

Topological and Functional Study of Subunit h of the F₁F₀ ATP Synthase Complex in Yeast *Saccharomyces cerevisiae*[†]

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Received July 18, 2003; Revised Manuscript Received August 20, 2003

ABSTRACT: Subunit h, a 92-residue-long, hydrophilic, acidic protein, is a component of the yeast mitochondrial F₁F₀ ATP synthase. This subunit, homologous to the mammalian factor F6, is essential for the correct assembly and/or functioning of this enzyme since yeast cells lacking it are not able to grow on nonfermentable carbon sources. Chemical cross-links between subunit h and subunit 4 have previously been shown, suggesting that subunit h is a component of the peripheral stalk of the F₁F₀ ATP synthase. The construction of cysteine-containing subunit h mutants and the use of bismaleimide reagents provided insights into its environment. Cross-links were obtained between subunit h and subunits α , f, d, and 4. These results and secondary structure predictions allowed us to build a structural model and to propose that this subunit occupies a central place in the peripheral stalk between the F₁ sector and the membrane. In addition, subunit h was found to have a stoichiometry of one in the F₁F₀ ATP synthase complex and to be in close proximity to another subunit h belonging to another F₁F₀ ATP synthase in the inner mitochondrial membrane. Finally, functional characterization of mitochondria from mutants expressing different C-terminal shortened subunit h suggested that its C-terminal part is not essential for the assembly of a functional F₁F₀ ATP synthase.

The F₁F₀ ATP synthase of *Saccharomyces cerevisiae* is a 600 kDa enzymatic complex of the mitochondrial inner membrane. This reversible enzyme couples the use of the H⁺ electrochemical gradient ($\Delta\mu\text{H}^+$) generated by the respiratory chain to ATP synthesis and can be described as a molecular rotary motor. The rotor, which is driven by the proton flux through the inner membrane, is composed of the subunit 9 oligomer (10 copies) and subunits γ , δ , and ϵ . The stator is composed of nine major subunits. Subunit β , which holds the catalytic sites, is organized with subunit α in a soluble $\alpha 3\beta 3$ hexamer. Subunits OSCP, 4, d, h, f, and 8 form a peripheral stalk linking the catalytic $\alpha 3\beta 3$ hexamer to subunit 6 (1). A specific proton-conducting pathway is formed by the subunit 9 oligomer and subunit 6, which interact in the membrane. The model for ATP synthase energy coupling is the binding change mechanism (2). The affinity change for substrates and products at catalytic sites is coupled to proton transport by the rotation of the γ subunit inside the $\alpha 3\beta 3$ hexamer. Historically, the ATP synthase complex has been separated into two different parts: (i) the

F₁ water-soluble sector (which is composed of the $\alpha 3\beta 3$ hexamer and the γ , δ , and ϵ subunits) retains the ability to hydrolyze ATP; (ii) the F₀ sector, which is embedded in the inner membrane, is composed of both membrane (9, 6, 4, 8, and f) and soluble (OSCP, h, and d) subunits. In addition, two stalks have also been described. The central stalk is composed of subunits γ , δ , and ϵ , while subunits 4, f, h, d, and OSCP form the peripheral stalk. This topological organization has been described in several organisms from bacteria to mammals. Whereas the F₁F₀ ATP synthase of *Escherichia coli* and yeast is very similar (3, 4), the number of proteins constituting the bacterial stator is different. Subunits 8, f, d, and h do not have any homologue in bacteria. In *E. coli*, the peripheral stalk is composed of two subunits b (homologue of yeast subunit 4), subunit δ (homologue of yeast OSCP), and subunit a (homologue of yeast subunit 6). In 1998, Spannagel et al. (5) showed that there is only one subunit 4 per the eukaryotic ATP synthase complex. Some authors hypothesized that the additional subunits (8, f, d, and h) present in the yeast peripheral stalk might compensate the absence of the second subunit b (4).

In 1999, Stock et al. (6) using X-ray diffraction determined at 3.9 Å resolution the structure of the main part of the F₁ sector associated with the subunit 9 oligomer of the F₁F₀ ATP synthase of baker's yeast. The difficulty of obtaining crystallographic or NMR data on isolated subunits of the complex has necessitated the development of alternative low-resolution methodologies such as electron microscopy (7),

[†] This work was supported by grants from the CNRS (AC Dynamique et Réactivité des Assemblages Biologiques and UMR5095), Université Victor Segalen Bordeaux 2, and the Conseil Régional d'Aquitaine. R.F. holds a research grant from the Ministère de la Recherche et la Technologie.

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chemical cross-linking, and disulfide bond formation experiments.

The experimental strategy used in this paper combines the introduction of a cysteine residue at a known position in a given subunit of the F₁F₀ ATP synthase with the use of homo- or heterobifunctional reagents. In this way, it is possible to cross-link this subunit to neighboring proteins that can be identified by Western blot analysis (for a review, see ref 8).

Subunit h, an acidic (pI 4.25) cysteine-less protein, was isolated in 1996 by Arselin et al. (9). It is encoded by the *ATP14* gene, and its mature form is 92 residues long (10.4 kDa). Inactivation of the *ATP14* gene leads to a lack of oxidative phosphorylation and a high instability of the mitochondrial DNA, thus suggesting the essential role of subunit h in the assembly and/or the stability of the F₁F₀ ATP synthase. Complementation experiments in *S. cerevisiae* showed that subunit h is the functional homologue of the mammalian F₆ subunit (10). Cross-linking experiments localized this hydrophilic subunit close to subunit 4, a well-known component of the peripheral stalk of the enzyme (11). Better knowledge of the topological environment of subunit h is essential to understand its role in the F₁F₀ ATP synthase. The present study was based on the combination of cysteine-generated mutants and the use of cross-linking reagents. The stoichiometry of this protein in the complex was determined by radiolabeling. New data showing the proximity of subunit h to subunits f, d, 4, and α were obtained. In addition, a functional analysis of the C-terminal end of the protein was performed. On the basis of these results, a structural and topological model for subunit h is proposed.

EXPERIMENTAL PROCEDURES

Materials. 1,6-Bis-maleimido-hexane (BMH)¹ was from Pierce. *N,N'*-o-Phenylene-dimaleimide (OPD) was obtained from Sigma. BMH and OPD were solubilized in dimethyl-formamide. Stock solutions (100 mM) were stored at -20 °C. Oligonucleotides were purchased from MWG-BIO-TECH. All other chemicals were of reagent grade quality.

Construction of Mutant Strains. The *S. cerevisiae* strain D273-10B/A/H/U (*MAT α* , *met6*, *his3*, *ura3*) was the control wild-type strain (12). The yeast mutants were named as (wild-type residue)(residue number)(mutant residues), where the residues were named by their single letter code. -H6 was added at the end of the name when a (His)₆-tag was added at the C-terminus. A stop codon was introduced in the *ATP14* gene sequence of the (wild-type residue)(residue number)-stop mutants. The strains containing modified versions of subunit h were obtained after complementation of the disrupted yeast strain JY1-2 (*MAT α* , *met6*, *his3*, *ura3*, *atp14::URA3*) by the monocopy vector pRS313 containing each different mutated *atp14* gene. Lysine to cysteine residue replacement, (His)₆-tag addition, or stop codon insertion were done by overlap extension using PCR (13) with the wild-type *ATP14* gene as a template. When the (His)₆-tag was added, a region coding for five histidine residues was inserted

between the 3' extremity of the *ATP14* gene and its stop codon, as the wild-type subunit h C-terminal residue is a histidine. Bacteria and yeast were transformed by electroporation and lithium chloride (14) methods, respectively.

Biochemical Procedures. Cells were grown aerobically at 28 °C in a complete liquid medium containing 2% lactate as a carbon source and harvested in logarithmic growth phase (8×10^7 cells/mL). Mitochondria were prepared as described previously (15) and suspended in the isolation buffer (0.6 M mannitol, 2 mM EGTA, 10 mM Tris-maleate, pH 6.8). The protein concentration was determined according to the Lowry method (16) in the presence of 5% SDS with bovine serum albumin as the standard protein. Specific ATPase activity was measured in the absence or in the presence of oligomycin (6 μ g/mL) at pH 8.4 according to Somlo (17) and modified as described in Velours et al. (10). When added, Triton X-100 was at 0.375% final concentration. Oxygen consumption rates were measured using a Clark electrode with NADH as the respiratory substrate (18).

Cross-Linking Experiments. For cross-linking experiments with OPD or BMH, mitochondria were washed twice with 0.6 M mannitol, 2 mM EGTA, 50 mM HEPES, pH 7.0 and resuspended in the same buffer at a protein concentration of 5 mg/mL. This suspension was incubated in the same buffer in the absence or in the presence of 300 μ M of either OPD or BMH for 1 h at room temperature. Reactions were stopped by the addition of 25 mM 2-mercaptoethanol. Oxidation experiments were performed in 0.6 M mannitol, 50 mM HEPES, pH 7.0 with 1 mM CuCl₂ and stopped after 30 min incubation at 4 °C by the addition of 5 mM EDTA and 5 mM NEM.

Triton X-100 Extracts. The mitochondrial Triton X-100 extracts were prepared as in Paumard et al. (19). At the Triton X-100 concentration used (0.375%), a functional ATP synthase is extracted from the inner membrane of the mitochondria, and the Fo sector keeps its sensitivity to the Fo inhibitors. In addition, the subunit composition of the extracted ATP synthase is the same as that present in the mitochondrial membrane, except for the additional subunits e, g, and k, which are specific to the dimeric form of the enzyme (20). For cross-linking experiments, each protein extract was incubated as described above in Cross-Linking Experiments.

Electrophoretic and Western Blot Analyses. SDS-PAGE was done with Tris-glycine or Tris-tricine 15% polyacrylamide slab gels according to Laemmli (21) or Schagger and von Jagow (22), respectively. Western blot analyses have been described previously (9). Evaluation of the apparent molecular mass of cross-linked products was done by using molecular masses of a prestained protein ladder from Invitrogen Corp. Proteins were electrotransferred onto nitrocellulose membranes (Membrane Protean BA83, Schleicher and Shuell). Primary antibodies were polyclonal rabbit antibodies. Secondary antibodies were peroxidase-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc.). Western blots were revealed using the Enhanced Chemiluminescence method (Amersham Pharmacia Biotech).

Anti-Subunit h Antibody Purification. Anti-h serum was prepared by rabbit immunization with subunit h purified by HPLC as described previously (9). Polyclonal anti-subunit h antibodies were purified by affinity FPLC using a 1 mL

¹ Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Ni-NTA, nickel nitrilotriacetic acid; SDS, sodium dodecyl sulfate; BMH, 1,6-bis-maleimido-hexane; OPD, *N,N'*-o-phenylene-dimaleimide; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight.

NHS-activated HiTrap column (Amersham) on which pure recombinant subunit h was immobilized, as described by the manufacturer. Purification was performed as follows (Lascu, I., personal communication): 20 mL of anti-subunit h serum was loaded onto the column (0.2 mL/min) followed by extensive washing (1 mL/min) with PBS (10 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.14 M NaCl). When the optical density at 280 nm of the eluate dropped below 0.01, the column was washed with 2 mL of 20 mM Tris-HCl (pH 7.4). The elution of the antibodies was performed with 20 mM NaOH, by inverting the flow through the column. Fractions of 0.5 mL were collected in tubes containing 0.5 mL of 0.5 M Tris-HCl (pH 7) for immediate neutralization. The fractions of interest were pooled and dialyzed against PBS and then PBS + 50% glycerol. The purified antibodies were stored at -20°C .

Stoichiometry Determination. F1Fo ATP synthase from the K51C subunit h mutant was purified as described previously (9). A total of 250 μg (0.4 nmol) of the purified enzyme was used for cysteine labeling. The reaction was done according to Collinson et al. (23) under reducing and denaturing conditions (100 mM Tris-HCl pH 8.0, 60 μM DTT, 1% SDS, under a continuous flow of argon) for 1.5 h using 40 nmol iodo[2- ^{14}C]acetate (53 mCi/mmol, Amersham). Proteins were separated by SDS-PAGE. The slab gel was dried and exposed on a phosphorimager plate (Molecular Dynamics) for 1 week. The recorded signal was analyzed with ImageQuant software.

Purification and Trypsic Digestion of Cross-Linked Products. Purification of H6-tagged proteins was done in denaturing conditions (mannitol 0.6 M, SDS 0.1%, 50 mM HEPES-NaOH, pH 7.0) as in Valiyaveetil and Fillingame (24). The purified proteins were submitted to SDS-PAGE, and the slab gel was stained with Amido Black. The selected bands were cut and destained. The proteins they contained were submitted in the gel slices to trypsin action in 0.2 M Tris-HCl, pH 9.0. Digestion was carried out for 4 h at 28°C and stopped by freezing the digests. The resulting peptides were extracted and concentrated in a vacuum centrifuge. The proteolytic digests were desalted and concentrated by microchromatography over $\mu\text{C}18$ ZipTips (Millipore). The peptides were eluted with 2 μL of the solvent, 70% acetonitrile/0.1% TFA in water. This treatment decreases the SDS concentration of the sample to a level compatible with mass spectrometry analysis.

MALDI Mass Spectrometry Analysis. Mass spectrometry analyses were performed on a Bruker REFLEX III MALDI-TOF in the reflectron mode with a 20 kV acceleration voltage and a 23 kV reflector voltage. α -Cyano-4-hydroxy-cinnamic acid (Sigma) was used as a matrix, prepared as a saturated solution in 50% acetonitrile/0.1% TFA in water. Peptide mixtures were mixed in equal volume with the matrix solution. Samples were prepared with the dried droplet method on a stainless steel target with 26 spots.

External mass calibration was achieved with a mixture of eight peptides having masses ranging from 961 Da (fragment 4–10 of adrenocorticotrophic hormone) to 3495 Da (β -chain of oxidized bovine insulin). For PSD (post source decay) experiments, the reflector voltage was stepped down in 10–12 steps, starting from 30 kV, to collect fragment ions from the precursor to immonium ions.

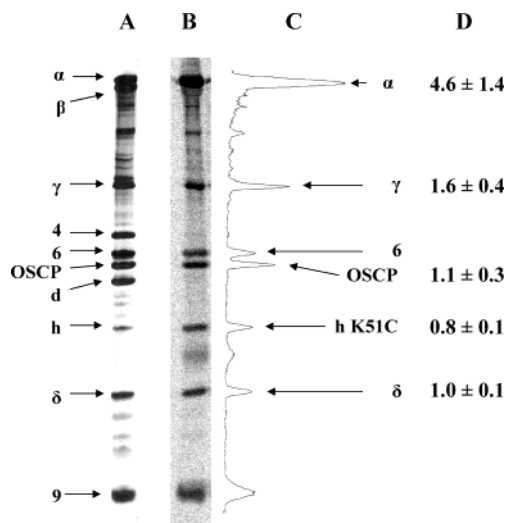


FIGURE 1: Stoichiometry of subunit h in the F1Fo ATP synthase. A total of 250 μg of purified K51C F1Fo ATP synthase was incubated at 1 mg/mL in 100 mM Tris-HCl pH 8 and 1% SDS in the presence of 60 μM DTT for 30 min at room temperature under argon. Forty nmol of iodo[2- ^{14}C]acetate (53 mCi/mmol) was added, and incubation was performed for 1.5 h. Samples were concentrated under vacuum, and Tris-glycine SDS-PAGE was performed. (A) Silver staining. Ten μg of purified K51C ATP synthase. (B) Autoradiography obtained after 7 days exposure on a phosphorimager plate. Fifty μg of labeled purified K51C ATP synthase. (C) Integration of the signal using ImageQuant software. (D) Apparent stoichiometry calculated using subunit 6 as standard (stoichiometry = 1). Labeling was performed on two different preparations of K51C F1Fo ATP synthase. For each preparation, three different samples containing 10, 20, and 50 μg , respectively, were separated by SDS-PAGE and analyzed by autoradiography. The values represent the mean of six different analyses.

RESULTS

Stoichiometry of Subunit h Is 1 per F1Fo ATP Synthase Monomer. An accurate determination of the stoichiometry of subunit h in the F1Fo ATP synthase complex was essential for further topological analysis.

For this purpose, the F1Fo ATP synthase complex of a cysteine containing the subunit h mutant (K51C) was purified and labeled by iodo[^{14}C]acetate. The Fo subunit 6 was used as internal standard (its stoichiometry is 1 per ATP synthase monomer, and it contains one cysteine residue in its sequence). Labeling was performed in reducing and denaturing conditions using a quantity of iodo[^{14}C]acetate in a 5-fold excess as compared to the total number of cysteine residues present in the yeast F1Fo ATP synthase. In these conditions, it can be assumed that all the cysteine residues present in the complex were accessible and were labeled by [^{14}C]iodoacetate. A stoichiometry of 0.8 ± 0.1 subunit h per ATP synthase monomer was determined by integrating the radioactivity bound to subunit h as compared to the standard (Figure 1). Thus, the exact stoichiometry of subunit h should be 1 per F1Fo ATP synthase monomer. Note that the stoichiometry found for α and γ was above the expected stoichiometry (3 and 1, respectively). This was due to the purification method used, which causes a partial dissociation between the F1 and Fo sectors. Free soluble F1 was copurified with F1Fo ATP synthase, thus resulting in the overestimation of the stoichiometry of F1 subunits.

Strategy for Topological Analysis. Preliminary work using dithiobis(succinimidylpropionate) (DSP), an amino reactive

Table 1: Western Blot Analysis of Cysteine Mutants of Subunit h^a

mutant	cross-linking reagent used	cross-link product apparent mass (kDa)	signal with antibody raised against					present in 0.375% Triton X-100 extract
			h	f	d	4	α	
wild-type	OPD	none						
	BMH	none						
K12C	OPD	24	++					no
		35	±			±		yes
		65	+++				+	yes
	BMH	24	+++					no
		35	±			±		yes
		65	+++				+	yes
K15C	OPD	35	±		±			yes
	BMH	24	++					no
		35	+++		±	++		yes
K29C	OPD	35	++		++			yes
	BMH	23	++					yes
		35	+++		++			yes
K36C	OPD	23	+++					yes
		38	+++		+++			yes
		23	++					yes
	BMH	38	++		+++			yes
		23	+++					yes
		24	+++					yes
K51C	OPD	38	+++		+++			yes
		23	+++	+				yes
		24	±					yes
	BMH	38	+++		+++			yes
		23	+++	+				yes
		24	+++					yes
K65C	OPD	38	+++		+++			yes
		24	±			±		no
		38	±		±	±		yes
	BMH	65	+++				+	yes
		24	+++					no
		38	±		±	±		yes
		65	+++				+	yes

^a Isolated mitochondria from yeast cysteine mutants were incubated with 300 μ M OPD or 300 μ M BMH reagents and analyzed by Western blot. Control experiments (no cross-linking reagent) were performed for every mutant and gave the same result as the wild-type strain. A 30 kDa band corresponding to a cross-reaction of the antibody raised against subunit h with an unidentified mitochondrial protein is not displayed in this table. Results obtained with the K89C mutant that displayed a nonreproducible large number of cross-link products are not shown in this table. +++: very strong signal; ++: strong signal; +: normal signal; ±: weak signal; and blank: no signal.

homobifunctional reagent, showed the existence of a large number of cross-linked products between lysine residues of subunit h and neighboring proteins (not shown). Most of these cross-links were still observed in Triton X-100 solubilized F₁F₀ ATP synthase. These results suggested that several proteins or ATP synthase subunits were in close proximity (<12 Å) to subunit h. However, a precise topological analysis was very difficult to obtain by using DSP, as subunit h contains seven lysine residues able to react with it.

To obtain a more precise insight into the topology of subunit h in the complex, each of the seven lysine residues (at positions 12, 15, 29, 36, 51, 65, and 89 of the mature sequence) was substituted by a cysteine residue. This cysteine residue represents a unique target able to react with homobifunctional sulfhydryl-reactive compounds such as bismaleimide reagents. For every mutant, the cell doubling time and the mitochondrial ATPase activities were measured. No significant difference with the wild-type strain was observed (data not shown). Thus, it was assumed that the structure of the mutated subunit h did not change dramatically and that F₁F₀ ATP synthase was correctly assembled.

OPD or BMH are able to covalently link two cysteine residues distant at a maximum of 9 or 15 Å, respectively (25). These two reagents were used to investigate the molecular environment of subunit h cysteine mutants in the F₁F₀ complex. Proteins were separated by SDS-PAGE, and

the cross-linked products were revealed by Western blot analysis.

Subunit h Cross-Linked with Several Unidentified Proteins. The left part of Table 1 shows that by using either OPD or BMH on mitochondria from each mutant, several cross-link products were revealed by subunit h antibodies. The number and the apparent molecular mass of these cross-link products varied from one mutant to another. No cross-link product was detectable with wild-type mitochondria, thus indicating that the adducts were formed in the mutants from the introduced cysteine residues. At the pH of the reaction (pH 7.0), maleimide reagents are assumed to react specifically with cysteine residues. Taking into account this specificity, we tested antibodies raised against F₁F₀ ATP synthase subunits, which contain a cysteine residue and have a molecular mass compatible with the mass of the cross-link products. None of the antibodies raised against subunits OSCP, 6, 9, δ , γ , e, or g were able to react with any of the band detected with anti-h antibodies (not shown). Therefore, these bands might be either unidentified cysteine-containing proteins that did not belong to the complex or cysteine-less subunits of the F₁F₀ ATP synthase cross-linked by another maleimide reactive residue. In control experiments (i.e.: without OPD or BMH), a 30 kDa band was revealed both in wild-type and in mutant mitochondria. Since this band was still present in mitochondria lacking subunit h (data not shown), it corresponds to a cross-reaction of the anti-subunit

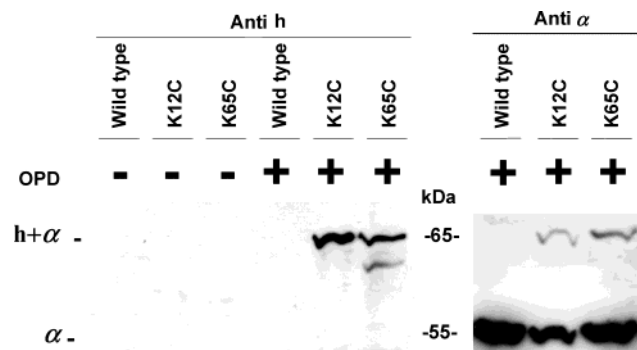


FIGURE 2: Cross-links between subunit h and subunit α . Isolated mitochondria from yeast mutants K12C and K65C were incubated with 300 μ M OPD reagent and analyzed by Western blot. A total of 50 μ g of mitochondrial proteins were separated by Tris-glycine SDS-PAGE. Blots were probed with anti-h purified polyclonal antibody (dilution 1:500) or anti- α polyclonal serum (dilution 1:100 000).

h antibody with an unidentified mitochondrial protein. The presence of this protein, which was able to react with bismaleimide reagents, made the analysis of Western blots difficult in a molecular mass range higher than 40 kDa. The K89C mutant displayed a nonreproducible large number of cross-link products. No further analysis was performed on this mutant.

Proximity Exists between Subunits h and α : K12C and K65C Mutants. The analysis of high molecular mass cross-link products was possible by changing the Tris-tricine migration buffer by Tris-glycine and by increasing the migration time. Figure 2 shows that mitochondria from both K12C and K65C mutants, incubated with 300 μ M OPD, displayed a 65 kDa band that reacted with both anti-subunit h and anti-subunit α antibodies. No cross-link between subunit α and subunit h was observed in wild-type mitochondria or in the other mutant strains (not shown). Thus, subunit α is likely to be in the vicinity of residues 12 and 65 of subunit h. These results are the first evidence of the proximity (9–10 Å) of subunit h and a subunit of the F1 sector, an interaction that could stabilize the link between the peripheral stalk and the F1 sector of the ATP synthase.

Identification of the Proteins Cross-Linked with Subunit h. To identify all the proteins involved in the cross-link products with subunit h, a (His)₆-tag was added at the C-terminus of all mutants except K89C. This tag allowed the purification of the cross-link products for further identification by mass spectrometry analysis. Addition of the (His)₆-tag did not change the cell doubling times or the ATPase activities of mitochondria from these strains as compared to the wild-type strain (data not shown). In the (His)₆-tagged version of the K12C, K15C, and K29C mutants, the same cross-link products were detected as compared to the nontagged mutants (not shown). As a result of the addition of the tag, a small increase of about 0.5 kDa in the apparent molecular mass was observed. In the case of the K36C-H6, K51C-H6, and K65C-H6 mutants, the introduction of the (His)₆-tag modified the pattern of the Western blots in the 23–25 kDa mass range (Figure 3). For the K36C-H6 mutant, an additional 25 kDa band appeared as compared to the K36C mutant. In addition, in this (His)₆-tagged mutant, the intensity of the 23.5 kDa band decreased as compared to that of the 23 kDa band in the K36C mutant. On the other

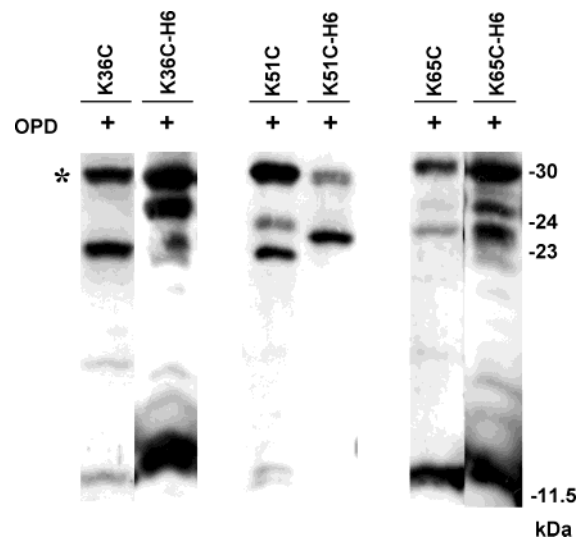


FIGURE 3: Introduction of a (His)₆-tag modified the cross-link pattern of some cysteine mutants. OPD cross-linking experiments were performed on mitochondria from the different mutant strains as described in Experimental Procedures. Tris-tricine SDS-PAGE was performed on 50 μ g of mitochondrial proteins, and blots were probed with anti-h purified polyclonal antibody (dilution 1:500). Results obtained with K12C-H6, K15C-H6, and K29C-H6 are not displayed since introduction of the (His)₆-tag did not modify the cross-link pattern as compared to the nontagged mutants, except for the small mass increment due to the tag. *This 30 kDa band corresponds to a cross-reaction of the antibody raised against subunit h with an unidentified mitochondrial protein.

hand, the 24 kDa band disappeared upon addition of the tag to the K51C mutant. In the case of the K65C mutation, a 23.5 kDa band was observed in the tagged version. These differences due to the introduction of the tag will be discussed next. Since most of the cross-link products were still present in the (His)₆-tag mutants, they were used for further purification and analysis of the cross-link products.

As shown in Figure 4A, for the K51C-H6 mutant, the cross-link products previously observed in the K51C mutant were still present and could be purified by Ni-NTA affinity chromatography. To obtain a sufficient amount of cross-linked products for mass spectrometry analysis, 100 mg of mitochondrial proteins was incubated with OPD and used for purification. The Ni-NTA purified fractions were separated by SDS-PAGE, and the gel was stained with Amido Black. Typical results of the purification obtained with the K51C-H6 mutant are displayed in Figure 4A. Three major bands of 12, 23.5, and 38.5 kDa, respectively, were revealed by Amido Black. The pieces of gel containing the purified product bands were cut, and the proteins they contained were submitted to trypsin digestion within the gel. The resulting peptides were extracted and analyzed by MALDI-TOF mass spectrometry (Figure 4B). Peptides from subunit f (seven peptides) and subunit h (four peptides) were present in the 23 kDa band. The 38 kDa band contained peptides from subunit d (14 peptides) and subunit h (five peptides). As the tryptic digestion was not complete, several peptides contained the same part of the sequence of these proteins. As expected, the 12.5 kDa band corresponded to (His)₆-tagged subunit h. The analysis of this band confirmed the presence of the tag in C-terminal (His)₆ and confirmed the substitution of the Asn 1 residue by an Asp residue (9). Note that this posttranslational modification was not complete

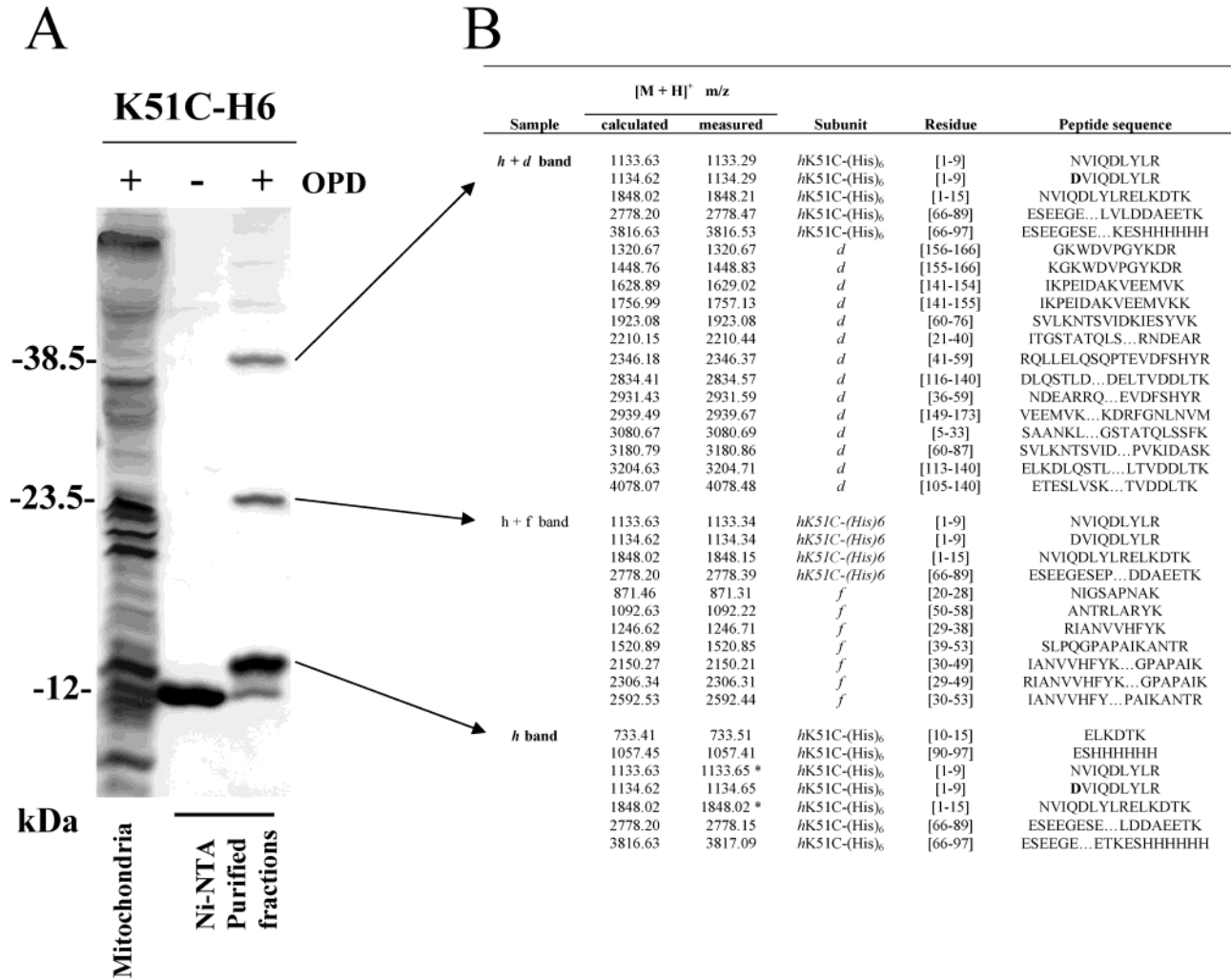


FIGURE 4: Mass spectrometric analyses of tryptic peptides of K51C-H₆. A total of 100 mg of mitochondria from the K51C-H₆ mutant were prepared and incubated for 1 h with 300 μ M OPD. Ni-NTA purification in denaturing conditions (SDS 1%) and Tris-tricine SDS-PAGE separation of the cross-link products were performed. The gel was stained with Amido Black (A). The revealed bands were cut and submitted to trypsin action in the gel. Peptides were extracted and analyzed by MALDI-TOF mass spectrometry as described in Experimental Procedures (B).

since both Asp and Asn residues containing the N-terminal peptide were detected. Except for the K89C substitution, such analyses were performed on the other mutants. As shown in Table 2, the cross-link partners of subunit h were subunits f, d, and 4 depending on the mutant. Note that some peptides were not found for the different proteins. In particular, the part of the sequence involved in the cross-link (adduct between a peptide from subunit h and a peptide from its partner) was not detected. This could be due to the partial tryptic digestion, selective extraction from the piece of gel, and/or the MALDI matrix. Unfortunately, the residue of the subunit h neighbors involved in the cross-link could not be identified by this method. Although no cysteine residue was present in the sequences of subunits f, d, and 4, the latter were linked to a cysteine of subunit h by bismaleimide reagents in the conditions used. This showed that the specificity of these reagents for cysteine was not that which is generally assumed in the conditions used (pH 7.0, 30 °C).

Confirmation of the mass spectrometry results was performed by Western blot analyses of the different cross-link products obtained from all the mutants using both OPD and BMH. Nitrocellulose membranes were revealed with anti-subunit h antibodies and with either anti-subunit d-, anti-

subunit f- (Figure 5), or anti subunit 4- (not shown) antibodies. Results are summarized in the right part of the Table 1. In addition, the presence of the cross-link products was tested in Triton X-100 extracts at a concentration that solubilizes an active form of ATP synthase that is fully sensitive to Fo inhibitors (Table 1).

Cross-Links Involving Subunits h and d Are Present. As shown in Table 2, subunit d was identified by mass spectrometry analysis as a partner involved in cross-linking reactions with subunit h. These results were obtained with four mutants (K29C-H₆, K36C-H₆, K51C-H₆, and K65C-H₆).

The proximity between subunit h and subunit d was also demonstrated by Western blot using 300 μ M OPD on mitochondria from the K12C, K29C, K36C, K51C, or K65C mutants (Figure 5 and right part of Table 1). The signal intensity of the h + d adduct was variable depending on the mutant and the cross-linking reagent. This might reflect a variable distance between the residues involved in the cross-link. Moreover, the apparent molecular masses of the different h + d cross-link products were slightly different from one mutant to the other. These different electrophoretic mobilities could be due to the different shapes of the cross-

Table 2: Mass Spectrometry Analysis of (His)₆ Mutants^a

mutant	apparent mass of cross-linked product (kDa)	identification of cross-linked product	number of detected peptides from subunit h	number of detected peptides from cross-linked subunits	detected sequence of cross-linked protein
K12C H6	65	h + α	2	9	68–77; 189–198; 411–421; 300–310; 511–521; 40–52; 329–341; ^c 128–143; 144–163
K29C H6	35	h + d		19	156–164; 165–173; 5–14; 155–164; 88–97; 77–87; 156–166; 21–33; 21–34; 64–76; 1–14; 21–35; 141–154; 141–155; 88–104; 42–59; ^c 41–59; 116–140; 113–140;
K36C H6	24 ^b		8		
	38	h + d	5	13	165–173; 5–14; 88–97; 77–87; 156–166; 155–166; 64–76; 141–154; 141–155; 42–59; 41–59; 116–140; 113–140
K51C H6	23	h + f	4	7	20–28; 50–58; 29–38; 39–53; 30–49; 29–49; 30–53
	38	h + d	5	14	156–166; 155–166; 141–154; 141–155; 60–76; 21–40; 41–59; 116–140; 36–59; 149–173; 5–33; 60–87; 113–140; 105–140
K65C H6	23	h + f	4	10	7–14; 20–28; 94–101; 20–29; 39–49; 30–38; 29–38; 7–19; 39–53; ^c 20–38
	35	h + d	4	17	71–76; 156–164; 165–173; 5–14; 77–87; 156–166; 21–34; 34–76; 21–35; 141–154; 141–155; 158–173; 60–76; 42–59; 41–59; 116–140; 113–140
	35	h + 4	3	11	187–194; 123–131; 122–131; 195–205; 107–119; 215–227; 50–65; 187–200; 228–242; ^c 228–244; 177–194

^a The samples were prepared as described in Figure 4. This table summarizes the results obtained for each (His)₆-tagged cysteine mutant. Sequences of the cross-linked product are indicated in increasing order of mass. Peptides from subunit h are not displayed for clarity. Molecular masses indicated are the apparent masses observed by Western blot for the nontagged subunit h. No protein was detectable after Amido Black staining for the K15C-H6 mutant. ^b This cross-link product is spontaneous. ^c Peptide was identified by analyzing post source decay fragment ions.

link products, which in turn might be due to the different cross-link positions on subunits h and d.

Subunits h and 4 Are Close. As shown in Table 2, the cross-link product between subunits 4 and h was only detectable by mass spectrometry analysis in the K65C-H6 mutant. On the other hand, as shown in Table 1, this result was confirmed by Western blot analysis of the K65C mutant using OPD and BMH. In addition, a h + 4 cross-link product was also detected in the K12C mutant (Table 1). This adduct was also observed but only with BMH as a cross-linking reagent on mitochondria from the K15C mutant. In the case of the K12C and K15C mutants, the quantity of the cross-link products was low and gave faint signals on the Western blot. These low quantities were a limiting factor for mass spectrometry analysis. In fact, Amido Black staining, a mass spectrometry-compatible method of gel staining, is not sensitive enough (limit of detection: 5–10 μ g of proteins), thus making the location of some cross-link products and their analysis impossible.

Proximity between Subunits h and f Exists. In the same conditions, an h + f adduct was clearly shown on Western blots revealed by anti-h and anti-f antibodies only in the case of the K51C mutant (Figure 5). As shown in Table 1, by using OPD on mitochondria from the K36C mutant and by using BMH on mitochondria from the K29C mutant, a band showing the same apparent mass (23 kDa) appeared on anti-h Western blots but not on the anti-f ones. Antibodies raised against the F1Fo ATP synthase subunits with the same range of apparent mass (subunits g, e, and δ) as subunit f were tested without success on these Western blots. Even so, these cross-link products had exactly the same apparent mass (23 kDa) (visible with longer migration times in SDS–PAGE) as the h + f cross-link product detected in the K51C mutant and were still present in 2% Triton X-100 mitochondrial protein extracts, thus indicating that the cross-linked protein is a protein of the ATP synthase. Identification of the cross-linked protein by mass spectrometry analysis was informative only for the K51C-H6 and K65C-H6 mutants. As shown in

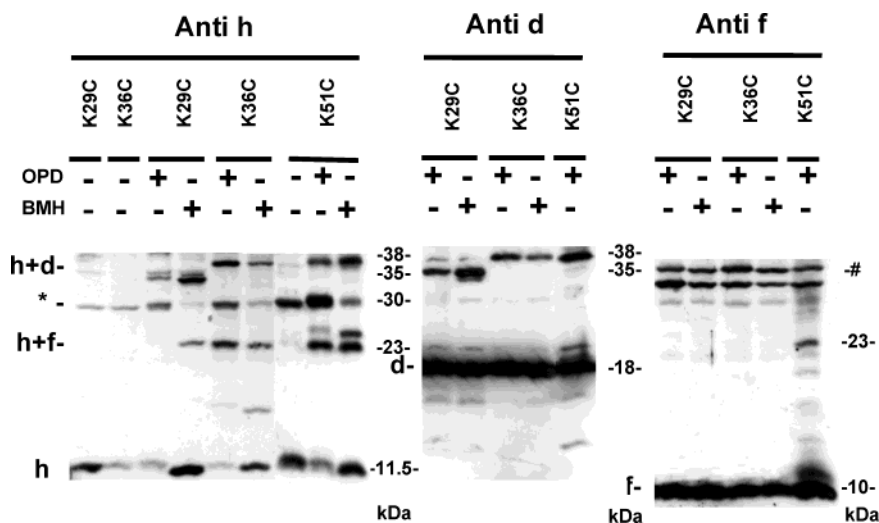


FIGURE 5: Identification of cross-link products in the K29C, K36C, and K51C mutants. Mitochondria from yeast mutants K29C, K36C, or K51C were incubated with 300 μ M OPD or 300 μ M BMH reagents. A total of 50 μ g of mitochondrial proteins were separated by Tris-tricine SDS-PAGE and analyzed by Western blot. Blots were probed with anti-h purified polyclonal antibody (dilution 1:500), anti-f polyclonal serum (dilution 1:10000), or anti-d polyclonal serum (dilution 1:10 000). *This 30 kDa band corresponds to a cross-reaction of the antibody raised against subunit h with an unidentified mitochondrial protein. #These two bands (34–35 kDa), also visible in wild-type samples (not shown), correspond to a cross-reaction of the anti-f polyclonal serum with unidentified mitochondrial proteins.

Table 2, subunit f was identified in association with subunit h in a 23.5 kDa cross-link product in these mutants. Thus, the proximity between subunits h and f was clearly demonstrated only in the K51C and K51C-H6 mutants.

The existence of a relevant h + f cross-link product was not shown in the K65C mutant by Western blot analysis. However, in the K65C-H6 mutant, a 23.5 kDa band appeared on Western blots (Figure 3) and was identified as an h + f cross-link product by mass spectrometry analysis but was not reactive with the anti-f antibodies. The absence of reaction of anti-f antibodies might be due to the inaccessibility of the anti-f epitopes in the cross-link product. This might also explain the fact that this subunit was not identified in the 23 kDa cross-link product obtained with the K29C and K36C mutants. In addition, the introduction of the (His)₆-tag modified the topological environment of position 65 of subunit h, making it closer to subunit f than to subunit α since the cross-link between subunits h and α found in the untagged K65C mutant (Figure 2) was no longer detected in mitochondria from K65C-H6 by mass spectrometry (Table 2) or on Western blots (not shown). Introduction of the (His)₆-tag also modified the topology of position 36 of subunit h. In the K36C-H6 mutant, the amount of the 23.5 kDa cross-link product was very low as compared to the 23 kDa band in the K36C mutant, thus rendering impossible its identification by mass spectrometry.

In the Mitochondrial Membrane, Two Subunits h Belonging to Two Different ATP Synthases Could Be in Close Proximity. Using OPD or BMH on isolated mitochondria from the K12C, K15C, K51C, and K65C mutants, an unidentified 24 kDa cross-link product (h + x) was found (Table 1). Longer migration times in SDS-PAGE emphasized the difference in apparent mass between this band and the 23 kDa band in K51C that corresponded to the h + f cross-link. In these conditions, the h + x cross-link product had exactly the same apparent mass in the K12C, K15C, K51C, and K65C mutants using OPD or BMH as the cross-linking reagent, and this mass was twice the apparent mass of subunit h. None of the antibodies raised against the F1Fo

ATP synthase subunits with a molecular mass compatible to this cross-link product were able to react with this 24 kDa band (not shown). A similar 24 kDa band reactive with anti-h antibodies was also detected after oxidation using CuCl₂ on mitochondria isolated from the K12C, K29C, K36C, K51C, and K65C mutants (Figure 6A), thus indicating that two cysteine residues were involved in this cross-link. In all the cases, when the cross-linking reaction done with either bismaleimide reagents or CuCl₂ oxidation was performed after protein extraction with 0.375% Triton X-100, this cross-link product was not present (not shown). Mass spectrometry analysis was performed on the Ni-NTA-purified putative h + h cross-link (24 kDa) product after oxidation by CuCl₂ on mitochondria from K36C-H6. Eight tryptic peptides from subunit h could be identified (Figure 6B). Therefore, this 24 kDa cross-link product could correspond to a dimer of subunit h. As we determined a stoichiometry of 1 subunit h per ATP synthase monomer, the subunit h dimer could be involved in the link between two ATP synthase monomers.

C-Terminal End (68–92) of Subunit h Is Not Essential for the Assembly and/or Activity of the F1Fo ATP Synthase. The C-terminal end of subunit h is remarkable. First, it is 15 residues longer than the C-terminal part of the F6 mammalian homologue, which is able to functionally complement the absence of subunit h in yeast mitochondria (10). Second, on the last 25 residues of the protein, 12 are acidic and only two are basic, it may be considered as a very acidic part of the protein. To determine the structural and functional role of this C-terminal end of subunit h, two truncated mutants named E68stop and D78stop were constructed. In E68stop and D78stop mutants, the glutamate 68 codon and the aspartate 78 codon were respectively replaced by a stop codon in the *ATP14* gene. The functional consequences of these two C-terminal deletions were analyzed.

The cell doubling times of these mutant strains were measured, and mitochondria were prepared to measure respiration rates, ATP/O ratios, and ATPase activities (Table 3). The deletion of the last 15 residues (D78stop mutant) had no significant influence either on cell growth or on

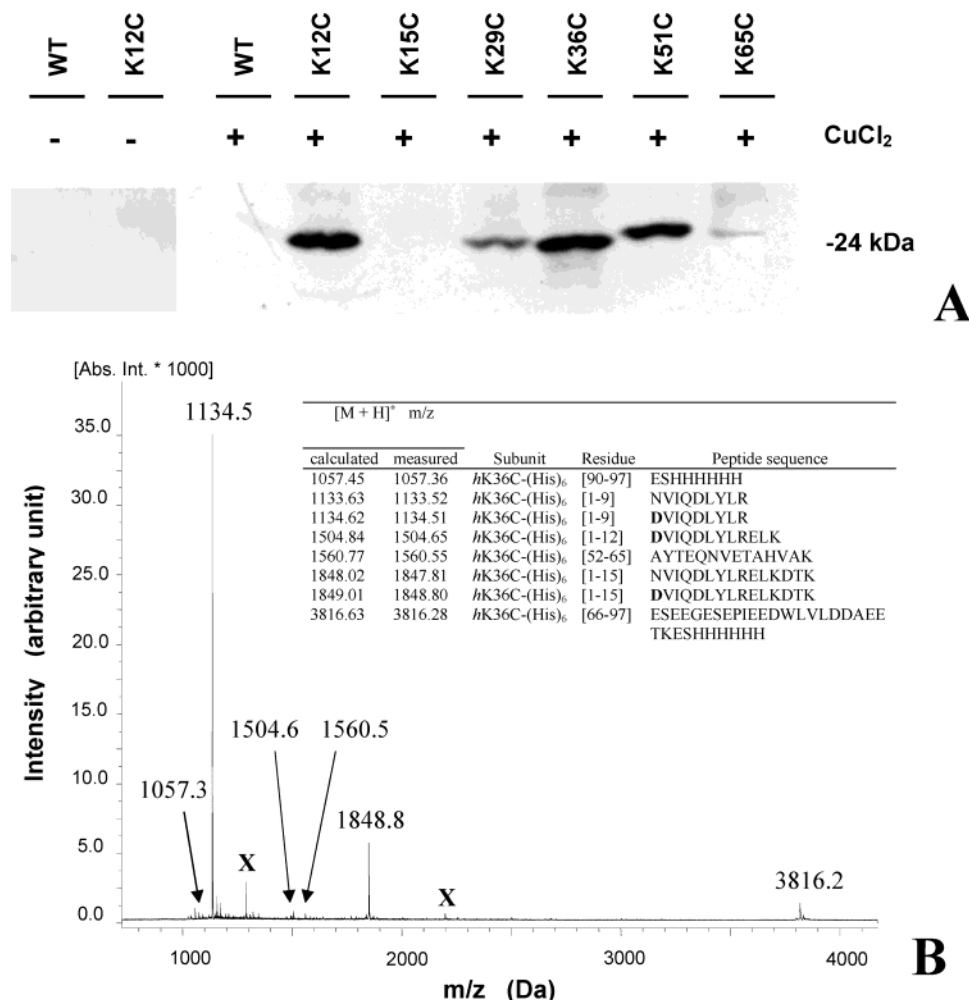


FIGURE 6: Cross-link product obtained by CuCl₂ oxidation. (A) Oxidation experiments on mitochondria from the cysteine mutants of subunit h. Mitochondria from the different strains were incubated or not with 1 mM CuCl₂ as described in Experimental Procedures. Samples (50 μ g of proteins) were separated by Tris-tricine SDS-PAGE and analyzed by Western blots revealed by purified anti-h antibodies (dilution 1:500). The results obtained in the absence of CuCl₂ are shown only for the wild-type and the K12C mutant since all the other mutants gave the same result. For clarity, only the part of the blot in the 22–26 kDa range is displayed. Since the oxidation reaction was not complete, free subunit h was still present and detected (12 kDa) on the Western blot (not shown). (B) Mass spectrometry analysis of the oxidation product in the K36C-H6 mutant. A total of 100 mg of mitochondria from the K36C H6 mutant was prepared and incubated for 30 min with 1 mM CuCl₂. Ni-NTA purification and Tris-tricine SDS-PAGE separation of the purified sample were performed. The gel was stained with Amido Black (not shown). The 25 kDa band was cut and submitted to trypsin action in the gel. Peptides were extracted and analyzed by MALDI-TOF mass spectrometry as described previously. Six peaks, with the measured mass indicated earlier, corresponded to subunit h peptides (inset) visible on the spectrum presented. The other peaks (noted X) could not be attributed to any protein.

oxidative phosphorylation. The respiration rates (in states 3 or 4), the respiratory controls, and the ATP/O ratio were almost similar to those measured in wild-type mitochondria. Thus, this deletion did not modify the stability or the ATP synthesis activity of the F1Fo ATP synthase. However, the ATPase activity measured at alkaline pH showed an increase in oligomycin insensitivity, thus suggesting a partial F1 uncoupling at this pH. This uncoupling was enhanced when the last 25 residues of subunit h were deleted (E68stop mutant). In this case, the effect of the deletion was observed on the efficiency of the oxidative phosphorylation since the state 3 respiratory rate, respiratory control, and ATP/O ratio decreased significantly. Note that in this mutant, F1Fo ATP synthase was still assembled and functional since mutant cells were able to grow on nonfermentable substrates with a similar cell doubling time to wild-type cells. From these data, it could be concluded that the C-terminal part of subunit h is not significantly involved in its structural role within the peripheral stalk but might contribute to correct F1 coupling.

DISCUSSION

Bismaleimide Nonspecificity. Combining cysteine site-directed mutagenesis with the use of thiol reactive homobifunctional reagents is an important alternative method for obtaining structural data on protein complexes or intrinsic membrane proteins. Despite the low resolution obtained, such a method has largely been used by Kaback and co-workers for studying the lactose permease and gave important structural and functional information on this carrier (26, 27). It has also proved to be useful for the topological analysis of multisubunit membrane protein complexes such as the F1Fo ATP synthase (1). Among the various thiol reactive homobifunctional reagents, bismaleimide reagents have been widely used, and the data have been interpreted by taking into account only the postulated specificity of these reagents for cysteine residues. The approach developed to analyze the environment and the topology of subunit h in the F1Fo ATP synthase was initially based on this specificity. Our

Table 3: Functional Analyses of the Subunit h C-Terminal Truncated Mutants^a

strain	τ 1/2 (min)	respiration			ATP/O	ATPase activity				
		respiration rate (natom of O) (min) ⁻¹ (mg of protein) ⁻¹		respiratory control		mitochondria (nmol of Pt) (min) ⁻¹ (mg of protein) ⁻¹		TX-100 0.375% extract (nmol of Pt) (min) ⁻¹ (mg of protein) ⁻¹		
		state 4	state 3			no addition	oligomycin	no addition	oligomycin	% oligomycin insensitivity
wild-type	194 ± 10	302 ± 39	759 ± 58	2.5 ± 0.4	1.09 ± 0.13	2696 ± 98	243 ± 105	4985 ± 101	669 ± 25	13
JVY1-2+pRS313	213 ± 8	418 ± 52	1080 ± 35	2.4 ± 0.4	1.03 ± 0.08	2897 ± 87	1658 ± 36	4941 ± 117	3063 ± 25	62
ATP14 D78stop										
JVY1-2+pRS313	201 ± 13	282 ± 55	558 ± 10	1.9 ± 0.3	0.80 ± 0.13	1971 ± 17	1505 ± 95	4376 ± 100	3036 ± 75	69
ATP14 E68stop										

^a Yeast cells were grown at 28 °C with 2% lactate as a carbon source. Growth was monitored by turbidimetry at 550 nm. Cell doubling time (τ ^{1/2}) was calculated in the exponential growth phase over an 8-h period. Mitochondria were isolated. Oxidative phosphorylation rates and the ATP/O ratio were determined as described in Experimental Procedures. ATPase assays were performed at 30 °C with the addition of 6 μ g of oligomycin/mL where indicated. The wild-type control strain was the D273-10B strain. Measurements were performed in triplicate.

^a Yeast cells were grown at 28 °C with 2% lactate as a carbon source. Growth was monitored by turbidimetry at 550 nm. Cell doubling time ($\tau_{1/2}$) was calculated in the exponential growth phase over an 8-h period. Mitochondria were isolated. Oxidative phosphorylation rates and the ATP/O ratio were determined as described in Experimental Procedures. ATPase assays were performed at 30 °C with the addition of 6 μ g of oligomycin/mL where indicated. The wild-type control strain was the D273-10B strain. Measurements were performed in triplicate.

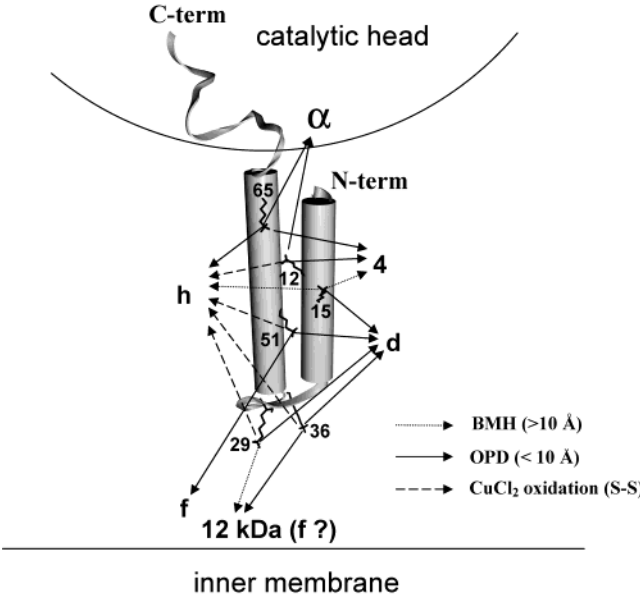


FIGURE 7: Structural and topological model of the subunit h. This model is based on secondary structure predictions and proximities to F1Fo ATP synthase subunits deduced from cross-link experiments in this work. Cylinders schematize the predicted α -helix 1 and α -helix 2.

present findings demonstrate the formation of cross-link products involving cysteine-containing subunit h and other subunits devoid of cysteine residue. Brewer and Riehm (28) showed a possible nonspecific reaction between *N*-ethylmaleimide (NEM) and proteins. Lysine residues could react with the maleimide group. Moreover, such a nonspecific behavior was described for the *p*-*N,N'*-phenylenebismaleimide (PPD) (29). When PPD, OPD, or BMH were incubated with wild-type mitochondria, no cross-linking product involving subunit h was detected. Thus, in the conditions used during this work, no lysine-lysine cross-linking occurred. Therefore, it can be concluded from the present data that the different cysteine-containing subunit h mutants are cross-linked between the introduced cysteine residue and one of the lysine residues of either subunit 4, subunit d, or subunit f. Unfortunately, the mass spectrometry analyses performed did not allow the identification of the lysine residues involved in the cross-links.

Owing to the possible nonspecificity of the bismaleimide reagents, the data obtained with such reagents have to be interpreted cautiously. Consequently, further investigations are necessary to discriminate specific (Cys-Cys) cross-links from nonspecific (Cys-Lys) cross-links. The use of CuCl_2 or bisthiosulfonate reagents that generate disulfide bridges can confirm the presence or not of the Cys-Cys cross-link observed with bismaleimide.

Structural Model of Subunit h. The structural model presented in Figure 7 is based on the present data and secondary structure predictions. First, most of the secondary structure prediction algorithms (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html) give a putative structural organization containing about 70% α -helix and 30% random coil. Three helices are predicted by these programs: helix 1 from Val 2 to Glu 25; helix 2 from Leu 39 to Ser 67; and helix 3 from Ile 75 to Ser 91. The sequence between residues 26 and 39 contains four

proline residues. This proline-rich loop could form a turn between helix 1 and helix 2. The proximity of residues 12 and 65 of subunit h with subunit α and subunit 4 is an indication of the relative proximity of these two residues in the folded protein and supports the existence of a turn between helix 1 and helix 2. The remaining structure of the protein is proposed as a random coil. The C-terminal extremity (residues 68–92) of subunit h has been represented as a random coil instead of the predicted α -helix. Functional studies on C-terminal truncated subunit h mutants indicated that this part of the protein is nonessential for the structural role of the protein. A very high number of cross-link products was obtained when the K89C mutant mitochondria were incubated with OPD and BMH (Table 1), thus suggesting a high mobility of the C-terminal part. Therefore, no precise structure can be proposed for this part of subunit h.

Subunit h in the F1Fo ATP Synthase Complex. In their structural model, Ko et al. placed the mammalian homologue of subunit h (F6) on top of the F1Fo ATP synthase as a component of a cap covering the F1 sector (30). However, they did not exclude other locations of subunit F6 in the Fo sector. The present data suggest that subunit h could be situated along the peripheral stalk between the mitochondrial inner membrane and the bottom of the F1 sector. In fact, the proximity between subunit 4 and subunit h, which was already shown by Soubannier et al. (11), is now confirmed since cross-links between these two subunits were obtained from residues 12, 15, and 65 of subunit h. Moreover, cross-links between subunit h and subunit d, a neighboring subunit of subunit 4 (11), were also found in the K29C, K36C, K51C, and K65C mutants. These data indicate that subunit h could be a constitutive element of the peripheral stalk of the F1Fo ATP synthase and might be involved in its stability. On the other hand, subunit h is close to subunit α . Cross-link products with this subunit were found in the K12C and K65C mutants, thus indicating that positions 12 and 65 of subunit h are in the vicinity of the F1 sector. As cysteine 203 of subunit α is buried in a β -strand, it might not be accessible to cross-linking reagents, thus suggesting that subunit α is cross-linked to subunit h through another residue, certainly a lysine residue. The absence of an h- α -adduct after CuCl_2 oxidation on mitochondria from K12C and K65C mutants favors this hypothesis. Finally, in the K51C mutant, subunit f was cross-linked to subunit h. Subunit f, an Fo component, is embedded in the membrane through its unique transmembrane domain, and its large N-terminal part (66 residues long) is located in the matrix (31). However, Stephens et al. (32) suggested that the N-terminal part of subunit f is located relatively close to the membrane, in close proximity to subunit 8. In both cases, this suggests that the turn between α -helix 1 and α -helix 2 is situated near the membrane (Figure 7). This central position of subunit h in the peripheral stalk might confer to it an important role in the physical coupling between the F1 and Fo sectors. More precise data are needed to confirm the topological model and the position of subunit h in the peripheral stalk. In particular, the identification of the residue of the neighboring proteins involved in the cross-links with subunit h will throw more light on the issue. A method coupling liquid chromatography purification of the cross-link products and mass spectrometry analysis is under development.

Is Subunit h Localized at the Dimerization Interface between Two F1Fo ATP Synthases? CuCl_2 oxidation of mitochondria isolated from the K12C, K29C, K36C, K51C, and K65C mutants resulted in the formation of a 24 kDa cross-link product revealed with anti-h antibodies (Figure 5). Further analysis either by immunodetection of F1Fo ATP synthase cysteine-containing subunits or by mass spectrometry did not allow the identification of the protein linked to subunit h through a disulfide bridge. Since the cross-link products displayed an apparent molecular mass twice that of subunit h, they might correspond to a dimer of subunit h. Moreover, these cross-link products were not detected when oxidation by CuCl_2 was performed on 0.375% Triton X-100 protein extracts. Such behavior has been described in the case of subunit 4 dimerization by disulfide bond formation (5). Since the subunit h stoichiometry is one per F1Fo ATP synthase, its dimerization might be possible only between two ATP synthase monomers that are close together in the mitochondrial inner membrane. Further work is under way to confirm this hypothesis.

ACKNOWLEDGMENT

The authors wish to thank Jacques Vaillier for helpful technical assistance and Christian Napias, Esther Marza, and Ray Cooke for critical reading of the manuscript.

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BI035270J