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LOCALISATION OF THE HYDROPHILIC C TERMINAL PART OF THE ATP SYNTHASE SUBUNIT 8 OF *Saccharomyces cerevisiae*

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The hydrophobic subunit 8 of the yeast ATP synthase was modified using the non-penetrating amino reactive specific reagent :isethionylacetimidate. The polypeptide was modified when using the isolated ATP synthase and sodium bromide-treated submitochondrial particules. It is shown that the only lysine of the protein was modified by the reagent. It is concluded that the hydrophilic C terminal part of the protein containing lysine 47 is located on the inner side of the inner mitochondrial membrane.

The membrane-embedded Fo part of the ATP synthase contains three hydrophobic subunits encoded by the mitochondrial DNA in yeast. The Oli1 and Oli2 loci code for proteins of 8000 Mr (subunit 9) and 21500 Mr (subunit 6) respectively (1,2,3). The third product of the mitochondrial ribosomes which belongs to the ATP synthase (4,5) is encoded by the aap1 gene (6). This polypeptide, (subunit 8), is soluble in organic solvents(4). In a preceeding paper we reported the isolation and the primary structure of this proteolipid which is 48 amino acids long(7). Its molecular weight is 5855 Da, and has a high α helical content (85%) as measured by circular dichroism spectroscopy (8). Prediction methods have shown that the subunit 8 contains a hydrophobic domain with which the protein may traverse the inner

ABBREVIATIONS : F1 and Fo : peripheral and integral membrane portions of the H+translocating ATP synthase,
IAI : isethionylacetimidate,
PMSF : phenylmethylsulfonyl fluoride,
PITC : phenylisothiocyanate,
PTC : phenylthiocarbamyl,
tlc : thin layer chromatography,

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mitochondrial membrane once (7). Furthermore the C terminal part of the protein contains a hydrophilic domain which may protrude outside the membrane. In this paper, we propose that the C terminus is localized on the inner side of the inner mitochondrial membrane.

MATERIALS AND METHODS

Isethionyl(1-^{14}C) acetimidate (2.1 GBq/mmol) and $^{35}SO_4^{2-}$ (0.9-1.5 TBq/mg) were obtained from the Radiochemical Centre, Amersham. IAI, Lysyl-lysine and PMSF were purchased from Sigma. PITC was from Pierce.

Preparations :

Cells of the diploid yeast strain "yeast foam" were grown aerobically with 2% galactose as carbon source(9). Mitochondria were prepared by the manual shaking method in the presence of 1 mM PMSF(10). In vivo labelled mitochondria were obtained from small scale experiments using $^{35}SO_4^{2-}$ as label, in the presence of cycloheximide(11). The ATP synthase was immunoprecipitated by F1 antiserum as in (12).

^{35}S-labelled mitochondria were suspended in the isolation buffer; 0.6M mannitol, 10mM sodium phosphate, 1mM PMSF pH 7.0. One half of the sample was centrifuged and the mitochondrial pellet was suspended in the following incubation buffer; 0.6M mannitol, 0.1M triethanolamine, 1mM PMSF pH 8.0 at a protein concentration of 2.7 mg/ml. The other half of the sample was sonicated for 3 min. at 80 volts (Hanemass sonicator). Sodium bromide was added at a final concentration of 4 M and the mixture was incubated for 1 hour at 0°C. The submitochondrial particules were recovered by centrifugation in a Beckmam airfuge at 100000g for 10 min. The floating layer was washed with the isolation buffer, and the pellet obtained after centrifugation was finally suspended in the incubation buffer at a protein concentration of 2.7 mg/ml.

Modification of the subunit 8 with IAI :

^{35}S-ATP synthase immunoprecipitates obtained from 2 mg of ^{35}S-labelled mitochondrial protein were suspended in 0.1 ml of 10 mM sodium phosphate pH 7.0. After centrifugation the pellets were incubated for 30 min. at 0°C in 0.05 ml of 0.1 M triethanolamine pH 8.0, some of which contained or not 4 M of sodium bromide. IAI was added at a final concentration of 50mM and incubation was carried out for 90 min. at 24°C. The reaction was stopped by addition of 0.01 ml of 1 M tris-HCl pH 7.0, and the suspension was extracted with 20 volumes of chloroform:methanol (2:1) for 2.5 hours at room temperature. 4 volumes of water were added for washing and the organic phase was concentrated under nitrogen. The dried residue was dissolved in 0.02 ml of chloroform:methanol (2:1) and analyzed by chromatography as in (7).

^{35}S-labelled mitochondria and submitochondrial particules (0.5 mg protein in 0.18 ml of incubation buffer) were incubated for 45min. at 24°C with 27 mM and 55 mM of IAI (10 μmoles IAI and 20 μ moles IAI/ mg of protein respectively). The reaction was stopped with tris-HCl as above . Mitochondria and submitochondrial particules were centrifuged. The pellet was suspended in 0.02 ml of incubation buffer and the proteolipids were extracted upon

addition of 1 ml of chloroform:methanol (2:1). The soluble fraction was washed with 0.25 ml of water, the aqueous phase was discarded and the organic phase containing the proteolipids was dried under nitrogen. The dried residue was solubilized in 0.02 ml of chloroform:methanol (2:1) and 0.08 ml of pre-cooled diethyl ether was added. The mixture was centrifuged and the pellet was dissolved in 0.02 ml of chloroform:methanol (2:1).

The labelling of mitochondria and submitochondrial particules by [^{14}C] -IAI was performed as above, but the proteolipids were precipitated twice with diethyl ether in order to remove most of the degradation products of [^{14}C]-IAI and labelled lipids.

Labelling of the subunit 8 by [^{14}C]-IAI

Unlabelled ATP synthase immunoprecipitates obtained from 1 mg of mitochondrial protein were dissociated with sodium bromide as above. They were incubated with 8.8 µ moles of [^{14}C]-IAI (18.5 MBq)(total volume:0.35 ml) for 90 min. at 24°C. The proteolipids were extracted with 7 ml of chloroform:methanol (2:1). The insoluble residue was then eliminated by centrifugation . After mixing with 1.5 ml of water, the aqueous phase was discarded and the organic phase was dried under nitrogen. Next, 80 µ g of unlabelled subunit 8 were added as carrier. The proteolipids were precipitated upon diethyl ether addition as above. The proteic pellet was solubilized in 0.1 ml of chloroform:methanol(2:1) and chromatographed as in (7). The area between Rf 0.6 and 0.7 was scraped and extracted with 10 ml of chloroform:methanol (2:1) for one hour at room temperature. The insoluble residue was eliminated by centrifugation. 4 volumes of water were added and the organic phase was concentrated to dryness. The dried residue was hydrolyzed with 6N HCl and the amino acids were coupled to PITC as in (13).

Preparation of PTC,N^{6}-acetamidino lysine

1 µ mole of lysyl-lysine in 0.2 M triethanolamine pH 9.0 was incubated with 5 µ moles of IAI for 2 hours at 24°C. After 10 min. of incubation the pH was adjusted to 9 by addition of triethanolamine (the total volume of the reaction was 0.3 ml). The mixture was dried and hydrolyzed with 0.1 ml of 6N HCl for 24 hours at 105°C. The amino acids were coupled with PITC as in (13). Analysis of PTC derivatives was performed by tlc on reverse phase plates (Whatman KC 18F) using methanol:water(1:1), 0.05 M ammonium acetate pH 6.8 as solvent.

RESULTS

Figure 1 shows the hydrophobic domain with which the protein could cross the membrane, presumably from residue 15 to residue 35. The hydrophilic domain which probably protrudes outside the membrane contains the only lysine residue. Since the N terminal residue is blocked, residue 47 is the only amino acid containing a free amino group. Thus, the ε amino group of lysine 47 appears to be an ideal target for a protein labelling reagent, which could provides information on the localisation of the C terminal part of the protein (matricial side or intermembrane side).

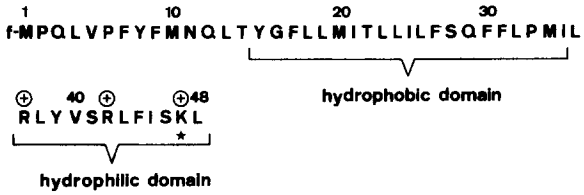


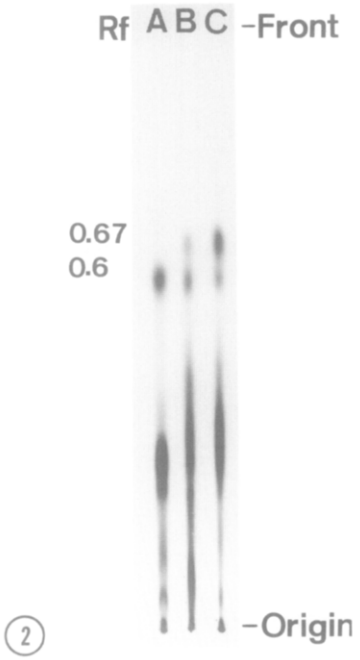
Figure 1 :
Amino acid sequence of the subunit 8. The sequence was described in (7). The amino acid residues are represented in the one-letter code as defined in (18).
+ basic residue; * The star indicates the only lysine residue of the subunit 8.

To this end, a polar, lipid insoluble non-penetrating reagent was chosen; this was IAI which reacts under physiological conditions with primary amines to form amidines and alcohols (14).

Figure 2 shows that addition of unlabelled IAI to an ^{35}S-immunoprecipitated ATP synthase led to a significant modification of the Rf of proteolipids on tlc plates (0.67 instead of 0.6 for subunit 8). A larger amount of subunit 8 was modified when the ATP synthase was dissociated upon sodium bromide addition. Densitometry of the autoradiography showed that 70% of the subunit 8 was modified in the sodium bromide-treated sample and only 34% in the untreated sample. This result could be explained by a steric hindrance due to F1.

Figure 3 shows that the subunit 8 was not modified by IAI when using whole mitochondria (Fig.3A-B) or mitoplast (not shown). Addition of IAI to F1 depleted submitochondrial particules modified subunit 8 (Fig.3C-D).A new spot appeared at Rf:0.67 as in Figure 2, and represented about 34% of the quantity of subunit 8. The intense spot at Rf 0.4 corresponded to subunit 9 (7).

We investigated the reaction of the labelled IAI to unlabelled membranes. In spite of diethyl ether precipitation steps contaminating lipids and degradation products of IAI were still



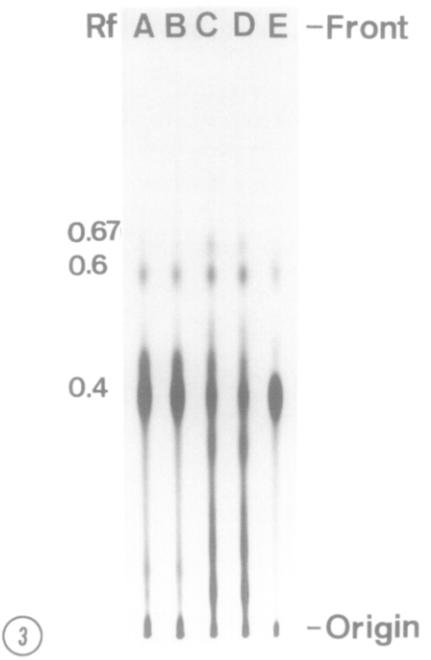


Figure 2 :
Modification of the proteolipids of [^{35}S]-ATP synthase by unlabelled IAI. ^{35}S-immunoprecipitates were incubated in the absence (A) or presence (B) of IAI. The proteolipids were extracted and chromatographed on tlc plates.
(C): 4M sodium bromide pretreated ATP synthase immunoprecipitate incubated with IAI.

Figure 3 :
Modification of the proteolipids at the mitochondrial and submitochondrial particule levels. The proteolipids were extracted, partially purified (see method section) and chromatographed as in Figure 2.
A and B : Proteolipids extracted from whole ^{35}S-labelled mitochondria incubated with 10 µ and 20 µ moles of IAI/ mg of protein respectively.
C and D : Proteolipids extracted from ^{35}S-labelled sodium bromide-treated submitochondrial particules incubated with 10 and 20 µmoles of IAI/ mg of protein respectively.
E: control; proteolipids extracted from ^{35}S-labelled mitochondria incubated without IAI.

present. Figure 4 shows a densitometric analysis of the labelled products . The submitochondrial particule fraction (Fig.4B) contains a shoulder at Rf 0.67. This shoulder appeared as a peak in the sodium bromide-depleted submitochondrial particule fraction (Figure 4 C).No spot was observed at Rf 0.67 in the whole mitochondrial fraction (Figure 4 A).The peak at Rf 0.6 was a non-proteic product, since it disappeared upon precipitation with diethyl ether (not shown).

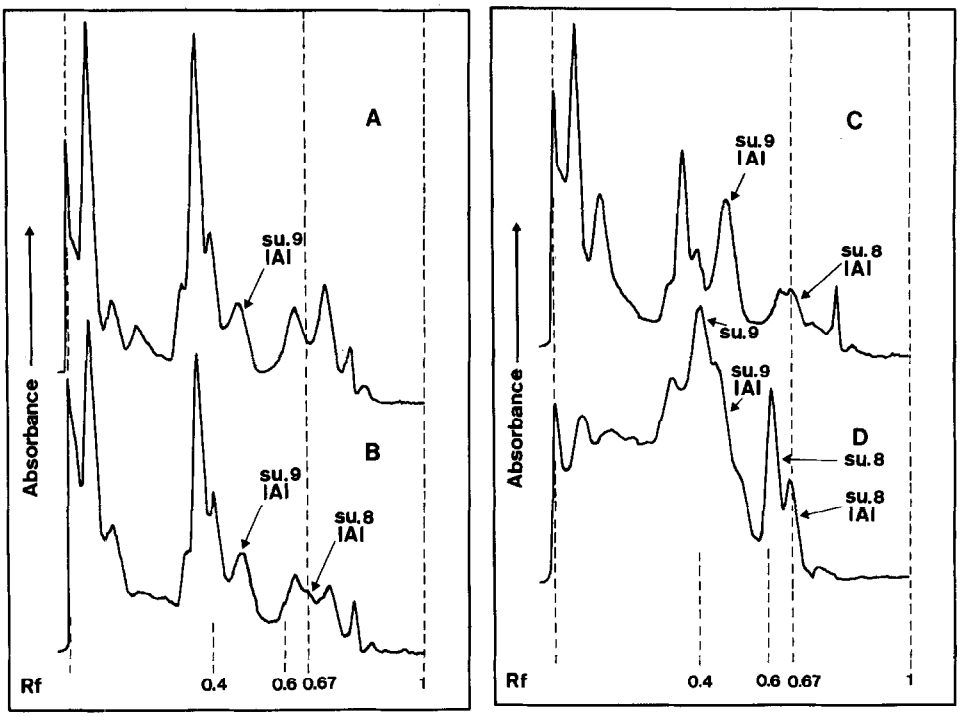


Figure 4 :
Labelling of proteolipids by $[^{14}C]$-IAI. Unlabelled mitochondria (A), submitochondrial particules (B) and sodium bromide-treated submitochondrial particules (C) were incubated with $[^{14}C]$-IAI. The proteolipids were analyzed by tlc as in Figure 2, and the autoradiography was scanned.
(D) : Control; densitometric analysis of Figure 3D.
su.9: subunit 9 ; su.9 IAI: subunit 9 modified by IAI
su.8: subunit 8 ; su.8 IAI: subunit 8 modified by IAI.

We verified that the target of IAI to the subunit 8 was the lysine 47. This was possible because of amidine formation, which is stable during hydrolysis (15). The experiments were as follows:

i) Unlabelled N^6-acetamidino lysine was prepared from the peptide lysyl-lysine and IAI, as described in the method section.

ii) Unlabelled subunit 8 was modified by $[^{14}C]$-IAI from sodium bromide-treated ATP synthase and the ^{14}C-labelled subunit 8 was extracted and hydrolyzed, thereby generating the $[^{14}C]$-N^6-acetamidino lysine. Figure 5B shows the migration of unlabelled PTC N^6-acetamidino lysine (Rf:0.6). The small amount of PTC lysine (Rf:0.26) was due to a loss of the acetamidino group under

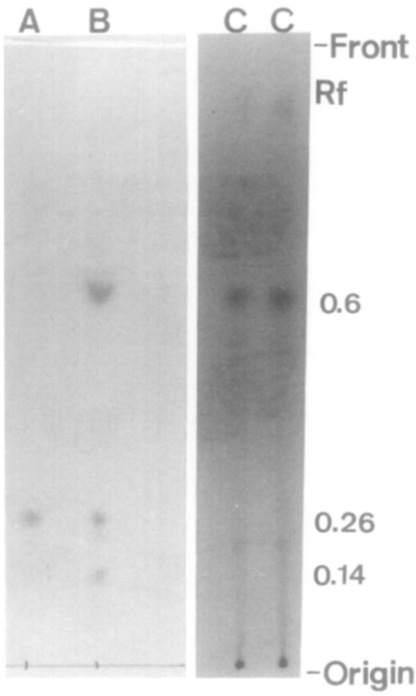


Figure 5 :
Labelling of lysine 47. The ATP synthase was incubated with [^{14}C]-IAI. The proteolipids were extracted and precipitated upon diethyl ether addition. The $N^{6,47}$-acetamidino subunit 8 was isolated and hydrolyzed by 6N HCl. The N^{6}-acetamidino lysine was coupled with PITC and the PTC,N^{6}-acetamidino lysine was analyzed by tlc. Unlabelled PTC lysine (A) and PTC,N^{6}-acetamidino lysine (B) were revealed upon iodine vapours. The labelled PTC,N^{6}-acetamidino lysine (C) was revealed by autoradiography.

acid hydrolysis. At Rf 0.14, there was a migrating contaminant caused by the solvents. Figure 5 C shows that lysine 47 was labelled by [^{14}C]-IAI (Rf:0.6).

DISCUSSION

This work demonstrates the accessibility of the C terminus of subunit 8 to amino group specific chemical non penetrating reagents such as IAI. The reagent binds to lysine 47 when using isolated ATP synthase, and does so preferentially when using pretreated sodium bromide ATP synthase. IAI also modifies subunit 8 in sodium bromide-treated submitochondrial particules as starting material. Only 34% of the ^{35}S-label was found at Rf

0.67. Assuming that 70% of the accessible subunit 8 reacted under our experimental conditions (Cf. sodium bromide-treated ATP synthase), the C terminal part of the protein protruding outside could represent about 48%. This value is in agreement with the amount of inverted submitochondrial particules widely obtained upon sonication. The possibility of an eventual decrease of the label amount on lysine 47 due to competition with other amino groups when using whole mitochondria was rejected, since the other proteolipid (subunit 9) was labelled whatever the starting material used. From these results, the location of the hydrophilic C terminal part of subunit 8 is on the matricial side of the inner mitochondrial membrane (F1 side). It is interesting to note that the orientation of subunit 8 led to a localisation of the three basic residues on the negative side of the mitochondrial membrane. This location may be related to the membrane potential.

Thus, although it cannot be concluded that location of the N terminus is on the outer side of the membrane, certain findings point to this fact: only one crossing was predicted from the primary sequence (7) ; a β turn was predicted around position 14 ; and a hydrophilic domain was presumably generated by unpaired backbone amide and carbonyl groups (due to proline residues 2 and 6), which is in favor of non-integration in the membrane. So, the N and C termini could be located, one on each side, of the inner mitochondrial membrane.

This chemical approach is facilitated when there is an asymmetry of the targets towards the reactive products. Subunit 9, the other proteolipid, contains 2 lysine residues (lys 8 and 44) (16). From prediction methods, Hoppe and Sebald (17) proposed that the E. coli subunit c, which is analogous to the subunit 9 of yeast mitochondria, could traverse the membrane twice in a

hairpin like structure. Thus, lysine 8 would be located on one side and lysine 44 on the other side of the membrane. Indeed, our experimental data (Figure 3) show a modification of the Rf of subunit 9 when using whole mitochondria and sodium-bromide treated submitochondrial particules as starting materials.

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