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EVIDENCE FOR THREE α SUBUNITS IN ONE MOLECULE OF F_1-ATPase FROM THERMOPHILIC BACTERIUM PS3

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Summary: The numbers of sulfhydryl residues in F_1-ATPase of thermophilic bacterium PS3 and its isolated subunits were analyzed with Ellman's reagent. This F_1-ATPase contained three sulfhydryl residues and no disulfide bridge. Of the five kinds of subunits of the F_1-ATPase, only the α subunit contained one sulfhydryl residue. So there are three α subunits in one molecule of the F_1-ATPase.

F_1-ATPase from mitochondria, chloroplasts, and bacteria is known to serve as the terminal trans-phosphorylating enzyme in oxidative phosphorylation and photophosphorylation (1,2). It consists of five different subunits (α, β, γ, δ and ε), however, there are several proposals on the stoichiometry of these subunits in an F_1-ATPase molecule (3-11). Our previous work suggested the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ for the F_1-ATPase (TF_1) from thermophilic bacterium PS3 (6,12), while others have proposed the structure $\alpha_2\beta_2\gamma_{1\sim2}\delta_{1\sim2}\varepsilon_{1\sim2}$ (7-11).

The studies on amino acid analysis have shown that only few cystein plus half-cystine residues are present in a TF_1 molecule (12). Thus the determination of distribution of them in TF_1 may offer further information on the subunit stoichiometry. This report shows the evidence for the presence of three α subunits in one molecule of TF_1.

Materials and Methods

TF_1 and its individual subunits were prepared as described in the previous papers (12, 13). Ellman's reagent, 5,5'-dithio-

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bis-(2-nitrobenzoic acid) (DTNB), and scintilator cocktail, Scintisol·500, were from Wako Chemicals. ^{14}C-DTNB was from CEA.

Titration of sulfhydryl groups with DTNB was carried out as described by Ellman (14). TF_1 or its purified subunits (0.2-1 mg) were dissolved in 0.3 ml of 50 mM Tris-sulfate (pH 8), containing 0.3 % sodium dodecylsulfate, and 20 µl of 2 mM DTNB solution was added. The change of the absorbance at 412 nm was followed to completion in a Shimazu UV-200 double beam spectrophotometer. Sulfhydryl residues in reduced samples were analyzed as follows. A solution of TF_1 or pufified subunits, in 1 % sodium dodecylsulfate and 50 mM dithiothreitol, was heated in a boiling water bath for about 10 min, and then rapidly passed through a column (0.5 x 20 cm) of Bio-gel P-6 equilibrated and eluted with 5 mM acetate buffer (pH 5.4) containing 0.2 % sodium dodecylsulfate. The eluted protein fraction (0.3 ml) was mixed with first 50 µl of 1 M Tris-sulfate (pH 8) and then 20 µl of 2 mM DTNB, and the increase of absorbance at 412 nm was measured. To avoid reoxidation, all the procedures were carried out within 20 min. A molar extinction coefficient of the 3-thio-6-nitrobenzoate ion was assumed to be 13.6 X 10^3 $M^{-1}cm^{-1}$ at 412 nm. Reduced glutathione was used as a standard to confirm this value under the conditions tested.

The distribution of sulfhydryl residues and disulfide bridges among the subunits was analyzed as follows : Native TF_1 (1.5mg/75µl) or reduced TF_1 solution (0.8mg/0.4ml prepared as described before) was mixed with 50 µl of 20 % sodium dodecylsulfate and 20 µl of 2 mM ^{14}C-DTNB (19.2 mCi/mmole). The mixture, which soon became yellow, was allowed to stand for 10 min at room temperature. Then free ^{14}C-DTNB and released ^{14}C-3-thio-6-nitrobenzoic acid were removed by passing the mixture through a Bio-gel P-6 column, and the fraction of eluate containing protein (about 30µg) was subjected to 10 % polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. The gel was sliced immediately after electrophoresis, and each piece was placed in a vial containing 1 ml of 5 mM ditiothreitol. The vials were closed with caps and incubated for 4 hours at 70°. Then a 10ml of Scintisol·500 was added and radioactivity was measured in a scintillation counter. Almost 90 % of the added radioactivity was recovered.

Protein concentrations were estimated by the method of Lowry *et al.* (15) using dry bovine serum albumin as a standard. Values for TF_1 and the α subunit were corrected by multipling them by a factor of 0.90, which was obtained by comparing the values with the dry weights of these proteins. Reduced proteins were assayed spectroscopically since a high concentration of Tris buffer caused interference in the method of Lowry *et al.* A solution of 1mg (dry weight) of TF_1 or the α subunit per ml had an absorption of 0.50 at 278nm, and the presence of a high concentration of Tris did not affect this value. The same factors of 0.90 for Lowry's method and 0.5 for absorption at 278nm, were adopted in assay of β subunits.

Results and Discussion

DTNB reacts with a sulfhydryl residue in a protein resulting in the formation of a modified protein leaving a

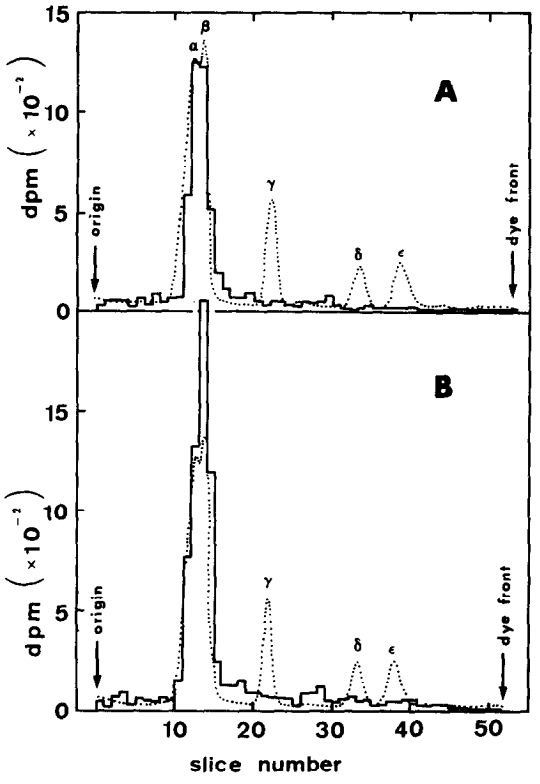

Fig.1: Distribution of radioactivity in subunits of TF_1 labeled with ^{14}C-DTNB. Sample of TF_1 or reduced TF_1 labeled with ^{14}C-DTNB was subjected to polyacrylamide gel electrophoresis in sodium dodecylsulfate and radioactivity of sliced gel was measured as described in Materials and Methods. Dotted lines are densitometric traces of stained gels. (A) unreduced TF_1 (B) reduced TF_1.

free anion of 3-thio-6-nitrobenzoate. ^{14}C-DTNB was used to modify TF_1 molecule determining the localization of sulfhydryl residues. The modified TF_1 containing ^{14}C-nitrocarboxyphenyl disulfide residues, was analyzed by polyacrylamide gel electrophoresis in sodium dodecylsulfate. Fig.1A shows that sulfhydryl residues are localized specifically in the fraction of α and/or β subunits, but not in other subunits. Localization of disulfide bridges could be also determined by the analysis of TF_1 treated with reducing

TABLE I

Titration of sulfhydryl groups in TF_1 and its α and β subunits

Experiment No.	Sample	Released TNB* (*n mole*)	Protein** (*n mole*)	-SH/protein
1	TF_1	6.88	2.23	3.09
	TF_1	12.67	4.47	2.84
	TF_1	19.30	6.70	2.88
2	TF_1(reduced)	3.81	1.21	3.14
	TF_1(reduced)	1.63	0.55	2.95
3	TF_1(reduced)	6.87	2.14	3.21
	TF_1(reduced)	2.08	0.67	3.09
4	α	4.88	6.84	0.70
	α(reduced)	2.48	2.58	0.96
	α(reduced)	2.34	2.31	1.01
	α(reduced)	8.16	6.88	1.19
5	α	2.39	3.93	0.61
	α(reduced)	4.14	4.46	0.93
	α(reduced)	2.58	2.78	0.93
6	β	0.31	9.92	0.03
	β(reduced)	0.16	3.22	0.05

* TNB : 3-thio-6-nitrobenzoate ion
** The molecular weights of TF_1, and the α and β subunits were assumed to be 385,000, 56,000, and 53,000, respectively.

reagent prior to ^{14}C-DTNB modification. The result shows that radioactivity is detected in the fraction of α and/or β subunits (Fig.1B). Thus the γ,δ, and ε subunits contain neither sulfhydryl residues nor disulfide bridges.

The number of sulfhydryl residues and disulfide bridges in TF_1,α or β subunit was determined by measuring the formation of 3-thio-6-nitrobenzoate anion. Without the reducing procedure, three sulfhydryl residues were found in TF_1 (Table I,Experiment No 1). These values were not significantly

different from those with the reducing procedures (Experiments No 2 and 3). Therefore TF_1 did not contain any disulfide bridge. Analyses of isolated α and β subunits showed that the α subunit (Experiments No 4 and 5), but not the β subunit (Experiment No 6), contained one sulfhydryl residue. Rather low values obtained for the α subunit without reduction could be explained by supposing that some isolated α subunits formed dimers linked by a disulfide bridge. This possibility was supported by the presence of a distinct band in a position corresponding to double the molecular weight of the α subunit on polyacrylamide gel electrophoresis in sodium dodecylsulfate, when the loaded sample had been denatured in the absence of any reducing reagent (data not shown). TF_1 did not contain any dimer of the α subunit, so it was probably an artefact formed during the isolation procedures of the α subunit.

Thus the numbers of sulfhydryl residues in TF_1 and the α subunit were determined to be three and one, respectively. The other subunits did not contain sulfhydryl residue. Disulfide bridges were absent in TF_1. These results strongly indicate that there are three α subunits in one molecule of TF_1. The very low content of cystein plus half-cystine residues in TF_1 made this conclusion more reliable than that from analysis of sulfhydryl residues in mitochondrial F_1 which contains twelve cystein plus half-cystine residues (7).

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