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MODIFICATION OF F_1-ATPase FROM YEAST SACCHAROMYCES CEREVISIAE WITH 5'-p-[^{3}H]FLUOROSULFONYLBENZOYL ADENOSINE

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F_1-ATPase was isolated from yeast S. cerevisiae. The constituent subunits 1 and 2 were purified by gel permeation chromatography, and their amino acid compositions determined. Both subunits have a similar composition except for ½ cystine, methionine, leucine, histidine, and tryptophan. When F_1 is treated for three hours with 5'-p-[^{3}H]fluorosulfonylbenzoyl adenosine in dimethylsulfoxide, 90% of the activity is lost. Disc gel electrophoresis of the modified complex showed that over 90% of the label was associated with subunit 2. A labelled peptide from a S. aureus digest of subunit 2 was isolated and sequenced. It had the following amino acid sequence: His-Try*-Asp-Val-Ala-Ser-Lys-Val-Gln-Glu, whereby Tyr* is the modified amino acid residue. This sequence shows homology to other sequences obtained from maize, beef heart, and E. coli F_1-ATPases.

Coupling factor complexes are present in a variety of membranes in which oxidative phosphorylation or photophosphorylation take place (1). The complex is composed of two multisubunit parts: One, F_1, is peripheral to the membrane, and has ATP hydrolytic activity; the remaining portion of the complex is designated as F_0, is integral with the membrane and mediates proton translocation (2).

In order to gain better understanding of the amino acid residue(s) involved in the ATPase activity, we have isolated F_1-ATPase, and its two major subunits 1 and 2 from yeast submitochondria. Chemical modification studies utilizing 5'-p-[^{3}H]fluorosulfonylbenzoyl adenosine were initiated, and the identity of the modified amino acid residue delineated.

Abbreviations: PA, performic acid oxidation; pCMB, p-chloromercuribenzoate; IAA, iodoacetic acid; [^{3}H]FSBA, 5'-p-[^{3}H]fluorosulfonylbenzoyl adenosine.

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MATERIALS AND METHODS

Organic and inorganic chemicals, solvents and enzymes. All chemicals used were of analytical grade purchased from Sigma Chemical Co., Fisher Scientific, American Scientific Co., or otherwise as indicated. Solvents and reagents for the Durrum D-500 amino acid analyzer, and Beckman 890C sequencer were purchased from Beckman Instruments Inc., or Pierce Chemical Co. Enzymes used for digestions were obtained from Boehringer Mannheim or Miles Laboratories.

Amino acid analysis and sequencing. Amino acid analyses were performed on a Durrum D-500 amino acid analyzer. Performic acid oxidation of some samples was according to the method of Hirs (3). Sequencing of peptides and proteins utilized 1/3 M and 1 M Quadrol respectively. Samples were analyzed on a Beckman 890C automatic sequencer.

Isolation of nucleotide-free F_1-ATPase. F_1-ATPase was isolated from commercially available sources of yeast S. cerevisiae according to published procedures (4). Briefly, the method involves breaking of cells using a Manton-Gaulon press followed by isolation of submitochondrial particles. F_1-ATPase is solubilized by treatment of particles with chloroform. The solubilized F_1 is then purified by chromatography on DEAE-cellulose. Fractions with enzymatic activity are pooled, and two ammonium sulfate cuts are made: 0-50% and 50-70%. The 50-70% ammonium sulfate fraction is dissolved in the minimum volume of buffer before application to an Agarose AcA-34 column equilibrated and eluted with 0.05 M Tris, 0.001 M EDTA and 0.1 M NaCl.

The isolated F_1 was freed of all contaminating nucleotides by passage over a Sephadex G-50SF column (100cm x 2.5cm) equilibrated and eluted with 0.05 M Tris and 0.001 M EDTA. Fractions with A 280/260 greater than 1.6 were taken to indicate a nucleotide-free fraction.

Titration of sulfhydryl groups. Free sulfhydryl groups were titrated using p-chloromercuribenzoate according to the procedure described by Benesch and Benesch (5). Unfolding of the protein was done in 6 M urea, and reduction of any disulfide linkages was with 1 mM dithiothreitol.

Recation of F_1-ATPase with [^{3}H]FSBA. [^{3}H]FSBA (50 mCi/mmol) was prepared by a procedure similar to that described by Colman et al. (6). About 100 µl of 100 mM [^{3}H]FSBA was mixed with 20 mM of cold FSBA in dimethylsulfoxide, and allowed to react with F_1-ATPase (300 mg in 5 ml of 0.05 M Tris, 0.001 M EDTA, pH 7) for three hours at room temperature. The mixture was then desalted on a Sephadex G-50SF column (100cm x 2.5cm) to get rid of excess reagent. The enzyme was then concentrated by ultrafiltration.

Identification of the labelled subunit and its isolation. A portion of the labelled F_1-ATPase was subjected to electrophoresis in 0.1% SDS following the general procedure described by Weber and Osborn (7). Bands corresponding to subunits 1, 2, and 3 were cut, and dissolved in a mixture of formic acid/perchloric acid, and incubated at 80° C for three hours. The solutions were then cooled, and counted for radioactivity. After identification of the labelled subunit, it was purified on large scale by gel permeation chromatography. The radioactive protein was then carboxymethylated using [^{14}C]iodoacetic acid according to published procedures (8). The alkylated protein was subjected to digestion by a protease isolated from S. aureus at pH 7.8 according to the method of Houmard and Drapeau (9).

RESULTS

Amino acid composition. The amino acid composition of purified subunits 1 and 2 of F_1-ATPase are shown in Table I. Subunit 1 has about twice the

Table I. Amino Acid Composition[a] of Subunits 1 and 2 of Yeast F_1-ATPase

Amino Acid	Subunit 1		Subunit 2	
	Mol%	Residues/55,000	Mol%	Residues/50,000
Aspartic Acid	10.53	52.65(53)	9.33	42.40(42)
Threonine[b]	4.98	24.90(25)	5.08	23.09(23)
Serine[b]	8.75	43.75(44)	8.23	37.40(37)
Glutamic Acid	11.51	57.55(58)	13.14	59.72(60)
Proline	4.21	21.05(21)	3.54	16.09(16)
Glycine	9.79	48.95(49)	11.70	53.18(53)
Alanine	7.60	38.00(38)	8.68	39.45(39)
1/2 Cystine[c]	0.55	2.75(3)	1.40	6.36(6)
Valine[d]	6.85	34.25(34)	7.82	35.54(36)
Methionine	0.60	3.00(3)	1.45	6.59(7)
Isoleucine[d]	5.61	28.05(28)	6.31	28.68(29)
Leucine[d]	10.78	53.90(54)	7.19	32.68(33)
Tyrosine	2.58	12.90(13)	3.31	15.04(15)
Phenylalanine[d]	3.44	17.20(17)	3.56	16.18(16)
Histidine	2.35	11.75(12)	1.36	6.18(6)
Tryptophan[e]	0.21	1.05(1)	0.48	2.18(2)
Lysine	6.05	30.25(30)	6.54	29.72(30)
Arginine	4.96	24.80(25)	4.52	20.54(21)

[a]Average of 24h, 48h, and 72h 6N HCl hydrolysates. In calculating the number of residues per mole, the mean residue molecular weight of an amino acid was assumed to be 110. [b]Determined by extrapolation to zero-time hydrolysis. [c]Determined after performic acid oxidation as cysteic acid. [d]72h hydrolysis value is reported. [e]Hydrolysates were in 4N methanesulfonic acid.

content of leucine , and one half the content of 1/2 cystine, methionine, and tryptophan as compared to subunit 2. The relatively low content of tryptophan of subunit 1 is also reflected in the elution profile shown in Fig. 1 whereby the absorbance at 280 nm of subunit 1 is much lower than that of subunit 2. The inserted disc gel electrophoresis patterns are those of purified subunits 1 and 2. The molecular weights of subunits 1 and 2 are about 55,000 and 50,000 respectively as determined by disc gel electrophoresis in 0.1% SDS.

Sulfhydryl content. F_1-ATPase has only two accessible pCMB-titratable sulfhydryl groups under non-denaturing and non-reducing conditions. One of these sulfhydryl groups is in subunit 2. Both subunits 1 and 2 have no disulfide linkages , but have free sulfhydryl groups.

Isolation of labelled peptide with [^{3}H]FSBA. Upon incubation of F_1-ATPase with [^{3}H]FSBA for three hours, about 90% of the activity is lost. Also 90% of the radioactivity is found associated with subunit 2, and only 10% with subunit 1 as evidenced by disc gel electrophoresis (see Materials and Methods).

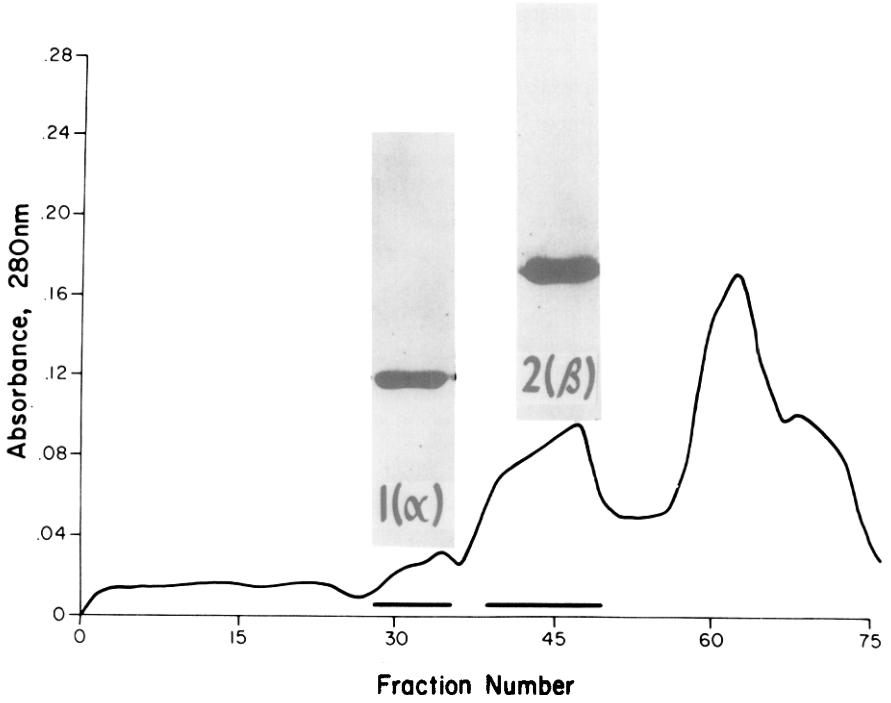

FIG. 1. Elution profile of the 50-70% ammonium sulfate fraction of chloroform F_1-ATPase on an Agarose AcA-34 column (150cm x 2.5cm) equilibrated and eluted with 0.05 M Tris, 0.001 M EDTA and 0.1 M NaCl. Fractions of about three ml were collected, and absorbance at 280nm determined. The inserted disc gel electrophoresis patterns are those of subunits 1 and 2.

The labelled subunit 2 was subjected to digestion by a protease isolated from S. aureus. One major radioactive peak was obtained that was purified to homogeneity by high performance liquid chromatography. The composition of the purified peptide was: $Asx_{0.83}$, $Ser_{0.71}$, $Glx_{1.92}$, $Ala_{0.95}$, $Val_{1.53}$, $His_{0.82}$, $Lys_{0.90}$, $Tyr_{(1)}$. 100 nmol of this peptide was subjected to automated sequence analysis, and the results are shown in Table II. Digestion of the radioactive peptide with a mixture of carboxypeptidase A + B showed the C-terminus to be Glu. The amino acid residue at position 9 was assigned as Gln based on the amino acid composition of the peptide, charge of the peptide, and structural homology considerations (see Table III). At each step of the Edman degradation, a portion of the ethyl acetate phase was counted for radioactivity. The majority of the label (about 90%) was found at position 2. The identity of this residue was determined to be tyrosine by comparison to an authentic sample of CBS-Tyr (O-4-carboxybenzenesulfonyl tyrosine).

Table II. Amino Acid Sequence[a] of Radioactive Peptide (100 nmol)

Step No.	Residue identified	Method of identification	Yield(nmol)
1	HIS	Spot test (Pauly)	ND[b]
2	TYR*	Compare to CBS-Tyr	ND
3	ASP	HPLC[c]	82
4	VAL	"	76
5	ALA	"	48
6	SER	"	ND
7	LYS	"	37
8	VAL	"	6

[a]Amino acid sequencing was done by the automated Edman degradation. The majority of radioactivity was recovered in cycle 2. [b]Not determined. [c]High performance liquid chromatography.

DISCUSSION

The amino acid compositions of subunits 1 and 2 of yeast F_1-ATPase are similar (Table I) except for few variations. This strengthens the hypothesis that these two proteins might be derived from each other (10), or from a common ancestral gene. Subunit 1 has a single tryptophan residue similar to the protein from E. coli which has a tryptophan located at the C-terminus of the molecule. Both subunits 1 and 2 are characterized by a low content of methionine, 1/2 cystine and tryptophan.

The presence of sulfhydryls in both subunits 1 and 2 contradicts a recent report (11) which found no disulfide linkages in F_1-ATPase or sulfhydryl groups in subunit 2.

The homology around the tyrosine residue modified with [^{3}H]FSBA (see Table IV) is striking. In a sequence stretch of 10 amino acid residues, homology runs well over 40%. This is also observed when the complete amino

Table III. Homologous Sequences Around the Tyrosine Residue Modified with [^{3}H]FSBA

MAIZE (13)	HIS-TYR*-GLU-THR-ALA-GLN-ARG-VAL-LYS-GLU
BEEF HEART (14)	HIS-TYR*-ASP-VAL-ALA-ARG-GLY-VAL-GLN-LYS
E. COLI (15,16)	HIS-TYR*-ASP-THR-ALA-ARG-GLY-VAL-GLN-SER
S. CEREVISIAE	HIS-TYR*-ASP-VAL-ALA-SER-LYS-VAL-GLN-GLU

acid sequences of subunit 2 from several species are compared. Identification of a tyrosine residue as the site of modification by [^{3}H]FSBA with the concomittant loss of activity might indicate that this residue is part of the active site. That 2 mM ADP or ATP protects against inactivation (personal observations) would indicate that the tyrosine residue modified by FSBA is likely to be at or near the nucleotide binding site. Esch and Allison (12) have suggested that the tyrosine residue modified by FSBA participates directly in catalysis possibly as a general base that increases the reactivity of the attacking water molecule.

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