Amino-terminal amino acid sequence of beef heart mitochondrial coupling factor B

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Bovine heart mitochondrial coupling factor B was isolated and purified to homogeneity in its active form. The amino-terminal amino acid sequence of the alkylated protein was determined. Two chains with exactly the same sequence except for the presence of an additional Phe at the amino-terminus on one of them were obtained. The 55 amino acid sequence appears to be largely hydrophilic with several charged amino acid residues. This sequence showed no homology with the *E. coli unc* operon, oligomycin sensitivity conferring protein, or coupling factor 6 or any protein in the data base.

1. INTRODUCTION

Bovine heart mitochondrial ATP synthase (H^+-ATPase or F_1-F_o) is an inner membrane multiprotein complex, concerned with the terminal reactions of oxidative phosphorylation leading to the synthesis of ATP. It is composed of two separable structures: F_1 is soluble, hydrophilic, comprises of 5 subunits and is directly involved in the hydrolysis of ATP; F_o, the membrane sector, is hydrophobic and is involved in H^+ conduction. While F_1 is preparations obtained from different sources exhibit certain similarities with respect to the number, size, function and primary structure of the subunits, the F_o appears to show large variations from one source to another. The *E. coli* F_o is composed of only three subunits, named a, b and c [1-3], and that of bovine heart is known to contain additional subunits including oligomycin sensitivity conferring protein (OSCP), coupling factor 6 (F_6), coupling factor B (F_B) and other poorly defined subunits [4]. The SDS gel electrophoresis patterns of most of the purified bovine heart mitochondrial F_o preparations thus show the presence of more than 4 proteins.

Coupling factor B has been shown to be a functional component of bovine heart mitochondrial H^+-ATPase required for P_i-ATP exchange in F_B-depleted submitochondrial particles but not for oligomycin-sensitive ATPase [5,6]. Subsequent work involving depletion-reconstitution and inhibition by Cd^{2+} established F_B as an essential component of F_o necessary for H^+ conduction [7,9]. Enzyme-linked immunosolvent assay using monoclonal antibody to F_B has also revealed F_B presence in F_o-F_1 in amounts stoichiometric with F_1 [10]. This report deals with the recently obtained information on the NH_2-terminal amino acid sequence of this protein.

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2. EXPERIMENTAL

Mitochondria were prepared as described earlier [9] and the ammonia-EDTA particles by a modification of the procedure described earlier [11].

3. RESULTS

3.1. *Preparation of alkylated F_B and sequence analysis*

The purification and assay of F_B were carried out essentially as described earlier [11,12] except for the following changes. The functionally active fractions

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from the DEAE-cellulose column chromatography were pooled, concentrated by ultrafiltration through a PM-30 membrane (Amicon). The pH of the concentrated fractions was adjusted to 5.3 by adding 6 M acetic acid when the solution became cloudy. It was allowed to stand on ice for 30 min and centrifuged twice, each time for 10 min at 19 000 × *g* in SS-34 Sorvall rotor, in order to remove the precipitated proteins. The pH of the clear supernatant was adjusted back to 7.0 using 1 M Tris base. Almost 90% of the activity was recovered in the supernatant. For the CM-cellulose chromatography, the column was equilibrated with 20 mM Hepes-KOH, pH 7.0; the F_B in Hepes buffer was brought to pH 7.5 and loaded on the column. The pH of 7.5 was used to avoid precipitation of F_B. The active protein was eluted from the column with 50 mM Hepes-KOH, pH 7.0. Between different steps of purification, the F_B containing fractions were concentrated by ultrafiltration through Amicon PM-30, and the activity was stabilized (for storage) by the addition of DTT and glycerol to 20 mM and 5–8%, respectively. Maintenence of a minimum concentration of 200 mM Tris-Cl, pH 7.5, was found to minimize the loss in activity of the concentrated preparations. The final purification step was carried out on a Sephadex G-75 column [11]. A typical fractionation pattern of the activity correlated with the protein staining of the individual fractions is shown in Fig. 1.

Fractions of F_B with the highest activity (Fig. 1) from two separate Sephadex G-75 column chromatography runs, i.e. from the last stage of purification containing a total of 160 units were pooled and lyophilized to reduce the volume to about 3–4 ml. The concentrated sample was dialysed against buffer containing 10 mM Tris-Cl, pH 7.5, 2% methanol and 0.1% 2-mercaptoethanol and later against water containing the above concentrations of methanol and 2-mercaptoethanol but no buffer. The dialysed sample was concentrated by centrifugation under vacuum and to this was added 100 μl of 7 M guanidine hydrochloride containing 0.5 M Tris-Cl, pH 8.5, and 14 mM 2-mercaptoethanol. The solution was incubated at 30°C for 1.5 h, then 1 μl of 4-vinylpyridine added and incubated at room temperature for 3 h. Then 5 μl of 5 M DTT was added, and the solution was incubated for 30 min at room temperature. Finally, the sample was dialysed against water, when the protein precipitated and it was transferred to a glass tube using a pasteur pipette. A small aliquot of this uniform suspension of the precipitate was analyzed by polyacrylamide gel electrophoresis (PAGE) [13] and silver staining [14]. As shown in Fig. 2, a prominent band at about 22 kDa was seen with negligible contamination. The suspension was centrifuged at 2000 rpm for 20 min and the precipitate was washed twice with water. The water adhering to the precipitate was removed by centrifugation under vacuum. The protein, 20 μg as estimated by analysis of the amino acid composition, was dissolved in 45% formic acid and analyzed for sequence using the Applied Biosystems Instruments, Model 470A Protein Sequencer, equipped with an on-line HPLC system (ABS 120 A PTH analyzer) using a reverse phase C_{18} (Brownlee) column. The Edman degradation was carried out to 55 cyles without any problem in detecting the amino acids. The initial yield was about 50% and the repetitive yield was >92%. It was clear from the data that two proteins which differed only by the presence of one phenylalanine at the NH_2-terminal were sequenced simultaneously (Fig. 3). The initial recovery of Phe was 661 pmol and of Trp 356 pmol. Except for the first Phe,

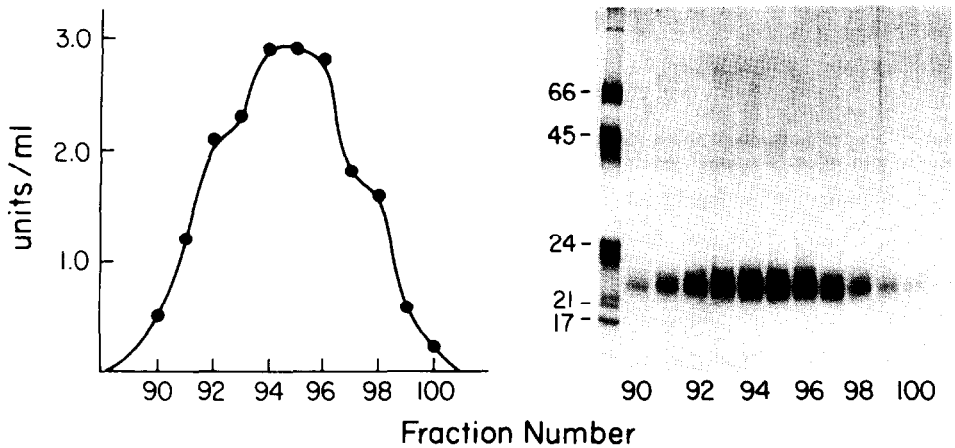

Fig. 1. Purification of F_B by Sephadex G-75 column chromatography: analysis by PAGE followed by silver staining of the active fractions. Sephadex G-75-40 column (2.5 × 75 cm) was equilibrated with buffer containing 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM DTT and 1% glycerol. Void volume of the column was 123 ml. The concentrated fraction from the CM-cellulose chromatography, 2.4 ml containing 85 units of activity, was loaded on the column. Fractions of 2.6 ml were collected. Most of the protein was recovered in the void volume with almost little or no F_B activity. The F_B activity was typically obtained at the end of the second void volume, i.e. at 220–240 ml with almost undetectably low protein levels. Left: 20 μl from each fraction was withdrawn and assayed for activity. Right: 50 μl from each fraction was lyophilized to dryness, and suspended in sample loading buffer and gel electrophoresis carried out under reducing conditions [7]. The gel was stained with silver [13,14].

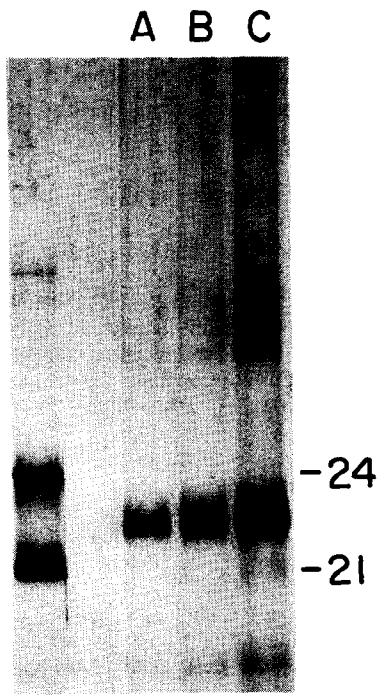

Fig. 2. Electrophoresis of purified F_B: approximately 160 units of purified F_B was alkylated, dialyzed and the precipitated F_B protein was washed in 0.5 ml of H_2O. Lanes A, B and C correspond to 0.4, 0.8 and 2.0 μl aliquots mized with the sample digestion mixture for PAGE and silver staining [13,14]. The remaining sample was used for sequence analysis. The protein standards in lane 1 were trypsinogen and soy bean trypsin inhibitor.

every amino acid appeared in two successive cycles.

In another experiment, preparative PAGE of purified alkylated F_B using 1.5 mm thick gel with a large well comb was carried out. After the usual staining and destaining of the gel with Coomassie brilliant blue, the protein band was sliced and the protein was electroeluted [15]. The extracted protein was analyzed for the sequence (data not shown). In spite of poor extraction of protein from the gel, 16 cycles were completed and the presence of two polypeptides with similar sequence except for a Phe at the NH_2-terminal on one of the chains was again evident.

The ragged NH_2-terminal ends were also noted for the ATPase inhibitor protein of bovine mitochondria [16] and the α, β and γ subunits of bovine F_1-ATPase [17]. The reason for occurrence of these frayed NH_2-terminal ends is not understood; it has been suggested that non-uniform processing of the precursor proteins during transport into mitochondria may have occurred. A second explanation may be cleavage of the NH_2-terminal residues from some of the chains by an aminopeptidase.

3.2. *Confirmation of F_B amino acid sequence*

The hydropathy plot [18] for the 55 amino acid peptide revealed extensive hydrophilic character except for a small stretch of hydrophobicity in the first few

NH_2-terminal sequence of coupling factor B

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1                5                10               15                20
Phe-Trp-Gly-Trp-Leu-Asn-Ala-Val-Phe-Asn-Lys-Val-Asp-His-Asp-Arg-Ile-Arg-Asp-Val
F  W  G  W  L  N  A  V  F  N  K  V  D  H  D  R  I  R  D  V

21               25               30               35               40
Gly-Pro-Asp-Arg-Ala-Ala-Ser-Glu-Trp-Leu-Leu-Arg-Gly-Gly-Ala-Met-Val-Arg-Tyr-His
G  P  D  R  A  A  S  E  W  L  L  R  G  G  A  M  V  R  Y  H

41               45               50               55
Gly-Gln-Gln-Arg-Trp-Gln-Lys-Asp-Tyr-Asn-His-Leu-Pro-Thr-Gly
G  Q  [Q]  R  W  [Q]  K  D  Y  N  H  L  P  T  G
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Fig. 3. The partial protein sequence of F_B. The NH_2-terminal sequence began with Phe as residue number 1 for two-thirds of the protein that was sequenced. The rest began with Trp as residue number 1. There may be a slight uncertainty regarding the identity of the amino acids in [ ].

residues at the NH_2-terminal end (data not shown). A strongly hydrophilic stretch of 13 amino acids, from 11–23, was selected for peptide synthesis. This stretch represents a hydrophilic region with clustered acidic and basic amino acids. This peptide was commercially synthesized and conjugated to KLH and ovalbumin (Immuno-Dynamics, LaJolla, CA). For immunization, 10 mg of each conjugated peptide was dissolved in 4 ml PBS and vortexed vigorously with 4 ml of complete Freund adjuvant until complete emulsification was achieved. The antigen (0.25 mg) was administered to each of the 10-week-old rabbits by intradermal injection at 4 places on the back. Rabbit serum obtained after the immunogenic response was established, as determined by enzyme-linked immunosorbent assay (ELISA) [10], was used at a dilution of 1:300 for Western blot [10] of highly purified (Sephadex G-5 step) F_B and partially purified F_B (CM-Sephadex fraction). Correspondence of bands detected by antibody reactivity with the previously reported monoclonal antibody to F_B (data not shown) established the presence of F_B-specific antibodies in the sera of rabbits immunized with the synthetic peptide. These data also support the validity of the amino acid sequence reported in Fig. 3, at least with respect to amino aids 11–23.

3.3. *Search for homologous sequences*

The database for proteins and nucleic acids, and the computer programs for sequence analysis were obtained through Bionet Services. The available F_B amino acid sequence as well as its reverse translated DNA sequence were used to align and search the complete protein database, bovine sequences of F_B and *E. coli unc* operon. Direct comparison was made with the amino acid sequence of OSCP [19] and that of F_6 [20]. None of the above revealed significant homology with the NH_2^+-terminal sequence of F_B. Fearnley and Walker [21] have claimed that the proteins encoded by mitochondrial genes A6L and ATPase 6 are bona fide components of ATP-synthase. They have also sequenced subunit b (24 kDa) and a protein named 'd' of molecular mass 18 600 which co-purified with the complex. The involvement of the A6L gene product and

subunits 'b' and 'd' in ATP synthesis has not been demonstrated. The F_B sequence did not show any resemblance to these sequences.

4. DISCUSSION

F_o preparations derived from ATP synthases of different organisms have passive proton conductance activity. However, there are differences between them in subunit composition. The F_o from prokaryotes has generally fewer protein subunits than F_o from the mitochondria of eukaryotes. *E. coli* F_o, which has been studied in the greatest detail, has three distinct subunits: subunit a (*unc* B) with amino acid sequence homology to yeast mitochondrial ATPase 6, subunit b (*unc* F) with no clearly identifiable sequence homology to any of the mitochondrial F_o subunits, and subunit c (*unc* E) with striking homology to ATPase 9 or the DCCD-inhibitable proteolipid [1-3]. All these three subunits are necessary and sufficient for the assembly of a functional F_o proton channel [22,23]. In the case of bovine heart mitochondria, preparations of F_o with an active H^+ channel show 8 or more prominent subunits [24] some of which have been identified and their role in H^+ conductance established. Genetic studies and experiments using DCCD or oligomycin as inhibitors have identified yeast mitochondrial ATPases 6 and 9 as functional components of the H^+ channel [25,26]. The functional role of the 24 kDa subunit (A6L gene product) in H^+ conduction in heart mitochondrial F_o remains to be established. Recent elegant studies of Joshi et al. [27,28] have shown that OSCP and coupling factor 6 (F_6) do not function in H^+ conduction although they appear in all mammalian F_o preparations.

Other than subunits 6, 8 and 9, F_B is thus the only other protein that has been unambiguously shown to be a functional component of bovine heart mitochondrial F_o by inhibitor as well as reconstitution studies [7-9]. Since its NH_2-terminal 55 amino acid sequence shows no homology with the *unc* operon, F_B may be unique to higher organisms, and thus, further sequence analysis and elucidation of its role become important.

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