Primary structure of a regulating factor, 15 kDa protein, of ATP synthase in yeast mitochondria

Yukuo Yoshida, Sadao Wakabayashi*, Hiroshi Matsubara*, Tadao Hashimoto and Kunio Tagawa+

Department of Physiological Chemistry, Medical School, Osaka University, Nakanoshima 4-3-57, Kita-ku, Osaka 530 and **Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan*

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The amino acid sequence of a factor, 15 kDa protein, which facilitates the formation of, and stabilizes the inactivated complex between mitochondrial ATPase and intrinsic inhibitor was established. The factor was found to be composed of 83 amino acid residues and to have an M_r of 9450, though the apparent M_r was 15000 when determined by SDS–polyacrylamide gel electrophoresis. The factor was characterized as a basic protein with 18 basic and 12 acidic amino acid residues. The amino acid sequence of the factor showed significant homology with those of yeast ATPase inhibitor and the 9 kDa protein which acts in concert with the 15 kDa protein in stabilizing the inactivated ATPase complex.

F_1F_0–ATPase *Stabilizing factor* *15 kDa protein* *9 kDa protein* *ATPase inhibitor*

1. INTRODUCTION

An intrinsic mitochondrial ATPase inhibitor has an inhibitory action on ATP-hydrolysis by F_1F_0–ATPase. The inhibitor forms an equimolar complex with the enzyme in the presence of ATP and Mg^{2+}, indicating that it is an essential subunit of F_1F_0–ATPase [1]. However, the inhibitor is readily dissociated from the complex in vitro and the enzyme becomes active when external ATP is removed [2]. In the previous report, it was shown that in the presence of two protein factors, named 15 kDa and 9 kDa proteins, the ATPase inhibitor inactivated F_1F_0–ATPase very efficiently and the enzyme–inhibitor complex was stable even in the absence of ATP and Mg^{2+} [3]. It was also shown that the factors had no effect on stabilization of the complex between purified F_1–ATPase and the inhibitor. Thus it is highly likely that the factors mediate the binding between the inactivated F_1–ATPase and the mitochondrial membrane, like the δ- and ϵ-subunits of bacterial [4] and chloroplast [5] F_1–ATPase which mediate binding of the $\alpha\beta\gamma$ complex to the membrane moiety, F_0.

The subunits, α, β and γ, of ATPases from various organisms correspond to each other. It has been shown, however, that bovine oligomycin sensitivity-conferring protein (OSCP) and the bovine δ-subunit have homologous sequences with those of *Escherichia coli* δ- and ϵ-subunits, respectively [6]. We recently determined the complete amino acid sequence of the small stabilizing factor, 9 kDa protein, and found that the sequence was closely similar to that of yeast ATPase inhibitor and that both proteins have high homology with *E. coli* ϵ- and a fragment of the bovine δ-subunit [7]. Thus, the relationship of the smaller subunits, δ, ϵ, ATPase inhibitor and OSCP is rather confusing.

Here, we determined the amino acid sequence of the large stabilizing factor, 15 kDa protein, purified from yeast, *Saccharomyces cerevisiae*, and compared its sequence with those of the other subunits of F_1F_0–ATPases of various organisms. It was found that the sequence of the factor showed significant homology with those of yeast ATPase inhibitor and the 9 kDa protein.

+ To whom correspondence should be addressed

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2. MATERIALS AND METHODS

The stabilizing factor, 15 kDa protein, was purified from yeast mitochondria as in [3]. The purified preparation gave a single band with an apparent M_r of 15000 on SDS–polyacrylamide gel electrophoresis (PAGE) with chymotrypsinogen (25000), myoglobin (17500), cytochrome *c* (12400) and insulin (5500) as marker proteins.

The procedures for sequence analysis were essentially the same as those in [7]. About 0.7 mg of the protein was subjected to automated Edman degradation in a solid-phase sequencer, LKB 4030, after activation of the protein with *p*-phenylenediisothiocyanate to achieve attachment of the aminopropyl glass beads [8]. The thiazolinone derivatives obtained at each step of degradation were converted to phenylthiohydantoin (PTH) amino acids and the PTH amino acids were analyzed by thin-layer chromatography (TLC) [9] and/or high performance liquid chromatography (HPLC) in an Irica model LC-300 apparatus [7]. The amino terminal amino acid residue and the sequences of the peptides obtained by treatment of the protein with various proteases were determined by manual Edman degradation [10].

About 10 mg of the protein were digested with 0.2 mg of staphylococcal V8 protease in 1.2 ml of 0.1 M Tris–HCl (pH 8.0) for 21 h at 40°C, and the digest was applied to a Bio-gel P-6 column (2 × 180 cm) equilibrated with 0.2 M NH_4HCO_3 buffer (pH 9.0). The fractions eluted from the column were each lyophilized and purified further by paper electrophoresis (Whatman 3 MM) at pH 6.5. A peptide (V-4) thus obtained was digested further with thermolysin and the resulting peptides were purified by HPLC as in [7].

Tryptic peptides of the succinylated 15 kDa protein were prepared as follows. About 10 mg of the protein was succinylated with 100 mg of succinic anhydride at pH 8 in 6 M guanidine–HCl containing 0.5 M $NaHCO_3$. The succinic anhydride was removed by dialysis against 0.1 M NH_4HCO_3 (pH 7.8) and the material was then hydrolyzed with 0.2 mg of trypsin for 3 h. The digest was chromatographed on a column (2 × 180 cm) of Toyopearl HW-40 and developed with 0.2 M NH_4HCO_3 (pH 9.0). The fractions were lyophilized and purified further by paper electrophoresis at pH 6.5 and 3.6.

In addition, about 10 mg of the protein was digested with *Achromobacter* lysyl endopeptidase. The digest was chromatographed on Toyopearl HW-40 as described above and fractions obtained were purified further by HPLC and paper electrophoresis.

3. RESULTS AND DISCUSSION

The amino acid composition of the 15 kDa protein is shown in table 1. The proteins contains no cysteine or methionine. The absence of cysteine was confirmed by colorimetric determination of sulfhydryl groups of the protein treated with dithiothreitol and 5,5′-dithio-bis-nitrobenzoic acid [11].

Solid-phase Edman degradation of 80 nmol (about 0.7 mg) of the protein showed that the

Table 1

Amino acid compositions of the yeast 15 kDa protein and the δ-subunit of F_1–ATPase

	15 kDa protein			δ-Subunit [14]
	mol/mol		Sequence analysis	
Asp	12.35	(12)	5	5
Asn			7	
Thr	3.71	(4)	4	7
Ser	5.06	(5)	6	18
Glu	11.93	(12)	7	15
Gln			5	
Pro	4.75	(5)	5	4
Gly	9.50	(10)	10	18
Ala	1.34	(1)	1	11
Val	2.70	(3)	2	7
Cys	–	–	0	1
Met	0	0	0	0
Ile	4.02	(4)	4	7
Leu	2.17	(2)	2	8
Tyr	1.93	(2)	2	1
Phe	2.77	(3)	3	3
Lys	10.17	(10)	11	9
His	3.48	(3)	3	4
Arg	4.13	(4)	4	3
Trp			2	
Total		(80)	83	121
Calculated M_r			9454	11600

amino-terminal sequence up to residue 25 was X-X-Thr-Asn-X-Trp-Thr-Glu-X-Glu-Gly-X-Ala-Asn-Pro-X-Tyr-Phe-X-X-Thr-Gly-Asn-Tyr-Gly-. The amino terminal residue was identified as threonine by manual Edman degradation. The carboxyl terminal amino acid was hardly detected by carboxypeptidase Y digestion.

The sequence study of the yeast 15 kDa protein is summarized in fig.1. There were 83 amino acid residues and basic residues exceeded acidic ones. The M_r calculated from the amino acid sequence was 9450, although it was estimated as 15000 by SDS–PAGE. Differences between the M_rs determined by SDS–PAGE and calculated from the composition have also been observed in the cases of ATPase inhibitor and the other stabilizing factor, 9 kDa protein.

Repeated sequences were clearly observed in both ATPase inhibitor and the 9 kDa protein, suggesting gene duplication in the evolutionary past [7]. But no obvious repeated sequence was found in the 15 kDa protein.

The secondary structure of the factor was deduced as in [12]. The α-helical contents of the ATPase inhibitor, 13 and 9 kDa protein [7], were found to be high, but that of the 15 kDa protein was only 27%. Basic, acidic and hydrophobic clusters were concluded to be localized in α-helical regions of both the inhibitor and 9 kDa protein [7]. Clusters of basic, acidic and hydrophobic residues were also found in the 15 kDa protein, e.g., residues 62–66, 46–50 and 57–61, respectively, and these may be involved in complex formation.

The stabilizing factor, 15 kDa protein, which has no inhibitory action on ATPase by itself, acts

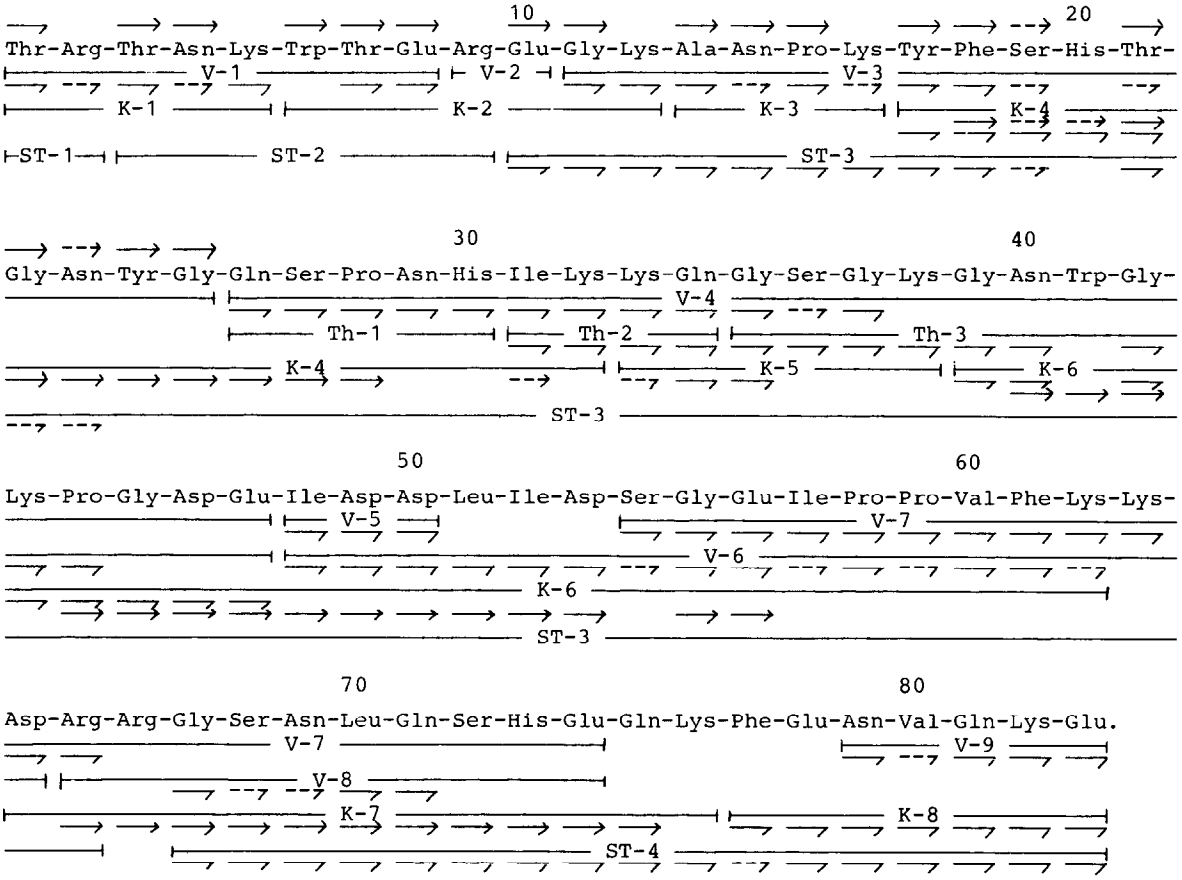

Fig.1. Summary of sequence studies on the 15 kDa protein. V-, Th-, ST-, and K- refer to the staphylococcal V8 protease peptides, thermolysin peptides derived from a V-peptide, tryptic peptides after succinylation, and lysyl endopeptidase peptides, respectively. (⟶) Solid-phase sequencing by Edman degradation of the protein and peptides, (⇢) below the sequence indicate manual Edman degradation on peptides. Dotted arrows indicate ambiguous assignments.

in concert with the other factor, 9 kDa protein, in the stabilization of the complex of F_1F_0–ATPase inactivated by the inhibitor, but not in the stabilization of the complex with soluble ATPase. The above results and considerations suggest that both stabilizing factors are involved in the binding of the F_1–ATPase–inhibitor complex to the mitochondrial membrane, as in the case of bacterial and chloroplast δ- and δ-subunits which mediate binding of the F_1-portion to the F_0-moiety [3]. The apparent M_r of the 15 kDa protein is very similar to that of the δ-subunit of yeast F_1–ATPase [3]. However, the amino acid composition of the factor is quite different from that of the δ-subunit of yeast F_1–ATPase [14], as shown in table 1, and the two are immunologically distinguishable [3]. The factor resembles the bacterial ϵ-subunit in both function and size, and the *E. coli* ϵ-subunit has been reported to have homologous sequences with the bovine δ-subunit [6]. Therefore, the structure of the factor was compared with those of other subunits of the F_1–ATPase complex including the inhibitor and the 9 kDa protein. The structure of the factor had no distinct homologous sequence with that of the *E. coli* δ- or ϵ-subunit [16], or a fragment of the bovine δ-subunit [6]. As shown in fig.2, however, it showed significant homology with the sequences of yeast ATPase inhibitor and 9 kDa protein, especially in the

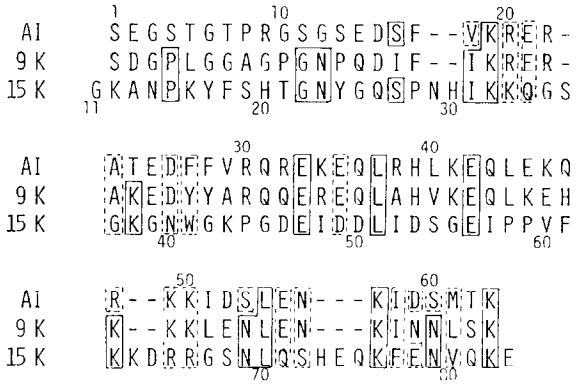

Fig.2. Comparison of the amino acid sequences of yeast ATPase inhibitor, 9 kDa and 15 kDa proteins. Identical and similar amino acid residues in the 15 kDa protein and ATPase inhibitor or 9 kDa protein are enclosed in solid and dotted boxes, respectively. — indicates a gap making alignments highly homologous. The sequence of ATPase inhibitor is from [13] and that of the 9 kDa protein from [7]. AI, 15K and 9K indicate the ATPase inhibitor, and the 15 kDa and 9 kDa proteins, respectively.

carboxyl terminal region. In a previous report, the inhibitor and 9 kDa protein were shown to be highly homologous with each other and also with the *E. coli* ϵ- and bovine δ-subunits, and it was suggested that these proteins were derived from a common ancestral gene and then diverged with different functions [7]. It seems likely that the 15 kDa protein was also derived from the common gene of these proteins and then evolved further.

The sequence homology between the 15 kDa protein and the yeast δ-subunit is unknown because the sequence of the δ-subunit is not yet established. We are now determining the sequences of the δ- and ϵ-subunits purified from yeast-F_1–ATPase.

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