mm) pore gradient gels was used as described in [7]; it was performed in a long linear gradient 10–16% polyacrylamide overlaid by a 2 cm stacking gel, using the buffer system according to Laemmli [8]. Gels were either stained with Coomassie blue or radioactivity was determined by fluorography with Hyperfilm-MP (Amersham Buchler) and intensifying screens (Kodak).

3. RESULTS

^{125}I-ASA-PE (fig.1) was obtained by simple modification of the corresponding phosphatidylethanolamine and easily radioiodinated in the subsequent step. Both the absorption maxima of ASA-PE at 282 nm and 312 nm decreased upon illumination with ultraviolet light (<300 nm) (fig.2). The maximum at 312 nm shifted to 308 nm. If $TF_0 \cdot F_1$ proteoliposomes were incubated with ^{125}I-ASA-PE, which was distributed on the surface of a microvial, apparently most of the radioactive, photoactive phospholipid was incorporated into the outer surface of the proteoliposomes. Photolabelling was performed with light > 300 nm. Horizontal SDS electrophoresis in ultrathin polyacrylamide pore-gradient gels (10–16%) was introduced, to detect all eight polypeptides by Coomassie blue staining without overloading the gel and to separate, therefore, the α- and β-subunits (fig.3). Additionally subunit b had an apparent molecular mass greater than δ and was therefore not located between δ and ϵ as described in [1]. The Coomassie blue staining profile in our gels gave the following pattern: α, β, γ, a, b, δ, ϵ, c.

After the photoreaction of ^{125}I-ASA-PE with $TF_0 \cdot F_1$ in proteoliposomes (lipid/protein, 100:1, w/w), α-subunits were predominantly labelled (fig.4). It is notable that in this case at least subunits a and b were not modified in the photoreaction, although hydrophilic parts of their polypeptide chains were located at least on the membrane surface in contact with TF_1. Because the photoactive reagent ^{125}I-ASA-PE was constructed to identify subunits in contact with

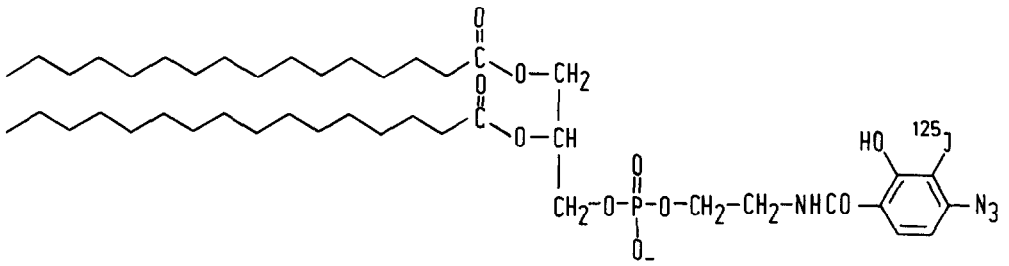

Fig.1. The radioactive, photoreactive phosphatidylethanolamine derivative ^{125}I-ASA-PE.

phospholipid head groups it was a remarkable finding that α-subunits were apparently close to the surface of polar head groups and covered at least this part of the outer surface of TF_1, which was in contact with TF_0. If a lipid to protein ratio of 2.4:1 (w/w) was chosen in the incubation procedure as in [2,3], the subunits α and ϵ of TF_1 and all three subunits of TF_0 were photolabelled (not shown).

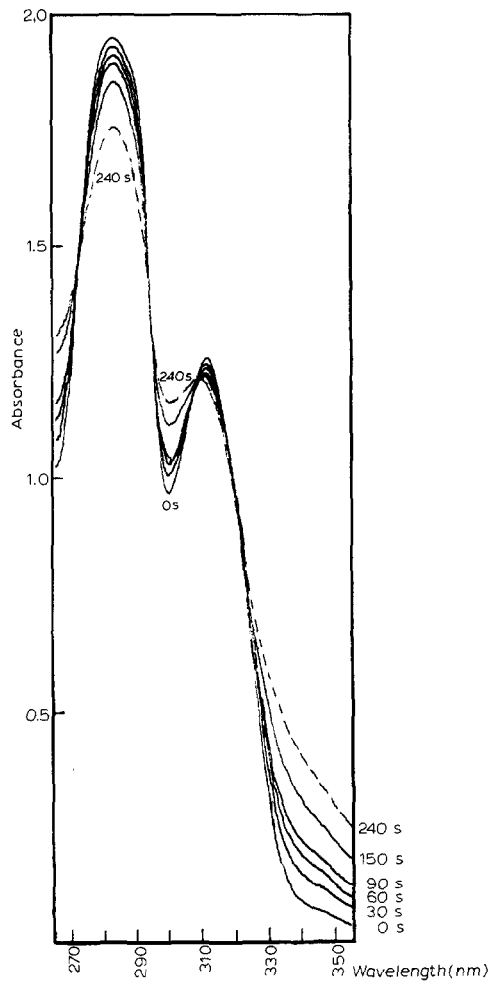

Fig.2. UV spectra of ASA-PE after various times of irradiation with ultraviolet light.

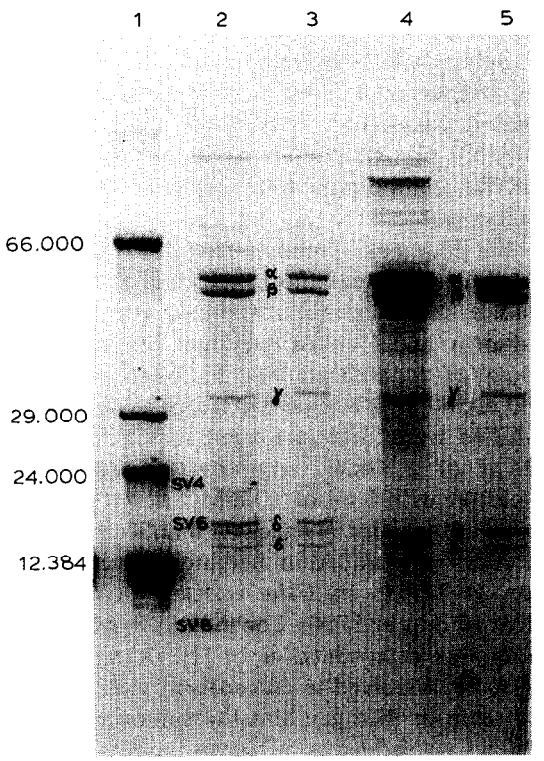

Fig.3. Horizontal SDS electrophoresis of TF_1 and $TF_0 \cdot F_1$ in ultrathin polyacrylamide pore-gradient gels, stained by Coomassie blue. Lane 1: M_r markers: bovine albumin, 66000; carbonic anhydrase, 29000; trypsinogen, 24000; cytochrome *c*, 12384. Lanes: 2, 3, $TF_0 \cdot F_1$, prepared as described in section 2; 4, TF_1, prepared by the chloroform method; 5, TF_1, prepared by the EDTA method.

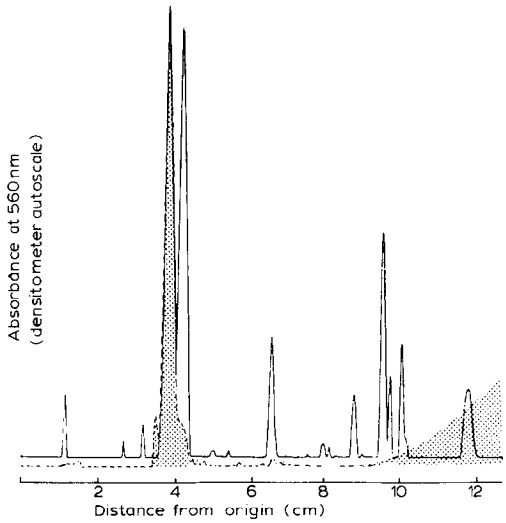

Fig.4. Labelling of thermophilic ATP synthase $TF_0 \cdot F_1$ with ^{125}I-ASA-PE. (a) Coomassie blue staining pattern of $TF_0 \cdot F_1$. (b) Corresponding pattern after labelling with ^{125}I-ASA-PE, as determined by fluorography.

4. DISCUSSION

Subunit α of $TF_0 \cdot F_1$ was identified to be in contact with phospholipid head groups of its proteoliposomes. This was possible because a new photoreactive and radioactive phosphatidylethanolamine derivative could be synthesized, of which the amino group was modified by the nitrene-generating 4-azido-3-[^{125}I]iodosalicylic function (fig.1). Though the positive charge of the protonated amino function disappeared by formation of an amide bond, both the phenolic OH group and amide bond made this photolabel obviously hydrophilic enough to be located near to the phospholipid-water interface. This view was supported by studies with head group labelled fluorescent probes of phosphatidylethanolamine, for example, NBD-PE [9]. Its fluorescence intensity was shown to be quenched by Ca^{2+} indicating its accessibilty to hydrophilic molecules in the aqueous medium [10], though the fluorescent NBD-group was a lipophilic heterocycle without hydrophilic functions. Photolabelling of subunit α suggested a model of $TF_0 \cdot F_1$, in which subunit α may be involved in binding of TF_1 to the membrane and could interact with a subunit or subunits of TF_0 (fig.5). This view was supported by an electron microscopic analysis of the subunit organisation of CF_1 [11]. As derived from Markham rotational analysis for elements of symmetry and tilting experiments of individual molecules in the microscope [12], the six globular masses of α- and β-subunits were located at the vertices of two parallel equilateral triangles, the planes of which were about 2 nm distant. Additionally these triangles and concomitantly the masses of α- and β-subunits were rotated by 60° relative to each other. Thus either all α- or all β-subunits of CF_1 were on one site of a hexagon, a model, which allowed one species only to be photolabelled as shown in our experiment.

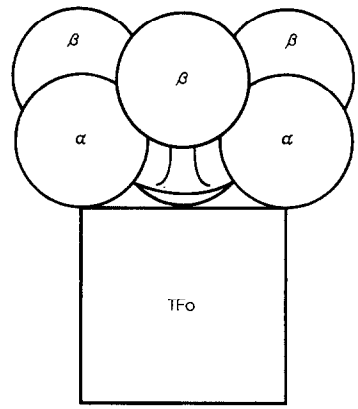

Fig.5. Proposed model for $TF_0 \cdot F_1$.

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