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THE PROXIMAL N-TERMINAL AMINO ACID RESIDUES ARE REQUIRED FOR THE COUPLING ACTIVITY OF THE BOVINE HEART MITOCHONDRIAL FACTOR B*

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Abstract

Treatment of the recombinant bovine factor B with trypsin yielded a fragment (amino acid residues 62-175) devoid of coupling activity. Removal of the N-terminal Trp2-Gly3-Trp4 peptide resulted in a significant loss of coupling activity in the FB Δ W2-W4 deletion mutant. Sucrose density gradient centrifugation demonstrated co-sedimentation of recombinant factor B with the ADP/ATP carrier, which is present in preparations of H⁺-translocating F₀F₁-ATPase, but not in preparations of complex V. The N-terminally truncated factor B mutant FB Δ W2-W4 did not co-sediment with the ADP/ATP carrier. Recombinant factor B co-sedimented with partially purified membrane sector F₀, extracted from F₁-stripped bovine submitochondrial particles with *n*-dodecyl- β -D-maltoside. Factor B inhibited the passive proton conductance catalyzed by F₀ reconstituted into asolectin liposomes. A factor B mutant, bearing a photoreactive unnatural amino acid *p*benzoyl-L-phenylalanine (*p*Bpa) substituted for Trp2, cross-linked with F₀ subunits *e* and *g* as well as the ADP/ATP carrier. These results suggest that the N-terminal domain and, in particular, the proximal N-terminal amino acids are important for the coupling activity and protein-protein interactions of bovine factor B.

Keywords

Factor B; Limited proteolysis; Cross-link; Energy coupling; Proton leak; Oxidative Phosphorylation; Mitochondria

INTRODUCTION

In living cells, synthesis of ATP from ADP and inorganic phosphate is catalyzed by the ATP synthase complex in the final step of oxidative phosphorylation or photosynthesis [1]. Historically, the basic architectural principles of the mitochondrial ATP synthase, which consists of catalytic sector F₁ and membrane sector F₀, connected by the central and peripheral stalks, have been established in the course of the studies aimed at resolution and reconstitution of oxidative phosphorylation or its partial reactions, using membrane fragments derived from bovine heart mitochondria [2]. These studies led to the identification and characterization of either single polypeptides or a multisubunit complex, ex. oligomycin sensitivity-conferring protein (OSCP), factor B, F₆ or F₁-ATPase, which restored the phosphorylation activity of appropriately depleted submitochondrial particles and which were called coupling factors [3-5]. In the ensuing years, a great deal of knowledge has been gained concerning the F₀F₁-

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ATPases, including high resolution crystal structures of the mammalian F₁-ATPase [6-8] and a subcomplex of the peripheral stalk [9,10]. In contrast, studies on coupling factor B, a “second energy-transfer factor,” which was identified by Sanadi and associates [11] forty years ago, have lagged behind.

In recent years, our studies on coupling factor B have aimed at closing a gap in the knowledge concerning its role in oxidative phosphorylation in animal mitochondria. We cloned and expressed in *Escherichia coli* human [12] and bovine [13] factor B and provided a biophysical characterization of recombinant bovine polypeptide [14]. We demonstrated that each polypeptide was able to restore oxidative phosphorylation and its partial reactions following reconstitution with “non-phosphorylating,” factor B-depleted submitochondrial particles (AE-SMP¹) [12,13,15]. We also reported a transient overexpression of human factor B in mitochondria of human HEK293 cells [16].

AE-SMP are inside-out vesicles produced by sonication of heavy bovine heart mitochondria at pH of ~8.8 in the presence of 0.6 mM EDTA. The loss of oxidative phosphorylation in AE-SMP could be attributed to a proton leak, which ensues following the removal of coupling factor B from their membranes. As a result, all energy-linked reactions that require the electrochemical proton gradient as an intermediate are suppressed in AE-SMP [4,12,15]. Fo inhibitors oligomycin and DCCD have been shown to block the proton leak and to partially restore the energy-linked reactions [4,12,13,17,18]. Because the pharmacological target of both inhibitors within mammalian mitochondria is well-defined when they are used in concentrations sufficient to recouple AE-SMP, these findings suggested a role of proton translocation pathway within membrane sector Fo in the proton leak observed in AE-SMP. It was further proposed that similar to other known coupling factors, factor B is a subunit of the mitochondrial FoF₁-ATPase [4,12].

In the present study, we characterized the structure-activity relationships in factor B and demonstrated that the N-terminal domain and, in particular, the extreme N-terminal amino acid residues are important for the coupling activity of the polypeptide, while the second half of the molecule, containing the leucine-rich repeat motif, exhibited no coupling activity. In a series of experiments, we demonstrated co-sedimentation of factor B with the ADP/ATP carrier and the membrane sector Fo. We provided evidence that coupling factor B inhibits the passive proton conductance catalyzed by Fo proteoliposomes. Finally, using a photocross-linking approach, we demonstrated proximity of the N-terminus of factor B to Fo subunits *e* and *g* as well as the ADP/ATP carrier.

¹The abbreviations used are:

FB	coupling factor B
SMP	bovine heart submitochondrial particles
AE-SMP	factor B-depleted SMP
DDM	<i>n</i> -dodecyl-β-D-maltoside
CCCP	carbonyl cyanide <i>m</i> -chlorophenylhydrozone
ACMA	9-amino-6-chloro-2-methoxy acridine
pBpa	<i>p</i> -benzoyl-L-phenylalanine
DCCD	<i>N</i> , <i>N</i> '-dicyclohexylcarbodiimide
AAC	ADP/ATP carrier

MATERIALS AND METHODS

Reagents

ATP, oligomycin, 2, 4-dithiothreitol, IPTG, β -D-octylglucoside, and *n*-dodecyl- β -D-maltoside were obtained from EMD Biosciences-Calbiochem (La Jolla, CA). Sephacryl S-200 HR and DEAE Sepharose FF were purchased from GE-Healthcare (Piscataway, NJ). Macro-Prep High Q anion exchange resin, 30% acrylamide/bis solution (37.5:1), broad and low molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA). DCCD, valinomycin, nigericin, CCCP were from Sigma-Aldrich (St. Louis, MO). ACMA was from Invitrogen (Carlsbad, CA). Asolectin (soy bean phospholipids extract containing 20% L- α -lecithin) was from Avanti Polar-Lipids, Inc. (Alabaster, AL). All other chemicals were reagent grade. The anti-peptide antibody against the ADP/ATP carrier, ANT (N-19), was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-AIF antibody was from Chemicon International (Temecula, CA). The antibody against human factor B has been described previously [12]. Antibodies against subunits of bovine FoF₁-ATPase were generously provided by Dr. Y. Hatefi (The Scripps Research Institute). AE-SMP were prepared from heavy bovine heart mitochondria, as described previously [12].

Recombinant DNA techniques and analytical procedures

Cloning, restriction enzyme analysis, plasmid preparation, and agarose gel electrophoresis were performed using standard molecular biology techniques. The N-terminal sequencing of the recombinant bovine factor B fragments, which were immobilized on an Immobilon-PSQ membrane (Millipore), was performed at the Center for Protein Sciences at the Scripps Research Institute (La Jolla, California) by Dr. Phillip Ordoukhanian. MALDI-TOF analysis was performed at the Center for Mass Spectrometry at the Scripps Research Institute.

Cloning and expression of FB₆₁₋₁₇₅ fragment and site-directed mutagenesis

The nucleotide sequence encoding amino acid residues 61-175 of factor B was PCR amplified with a pair of primers GAAGCTTCAAGATTCAGGCGATTGACGCCACCGATTCC (GB153, forward) and TTAATTCAAGTCCAATTTTAGCTCCAG (GB90, reverse) using plasmid pET43-bFB as a template. Plasmid pET43-bFB was previously prepared and contains the full-length bovine factor B [13]. The PCR product was cloned into the *Sma*I site of the pET43-1a expression vector. The 5' end of the PCR product included nucleotides encoding Ser-Phe residues, which were added to facilitate the subsequent cleavage of the recombinant fusion polypeptide NusA-FB₆₁₋₁₇₅ with thrombin. The recombinant FB₆₁₋₁₇₅ fragment was expressed and purified following procedures developed previously for the purification of full-length recombinant factor B [13]. The calculated mass of the FB₆₁₋₁₇₅ fragment, including the N-terminal Ser and Phe residues, is 13,391.72 Da.

To prepare the FB Δ W².W⁴ deletion mutant, the Trp², Gly³ and Trp⁴ residues were deleted in a step-wise manner using the QuikChange method and the pET43-bFB plasmid as a template. The following pairs of primers were used to introduce the desired deletions: FB Δ W², CCAACTGGTCTGGTCCCCCGAAGCTTCGGCTGGTTGAATGCAGTGTTTAACAAAGTGG (GB160, forward) and CCACTTTGTAAACACTGCATTCAACCAGCCGAAGCTTCGGGGGACCAGACCAGTTGG (GB160r, reverse); FB Δ W².G³, CCAACTGGTCTGGTCCCCCGAAGCTTCTGGTTGAATGCAGTGTTTAACAAAGTG (GB161, forward) and CCACTTTGTAAACACTGCATTCAACCAGAAGCTTCGGGGGACCAGACCAGTTGG (GB161r, reverse); FB Δ W². Δ ⁴, CCAACTGGTCTGGTCCCCCGAAGCTTCTTGAATGCAGTGTTTAACAAAGTGG (GB162, forward) and

CCACTTTGTAAACACTGCATTCAAGAAGCTTCGGGGGACCAGACCAAGTTGG (GB162r, reverse). The deletion mutant FB Δ W²-W⁴ was expressed and purified following procedures developed previously [13]. FB Δ W²-W⁴ contains Ser-Phe residues at the N-terminus and its calculated mass is 19,982.15 Da. The sequences of all the plasmid constructs and mutants prepared in this study were confirmed by DNA sequencing, performed at the DNA sequencing facility at the Department of Human Genetics at UCLA.

Preparation of F₀F₁-ATPase

F₀F₁-ATPase was purified from bovine heart AE-SMP according to a previously described protocol [19]. Bovine heart mitochondrial complex V [20] was kindly provided by Dr. Y. Hatefi (TSRI).

Preparation of the membrane sector F₀

F₁-stripped submitochondrial particles were prepared from bovine heart SMP treated with guanidine hydrochloride as described previously [21]. The guanidine hydrochloride-treated membranes were extracted with 1% DDM as described previously [22]. Membranes were diluted to a protein concentration of 2.5 mg/ml with a buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA and 10% DDM was added to a final concentration of 1%. Following a 30 min incubation on ice, the mixture was centrifuged in a Beckman type 70 Ti rotor for 1 h at 50,000 rpm, and the supernatant was applied to a column (1.5 × 8 cm) filled with a Macro-Prep High Q support resin. After washing off the un-bound proteins, a gradient of 0-0.5 M NaCl in a buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA and 0.02 % DDM was developed. The polypeptide composition of individual fractions was characterized by SDS-PAGE and Western blot analysis. Membrane sector F₀ eluted at salt concentrations between 0.2-0.3 M. Fractions containing F₀ were combined and concentrated using an Amicon Ultra-15 centrifugal device (Millipore Inc.). The partially purified F₀, concentrated up to 3-3.5 mg/ml, was stored in small aliquots at -80°C.

Sucrose density gradient fractionation

The sucrose density fractionation was performed essentially as described previously [23]. Sucrose step gradients were prepared in a 10 ml tube by applying 1 ml each of the following sucrose concentrations: 35, 32.5, 30, 27.5, 25, 22.5, 20, 17.5, and 15%. The sucrose solutions were prepared in 100 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer containing 0.05% DDM. In a routine experiment, 0.2-0.3 mg of recombinant bovine factor B were incubated with 4.8 mg of F₀F₁-ATPase or 2.5 mg of F₀ on ice for 30 min, and then applied to the top of a freshly prepared gradient. The tubes were centrifuged in a SW41 rotor for 17 hrs at 38000 rpm, 4°C. The sucrose gradients were fractionated manually from top to bottom in 1 ml fractions, from which a 100 µl aliquot was immediately withdrawn for SDS-PAGE analysis, and the rest of each fraction was snap-frozen in liquid nitrogen and stored at -80°C.

Assay of membrane vesicles energization

ATP-driven quenching of 9-amino-6-chloro-2-methoxy acridine (ACMA) was measured as described previously [13], using Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon Inc.) at an emission wavelength of 485 nm, following excitation at 415 nm at 30°C. The excitation and emission slits were adjusted to 1 nm. AE-SMP were diluted into 2.9 ml of a pH 7.5 buffer containing 0.25 M sucrose, 50 mM Tris-HCl, 5 mM MgCl₂, and 50 mM KCl, under constant stirring, to a concentration of 0.115 mg of membrane protein per milliliter. Full-length factor B (Fig. 1D, trace 2), FB₆₁₋₁₇₅ fragment (Fig. 1D, trace 3) or FB Δ W²-W⁴ deletion mutant (Fig. 2C, trace 3) were added at final concentrations of 10, 10 and 15 µg/ml, respectively. These concentrations were found to allow for a maximal stimulation of proton-pumping activity of AE-SMP. The assay buffer also included 1 µg/ml valinomycin and 0.7 µM ACMA. ACMA

fluorescence quenching was initiated with 1 mM ATP and terminated by the addition of the ATP synthase complex inhibitor oligomycin (final concentration 8 μ g/ml).

Preparation of Fo proteoliposomes and measurement of passive proton conductance

F_O proteoliposomes were prepared and F_O passive proton conductance was measured as described previously [21,24]. A mixture of asolectin (10 mg/ml) and β -D-octylglucoside (1.6%) in a 50 mM Tricine-NaOH, pH 7.5, 1 mM EDTA buffer was sonicated on ice slurry until the solution became clear. The F_O preparation (0.2-0.35 mg/ml) was added to the mixture and incubated on ice for 30 min. Excess detergent was removed by adding Bio-Beads SM-2 resin (70-80 mg/ml), and the mixture was rotated at 4°C for 1 h. The Bio-Beads were added four times. After removal of the Bio-Beads, the proteoliposomes were diluted 10 times in a 210 mM KCl, 10 mM Tricine-KOH, pH 7.5 buffer and centrifuged for 1 h at 4°C at 50,000 rpm in the Beckman type 70 Ti rotor. The pelleted vesicles were gently resuspended in 400 μ l of a buffer, containing 0.25 M sucrose, 10 mM Tricine, pH 7.5, and were kept on ice for the duration of the experiments. Proteoliposomes were stable on ice for at least 3 hrs. The vesicle suspension (15 μ l) was added to 2 ml of a 200 mM NaCl, 10 mM Tricine, pH 7.5 buffer, supplemented with 50 nM ACMA. Passive proton translocation into the vesicles was monitored via ACMA quenching following addition of valinomycin (10 ng), which elicited K⁺ efflux from the vesicles' lumen. Maximal ACMA quenching was obtained by adding 1 μ M protonophore CCCP. To analyze the effect of the wild type and mutant factor B, they were added to F_O proteoliposomes in the assay cuvette 3-5 min prior to addition of valinomycin in concentrations indicated in the Fig. 8 legend.

Preparation of the FB_{W2Bpa/G3E} double mutant

The factor B mutant harboring substitution of an unnatural photoreactive amino acid analog, *p*benzoyl-L-phenylalanine (*p*Bpa), for Trp2 was prepared according to a procedure developed in the laboratory of Dr. Peter G. Schultz [25]. Briefly, the tryptophan codon TGG at residue 2 was mutated to an amber codon TAG by QuikChange site-directed mutagenesis using plasmid pET43-bFB as the template. Subsequently, a Gly3Glu substitution, which occurs in the rat factor B amino acid sequence, was introduced. The FB_{W2Bpa/G3E} double mutant was co-transformed with the pSup-BpaRS-6TRN plasmid into the *E. coli* strain BL21 (DE3). The pSup-BpaRS-6TRN plasmid, which was kindly provided by Dr. Peter G. Schultz (The Scripps Research Institute), harbors six copies of a gene encoding an amber suppressor tRNA derived from *Methanocaldococcus jannaschii* tyrosyl-tRNA (*Mj*tRNA^{Tyr}_{CUA}) and a gene encoding a mutant *M. jannaschii* tyrosyl-tRNA synthetase (*Mj*TyrRS). The co-transformed bacterial colonies were selected following 2 days growth at 37° C on solid Luria-Bertani plates containing 100 μ g/ml carbenicillin and 50 μ g/ml chloramphenicol. For growth in a liquid culture, 5 ml of an overnight culture of each bacterial strain were inoculated into a 2 l flask containing 500 ml 2xYT medium in the presence of 100 μ g/ml carbenicillin, 50 μ g/ml chloramphenicol and 1 mM *p*Bpa and grown at 37° C in a shaker. At OD₆₀₀= 0.6, cells were cooled off to 25°C and protein expression was induced with 1 mM IPTG for 18 hrs. The purification procedure for the bovine factor B *p*Bpa-substituted mutant was identical to that described previously [13] and yielded the mutant at ~ 1/10 of the yield described for the wild type recombinant polypeptide.

Photocross-linking

The FB_{W2Bpa/G3E} mutant was incubated either alone or with FoF₁-ATPase in a buffer, containing 50 mM sucrose, 10 mM Tris-HCl, pH 7.5, and 0.02% DDM, on ice for 30 min. Samples were transferred to a 96 well plate and UV-irradiated using an 8-watt longwave UVP lamp, at a distance of ~5 cm, for 20-40 min on ice. Samples were denatured, separated by either 12 or 15% SDS-PAGE and subjected to Western blot analysis.

Extraction of heavy bovine heart mitochondria with DDM

Heavy bovine heart mitochondria were resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.7, and 1 mM EDTA to a concentration of 10 mg/ml, and DDM was added at detergent: protein ratio of 1.6:1 (w/w). After incubation on ice for 30 min, the mixture was centrifuged in a Beckman type 70.1 Ti rotor for 1 h at 50,000 rpm. The supernatant fraction was applied to the top of a 15-35% sucrose step gradient, prepared using 100 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer containing 0.05% DDM, and centrifuged in a SW41 rotor for 17h, at 38,000 rpm, 4°C. The final sucrose gradient was manually fractionated from top to bottom in 1 ml aliquots and analyzed by SDS-PAGE.

Western blot analysis

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat dry milk in TBS at room temperature for 1 h. The membranes were incubated with primary antibodies for 1 h at room temperature using the following dilutions: anti-F₁ α , 1:2,000; anti-subunit *b* and anti-subunit *d*, 1:1,000; anti-subunit *a*, 1:500; anti-subunits *e*, *f* and *g*, 1:100; anti-subunit *c*, 1:50; anti-human factor B, 1:10,000 (Figs. 9 and 10) and 1:500 (Fig. 11); anti-AIF, 1:1,000; anti-AAC, 1:100. Horseradish peroxidase-conjugated anti-rabbit and anti-goat antibodies were used as the secondary antibody. Immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent substrate (Pierce).

RESULTS

The structure-activity relationships in coupling factor B

Fig. 1A shows an SDS-PAGE analysis of the time course of proteolytic degradation of recombinant factor B with trypsin (protein:enzyme ratio, 200:1, w/w; room temperature, 0-60 min). The N-terminal sequence of the major cleavage fragment was determined to be I-Q-A-I-D-A-T-D, while the N-terminal sequence of the minor proteolytic fragment was D-Y-N-H-L-P-T. Analysis of amino acid sequence of bovine heart mitochondrial coupling factor B [13] indicated that the faster migrating band on SDS-PAGE was produced by the Lys61-Ile62 peptide bond cleavage, while cleavage of the Lys47-Asp48 peptide bond produced a slower migrating fragment with a significantly lower yield. No bands corresponding to the N-terminal factor B fragments could be identified under these conditions.

We cloned a factor B fragment comprised of amino acid residues 61-175 and additional Ser-Phe residues at the N-terminus, into the pET43-1a vector, behind the NusA polypeptide. The molecular mass of the purified recombinant factor B fragment, 13,380 Da, determined by MALDI-TOF (Fig. 1C), was in agreement with the calculated mass of 13,391.72 Da. We tested its coupling activity by measuring ATP-driven proton pumping activity of reconstituted AE-SMP. AE-SMP alone showed little ATP-driven proton pumping activity (Fig. 1D, trace 1). Full-length recombinant factor B significantly increased the steady state level of ACMA fluorescence quenching, which reflects formation of a Δ pH, acidic inside, across membranes of reconstituted AE-SMP (Fig. 1D, trace 2). No significant enhancement of ATP-driven proton pumping activity was observed after addition of FB₆₁₋₁₇₅ fragment to AE-SMP (Fig. 1D, trace 3).

To probe the role of the factor B N-terminal residues in its coupling activity, we prepared a series of deletion mutants in which amino acids Trp2, Gly3, and Trp4 were removed in a step-wise manner, and results concerning the FB _{Δ W2-W4} deletion mutant, in which all three residues were deleted, are shown in Fig. 2. On SDS-PAGE, the mutant migrated with a slightly increased electrophoretic mobility compared to the full-length recombinant factor B (Fig. 2A, lanes 2 and 1). The molecular mass of the mutant, 19,981 Da, determined by MALDI-TOF (Fig. 2B),

was in agreement with the calculated mass of 19,982.15 Da. The factor B mutant showed a significantly impaired ability to stimulate the ATP-driven proton pumping activity in reconstituted AE-SMP (Fig. 2C, trace 3). This result suggests that the N-terminal domain and the deleted residues, two of which are aromatic amino acids, are important for the coupling activity of factor B.

Analysis of interaction between factor B and FoF₁-ATPase

Bovine heart mitochondrial FoF₁-ATPase was prepared by extracting AE-SMP with sodium cholate [19]. Western blot analysis with anti-factor B polyclonal antibodies revealed no presence of endogenous bovine factor B in this preparation (data not shown). To probe whether the recombinant bovine factor B could stably associate with FoF₁-ATPase, the enzyme alone (Fig. 3A), factor B alone (Fig. 3B) or mixture of both (Fig. 3C) were centrifuged in a 15-35% sucrose density gradient, in the presence of 0.05% DDM, for 17 hrs at 4° C. The gradients were fractionated from top to bottom and the polypeptide composition of each fraction was analyzed by 15% SDS-PAGE followed by Coomassie Blue staining. FoF₁-ATPase, with a calculated molecular mass of ~600 kDa, was found in fractions 6 and 7 (Figs. 3A and C), which probably represent its monomeric and dimeric forms, respectively. The band with M_r~50 kDa seen in fractions 2 of panels A and C is a dissociated β subunit of the catalytic sector F₁. A prominent band with M_r~31 kDa seen in fractions 2-5 in panels A and C was identified as the ADP/ATP carrier by Western blot analysis (data not shown). This band was also present in fraction 6, which contains monomeric FoF₁-ATPase, and to a lesser extent in fraction 7. The co-sedimentation of the ADP/ATP carrier with FoF₁-ATPase is consistent with the recently characterized form of the rat liver mitochondrial enzyme, the ATP synthasome [26,27]. Mass spectrometry analysis of the peptides derived from trypsin digestion of the 31 kDa band excised from lanes 4 and 6 of panel A, identified this band as isoform 1 of the ADP/ATP carrier (data not shown), which is a major isoform expressed in the heart and skeletal muscles [28]. Recombinant factor B (panel B) was mostly found in fraction 2 (a band with M_r~22 kDa), in agreement with a previous report that at high protein concentrations, factor B forms oligomers, presumably trimers [14]. However, after incubation with FoF₁-ATPase, the factor B band was also detected in fractions 4 and 5 of panel C, which correspond to high molecular weight polypeptides or their assemblies. Because FoF₁-ATPase subunits *b*, OSCP and *d*, with M_r of ~25, 23 and 22 kDa, respectively, are difficult to resolve by conventional SDS-PAGE, we blotted samples identical to those shown in panel C with factor B antibody. The Western blot experiment did not detect recombinant factor B in either fraction 6 or 7 of panel C (data not shown).

The presence of recombinant factor B in fractions 4 and 5 of Fig. 3C could be due to its interaction with the ADP/ATP carrier. To test this hypothesis, we analyzed the sedimentation of factor B in the sucrose density gradient fractions after preincubation with complex V (Fig. 4). The latter preparation contains significantly lower amounts of the carrier than FoF₁-ATPase. Accordingly, no difference was noted between the sedimentation profiles of factor B centrifuged alone or in the presence of complex V (Figs. 4B and C).

Because deletion mutant FB $_{\Delta W2-W4}$ showed impaired coupling activity in the ATP-driven proton pumping assay (Fig. 2C, trace 3), we tested its distribution during centrifugation in a sucrose density gradient under conditions identical to those shown in Fig. 3. The results presented in Fig. 5B demonstrate that in contrast to the full-length factor B, the FB $_{\Delta W2-W4}$ mutant did not co-sediment with the ADP/ATP carrier.

Analysis of interaction between factor B and membrane sector Fo

We tested whether recombinant factor B could form a complex with membrane sector Fo, which could be resolved using sucrose density gradient centrifugation. Membrane sector Fo

was extracted with 1% DDM from guanidine hydrochloride-treated bovine heart submitochondrial particles [21]. The detergent extract, which is enriched in Fo, was clarified by a high speed centrifugation and used without further purification. Fig. 6 shows a 15% SDS-PAGE analysis of the fractions collected following centrifugation in a 15-35% sucrose gradient of DDM extract alone (Fig. 6A), factor B alone supplemented with 1% DDM (Fig. 6B) or mixture of both factor B and DDM extract (Fig. 6C). Fo subunits were detected in fractions 4 and 5 of both panels A and C in Fig. 6 by Western blot analysis (data not shown). The major Coomassie-stained band ($M_r \sim 31$ kDa) seen in fraction 3 in both panels was identified as the ADP/ATP carrier. Addition of 1% DDM had no effect on factor B oligomerization state, i.e. in Fig. 6B, as in Figs. 3B and 4B, factor B was mostly present in fraction 2. Strikingly, in a sample reconstituted with Fo (Fig. 6C), the factor B band was detected in fractions 3, 4 and even 5. To confirm this result, we concentrated fractions 4 and 5, which are shown in Fig. 6A and C, respectively, and re-analyzed their polypeptide composition in a second SDS-PAGE (Fig. 7). The identity of most polypeptides in the Coomassie-stained gel shown in Fig. 7 was established by Western blot analysis using antibodies against Fo subunits *a*, *b*, *d*, *e*, *f*, *g*, and *c*, as well as AAC. As noted previously [29], the anti-subunit *a* antiserum recognized two subunit *a* species. In lanes 1 and 2 of Fig. 7, which were loaded with concentrated fractions 4 and 5 of Fig. 6A, a poorly stained band ($M_r \sim 22$ kDa) corresponds to the faster migrating species of Fo subunit *a*. An intensely stained band, which belongs to the recombinant factor B, whose electrophoretic mobility is close to that of subunit *a*, could be seen in lanes 3 and 4. A lower amount of factor B is present in lane 4 (Fig. 7) where it is better separated from the slower migrating Fo subunit *d*. Notably, the staining intensity of the factor B band parallels fairly well to the intensity of the AAC band.

Next, we examined the functional consequence of factor B co-sedimentation with Fo by analyzing the effect of factor B on the passive proton conductance catalyzed by Fo proteoliposomes (Fig. 8). The membrane sector Fo used in these experiments was subjected to ion-exchange chromatography on DEAE-Sepharose, which removed bulk of detergent along with some protein contaminants. The protein fractions eluting from the ion-exchange resin within 0.2-0.3 M NaCl concentrations were used for reconstitution into asolectin liposomes. The polypeptide composition of partially purified Fo was similar to that shown in Fig. 7, lane 2. Fig. 8, trace 1 shows quenching of ACMA fluorescence following addition of valinomycin to K^+ -loaded Fo proteoliposomes. Subsequent addition of protonophore CCCP bypasses Fo, allowing for the protons entry into the vesicles at enhanced rate and the maximal ACMA quenching attainable under experimental conditions. The passive proton permeability catalyzed by Fo proteoliposomes was sensitive to oligomycin (Fig. 8, trace 5). Wild type recombinant factor B inhibited the passive proton conductance of Fo proteoliposomes in a concentration-dependent manner (trace 2, addition of 3.5 μ M of factor B, and trace 3, addition of 0.75 μ M of factor B). The $FB_{\Delta W2-W4}$ mutant (trace 4, 5 μ M) inhibited passive proton conductance to a less extent than wild type polypeptide. Together, these data demonstrate the ability of factor B to inhibit Fo-catalyzed passive proton conductance.

Photocross-linking of the $FB_{W2Bpa/G3E}$ mutant

To probe near-neighbor relationships of bovine factor B, we prepared a double mutant, $FB_{W2Bpa/G3E}$, which harbors a substitution of unnatural photoreactive amino acid *p*benzoyl-L-phenylalanine (*p*Bpa) for Trp2 in a G3E mutant background. Amino acid residue Glu3 is present in the rat factor B homolog [13]. Fig. 9, left panel, shows a Coomassie Blue-stained 12% SDS-PAGE, which contains the purified mutant alone (lanes 1-6) or the mutant mixed with FoF_1 (lanes 7-12) that were either UV- irradiated (lanes 4-9) or not irradiated (lanes 1-3, 10-12). Western blotting with anti-factor B antiserum (Fig. 9, right panel) demonstrated formation of factor B cross-linked products in the UV- irradiated samples. In addition to dimers and trimers of factor B (lanes 4-6), UV irradiation of $FB_{W2Bpa/G3E}$ in the presence of FoF_1

produced a series of distinct cross-linked products ($M_r \sim 50, 30$ and 26 kDa), which are labeled with arrowheads (Fig. 9, right panel, lanes 7-9). The ~ 50 kDa cross-linked product is located immediately below $F_1 \beta$ subunit and its position in UV- irradiated lane 9 of the left panel is indicated by arrowhead. Although it did not react with the commercial anti-peptide antibody against AAC, MS/MS analysis identified AAC peptides in this band. We therefore assigned the ~ 50 kDa band as a cross-linked product between factor B and the ADP/ATP carrier. The identity of a component participating in the formation of the ~ 26 kDa band, labeled as FB-x in right panel of Fig. 9, is unknown. This cross-linked product did not react with anti-peptide antibody directed against Fo subunit *c*, and the lack of an antibody against the A6L polypeptide prevents us from ruling this Fo subunit out as a potential factor B cross-linking partner. A band with $M_r \sim 30$ kDa was recognized by antibodies against factor B and Fo subunits *e* and *g* and therefore represents their cross-linked products (Fig. 10). Together, these data demonstrate that following reconstitution with F_0F_1 -ATPase, the N-terminus of factor B is located in proximity to Fo subunits *e* and *g* as well as AAC.

Identification of endogenous factor B in bovine heart mitochondria

Fig. 11A shows a 15% SDS-PAGE loaded with fractions collected after centrifugation of 1% DDM extract of heavy bovine heart mitochondria in a 15-35% sucrose gradient. Western blotting detected factor B in fractions 2-4, while antibodies against F_0F_1 subunits confirmed presence of the enzyme in fractions 5-7 (Fig. 11B). AAC was detected in fractions 2-5, while apoptosis-inducing factor (AIF), a mitochondrial intermembrane space polypeptide, which is responsible for execution of the caspase-independent apoptotic program [30], was detected in all fractions of the sucrose gradient. Proteomics analysis (data not shown) of the gel region enclosed by a rectangle in lane 3 of Fig. 11A identified 5 tryptic peptides, shown in bold in Fig. 11C, which derived from bovine factor B [13]. The amino acid sequence coverage of the mature bovine factor B achieved by MS/MS analysis was 29%. Other polypeptides identified in this same gel region included mitochondrial superoxide dismutase 2, peptidyl-prolyl cis-trans isomerase (cyclophilin F), as well as CoQ7, a component of the ubiquinone biosynthetic pathway.

DISCUSSION

In the present study we demonstrate that deletion of the Trp2-Gly3-Trp4 sequence in recombinant bovine factor B significantly impaired its ability to stimulate the ATP-driven proton pumping activity of AE-SMP (Fig. 2). Provided that deletion mutant folds correctly, this result suggests that the N-terminal domain, and in particular, the extreme N-terminal amino acid residues are important for the coupling activity of factor B. In contrast, a factor B fragment, comprised of amino acids 61-175, was inactive in a similar activity assay (Fig. 1). In integral membrane proteins, aromatic residues, especially tryptophans, have a preference for the interfacial region of lipid bilayers [31]. An algorithm for calculating the partitioning free energy of unfolded peptides into the phosphatidylcholine bilayer interface has been described [32, 33], and the free energy value for tryptophan residue was determined to be -1.85 kcal mol $^{-1}$. Using this value, the reduction in the free energy of binding the $FB_{\Delta W2-W4}$ deletion mutant to AE-SMP could be as much as $2 \times (-1.85)$ kcal mol $^{-1}$. At present, however, the relative contribution of protein-protein and protein-lipid interactions that govern the binding of factor B to AE-SMP is not known.

We have previously demonstrated that recombinant human factor B stimulates by 2.5-fold the ATP- $^{32}P_i$ exchange activity of complex V [15]. Here, we analyzed the sucrose density gradient sedimentation profiles of factor B alone or factor B recombined with either bovine mitochondrial F_0F_1 -ATPase or complex V in the presence of 0.05% DDM (Figs. 3 and 4). We could not detect co-sedimentation of factor B with either enzyme preparations. This could be

due to a) low affinity of protein-protein interactions between factor B and FoF₁, b) a relatively high dissociation rate constant of the preformed complex and/or c) dependence of these interactions on the presence of exogenous phospholipids, similar to demonstrated recently interactions between bovine FoF₁-ATPase and two proteolipids [34]. Instead, these experiments suggest the interaction between factor B and ADP/ATP carrier and its dependence on the presence of the factor B N-terminal Trp2-Gly3-Trp4 tripeptide (Fig. 5).

Factor B was also found to co-sediment with membrane sector Fo in a sucrose density gradient (Figs. 6 and 7). To probe the functional consequence of this association, we measured the effect of factor B on passive proton conductance catalyzed by Fo reconstituted in asolectin liposomes. Similar to previously described functionally active membrane sector Fo isolated from rat liver mitochondria [21], the partially purified bovine heart mitochondrial Fo used in the present study was shown to be active in the passive proton conductance assay (Fig. 8). The incorporation of Fo in phospholipid vesicles is expected to yield a mixture of vesicles in which with equal probability the lipid-inserted Fo would face either the vesicles lumen or the external medium. The former topological orientation would exclude Fo molecules from binding to externally added factor B. Indeed, the data presented in Fig. 8 show that factor B inhibition of passive proton conductance approached but did not exceed 50%. Fo molecules in both orientation are expected to be competent in passive proton conductance, the directionality of which is determined by an inwardly negative $\Delta\psi$ that is established following valinomycin-mediated K⁺ ions efflux. However, the conductance mechanism could be quite distinct. The Fo molecules facing the vesicular lumen rotate in the ATP synthesis direction, while the Fo molecules facing the external medium would be expected to rotate in the ATP hydrolysis direction. Only Fo molecules that face the external medium respond to addition of factor B. This is in contrast to the topological arrangement existing in intact mitochondria and in AE-SMP, where the binding of factor B to the matrix side of the mitochondrial inner membrane blocks a proton leak. This proton leak is presumably mediated by Fo rotating in the ATP synthesis direction. The above reasoning, along with the possibility of local alkalization in vicinity of Fo molecules due to inward proton conductance, could explain the high concentrations of factor B that were required to inhibit Fo. Our data demonstrate for the first time the ability of factor B to inhibit Fo proton conductance. This result is in agreement with our previous data, which showed that in factor B-depleted AE-SMP that were energized with NADH, succinate or ATP, the backflow of protons could be blocked by addition of factor B, as well as oligomycin and DCCD [12,13]. However, these data are in contrast with an earlier report, which demonstrated stimulation, not inhibition, of the Fo passive proton conductance by factor B [35]. In the latter report, however, F₁-stripped AE-SMP were used for reconstitution into asolectin vesicles.

Because the membrane sector Fo still contained the ADP/ATP carrier, it was unclear whether co-sedimentation reflected direct protein-protein interaction between factor B and Fo or was mediated by the carrier. This issue was addressed in cross-linking experiments using a factor B mutant in which an unnatural photoreactive amino acid *p*Bpa was site-specifically inserted in the position occupied by Trp2. Upon UV-irradiation, the keto group of benzophenone moiety is excited to a diradicaloid triplet state, in which the electron-deficient electrophilic oxygen is highly reactive toward C-H bonds of protein backbones [36]. The result is the formation of a covalent C-C bond with neighboring polypeptides. UV-irradiation of the factor B mutant in the presence of FoF₁ produced three major cross-linked products, with *M_r* of ~50, 30 and 26 kDa (Fig. 9). The same cross-linked products were obtained following UV-irradiation of FB_{W2Bpa/G3E} reconstituted with AE-SMP (data not shown). Using mass spectrometry, we detected presence of the ADP/ATP carrier in the ~50 kDa cross-linked product. Western blot analysis unambiguously established that the ~30 kDa band included two cross-linked products. One contained factor B cross-linked with Fo subunit *e*, and the other product was factor B

cross-linked with Fo subunit *g* (Fig. 10). The identity of the polypeptide to which factor B cross-linked to yield the ~26 kDa cross-linked product remains to be identified.

Previously, we demonstrated that the N-termini of Fo subunits *e* and *g* face the matrix side while their respective C-termini face the cytosolic side of the mitochondrial inner membrane [37]. Cross-links between two copies of *e* and cross-links of *g* with both *e* and *f* were also reported [37]. Analysis of animal subunit *g* revealed the presence of a completely conserved acidic residue (Glu82) in the middle of its single transmembrane helix [38]. The only other acidic residue with such a unique location in animal ATP synthase is the Glu58 residue of Fo subunit *c*. Deletion of the yeast counterparts of either bovine subunits *e* or *g* affected the oligomerization of yeast ATP synthase and changed the mitochondrial cristae morphology [39,40].

The current view on mechanism of proton translocation elaborated for bacterial Fo sector assigns a role to subunit *a* in formation of two aqueous half channels: one half channel directs protons from the outside to the Asp61 residue of subunit *c* located in the middle of the lipid bilayer, and the other half channel directs protons to the other side of the membrane into the bulk medium following the *c* ring rotation [41]. The role of bacterial subunit *a* charged residue Arg210 is thought to prevent the short-circuiting of both channels and to facilitate deprotonation of Asp61 at the mouth of exit half channel [41,42].

The mechanism of Fo inhibition by DCCD is well understood and is due to covalent modification of a crucial Asp61 (prokaryotes) or Glu58 (eukaryotes) in Fo subunit *c*. In the eukaryotic ATP synthase, the binding of oligomycin and related macrolide antibiotics at subunit *a* and subunit *c* ring interface could interfere with rotary motions of the subunit *c* ring [43].

The site at which factor B could inhibit a futile proton leak in mammalian Fo sector is proposed to lie downstream of the sites affected by DCCD and oligomycin, closer to the matrix surface of the mitochondrial inner membrane. Cross-linking of factor B with both Fo subunits *e* and *g* as well as the ADP/ATP carrier suggests that these polypeptides may assemble an aqueous proton exit pathway, which is occluded in the coupled ATP synthase by the proximal N-terminal residues of factor B.

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Fig. 1.

Loss of coupling activity in a factor B fragment comprised of amino acid residues 62-175. **A**, SDS-PAGE analysis of the recombinant bovine factor B treated with trypsin for 20 min (lane 2), 40 min (lane 3), and 60 min (lane 4) at the protein: enzyme ratio 200: 1, w/w. Lane 1 contains factor B in the absence of trypsin. Each lane was loaded with 5 μ g of protein. A gel similar to that shown in the figure was electroblotted to Immobilon-P^{SQ} membrane and the indicated fragments were subjected to the N-terminal sequence analysis. **B**, SDS-PAGE analysis of the recombinant factor B₆₁₋₁₇₅ fragment cloned into the pET43-1a vector, expressed and purified from the soluble fraction of *E. coli* lysate. Lane 1 contains 4 μ g and lane 2 contains 8 μ g of the recombinant FB₆₁₋₁₇₅ protein. Gels shown in **A** and **B** were stained with Coomassie Brilliant

Blue. Bars in panels *A* and *B* and the left lane in panel *B* show the positions of broad molecular weight standards (Bio-Rad). The molecular weights (in kDa) from top to bottom are as follows: myosin, 200; β -galactosidase, 116.3; phosphorylase b, 97.4; bovine serum albumin, 66.2; ovalbumin, 45; carbonic anhydrase, 31; trypsin inhibitor, 21.5; lysozyme, 14.4, and aprotinin, 6.5. *C*, Molecular mass determination of the recombinantly expressed FB₆₁₋₁₇₅ fragment using MALDI-TOF. Singly- and doubly-charged molecular ions, as well as a peak attributed to the FB₆₁₋₁₇₅ dimer, had masses of 13,380, 6,691 and 26,760 Da, respectively. *D*, ATP-driven ACMA fluorescence quenching in reconstituted AE-SMP. Proton-pumping activity of AE-SMP alone (trace 1), AE-SMP reconstituted with either 10 μ g/ml full-length recombinant factor B (trace 2) or 10 μ g/ml recombinant FB₆₁₋₁₇₅ fragment (trace 3). The concentration of AE-SMP was 115 μ g of protein/ml.

Fig. 2.

Deletion of the N-terminal Trp2-Gly3-Trp4 tripeptide impairs the coupling activity of bovine factor B. **A**, SDS-PAGE analysis of the full-length recombinant bovine factor B (lane 1, 5 μ g) and the FB Δ W²-W⁴ mutant (lane 2, 5 μ g). The left lane contains low molecular weight standards. **B**, Molecular mass determination of the recombinantly expressed FB Δ W²-W⁴ mutant using MALDI-TOF. Singly- and doubly-charged molecular ions with masses of 19,981 and 9,991 Da, respectively, are shown. **C**, ATP-driven ACMA fluorescence quenching in reconstituted AE-SMP. Proton-pumping activity of AE-SMP alone (trace 1), AE-SMP reconstituted with either 10 μ g/ml full-length recombinant factor B (trace 2) or 15 μ g/ml FB Δ W²-W⁴ mutant (trace 3). The concentration of AE-SMP was 115 μ g of protein/ml.

Fig. 3.

Analysis of interaction between recombinant bovine factor B and purified bovine heart mitochondrial F_0F_1 -ATPase using sucrose density gradient centrifugation. Panels A, B and C show Coomassie Blue-stained SDS polyacrylamide gels depicting the polypeptide composition of the 15-35% sucrose density gradient fractions collected from top (lane 1) to bottom (lane 9) following centrifugation of F_0F_1 -ATPase alone (A), factor B alone (B) or factor B mixed with F_0F_1 -ATPase (C). Positions of the mitochondrial F_0F_1 -ATPase subunits and factor B are indicated by bars on the right. Low molecular weight standards (Bio-Rad) are shown in the utmost left lane of each panel.

Fig. 4. Analysis of interaction between recombinant bovine factor B and purified bovine heart mitochondrial complex V using sucrose density gradient centrifugation. Panels A, B and C show Coomassie Blue-stained SDS polyacrylamide gels depicting the polypeptide composition of the 15-35% sucrose density gradient fractions collected from top (lane 1) to bottom (lane 9) following centrifugation of complex V alone (A), factor B alone (B) or factor B mixed with complex V (C). Positions of the mitochondrial complex V subunits and factor B are indicated on the right. Low molecular weight standards (Bio-Rad) are shown in the left lane of each panel.

Fig. 5.

Analysis of interaction between the FB $_{\Delta W^{2-W^4}}$ mutant and purified bovine heart mitochondrial F₀F₁-ATPase using sucrose density gradient centrifugation. Panels A and B show Coomassie Blue-stained SDS polyacrylamide gels depicting the polypeptide composition of the 15-35% sucrose density gradient fractions collected from top (lane 1) to bottom (lane 9) following centrifugation of the FB $_{\Delta W^{2-W^4}}$ mutant alone (A) or the FB $_{\Delta W^{2-W^4}}$ mutant mixed with F₀F₁-ATPase (B). Positions of the mitochondrial F₀F₁-ATPase subunits and factor B are indicated on the right. Low molecular weight standards (Bio-Rad) are shown in the left lane of each panel.

Fig. 6.

Analysis of interaction between recombinant bovine factor B and membrane sector F_0 using sucrose density gradient centrifugation. Panels A, B and C show Coomassie Blue-stained SDS polyacrylamide gels depicting the polypeptide composition of the 15-35% sucrose density gradient fractions collected from top (lane 1) to bottom (lane 9) following centrifugation of F_0 alone (A), factor B alone (B) or factor B mixed with F_0 (C). Positions of the membrane sector F_0 subunits b and d, the ADP/ATP carrier and factor B are indicated on the right. Low molecular weight standards (Bio-Rad) are shown in the left lane of each panel.

Fig. 7.

The polypeptide composition of the membrane sector F_o reconstituted with recombinant factor B. Fractions 4 and 5 from the experiment shown in Fig. 6, panels A and C, were concentrated and their equal aliquots were separated by 15% SDS-PAGE followed staining with Coomassie Brilliant Blue. Lanes 1 and 2 contain the concentrated fractions 4 and 5 of panel A, Fig. 6, respectively. Lanes 3 and 4 contain the concentrated fractions 4 and 5 of panel C, Fig. 6, respectively. Recombinant bovine factor B ($M_r \sim 22$ kDa) is present in lanes 3 and 4 and absent in lanes 1 and 2, as shown. The identity of the indicated membrane sector F_o subunits was confirmed by Western blot analysis.

Fig. 8.

Effect of recombinant bovine factor B on the passive proton conductance of Fo proteoliposomes. Fo was incorporated into K^+ -loaded asolectin vesicles and assayed as described under "Materials and methods". Shown is ACMA fluorescence quenching after addition of valinomycin (Val) or protonophore CCCP. 1. control Fo; 2. Fo plus FB (3.5 μ M); 3. Fo plus FB (0.75 μ M); 4. Fo plus FB $_{\Delta W2-W4}$ (5 μ M); 5. Fo plus oligomycin (7.5 μ g/ml). CCCP was added at a concentration of 1 μ M.

Fig. 9.

Photocross-linking of the $FB_{W2Bpa/G3E}$ mutant alone or in the presence of F_oF_1 -ATPase. $FB_{W2Bpa/G3E}$, at concentrations of 1 μ M (lanes 1, 4, 7, 10), 3 μ M (lanes 2, 5, 8, 11) and 6 μ M (lanes 3, 6, 9, 12), alone (lanes 1-6) or in the presence of 1.7 μ M F_oF_1 (lanes 7-12) was either UV-irradiated (lanes 4-9) for 30 min on ice or not irradiated (lanes 1-3, 10-12). Samples were separated by 12% SDS-PAGE and either stained with Coomassie Brilliant Blue (left panel) or electrotransferred to nitrocellulose and blotted with antibody against factor B, 1:10,000 dilution (right panel). The utmost left lane in each panel contains low molecular weight standards (Bio-Rad).

Fig. 10.

The $\text{FB}_{\text{W2Bpa/G3E}}$ mutant photocross-links with Fo subunits e and g . FoF_1 ($1.7 \mu\text{M}$) was preincubated with 2-fold (lanes 1, 2, 4, 5, 7, 8, 10, 11) or 4-fold (lanes 3, 6, 9, 12) molar excess of $\text{FB}_{\text{W2Bpa/G3E}}$ and mixture was either UV-irradiated (lanes 2, 3, 5, 6, 8, 9, 11, 12) for 30 min on ice or not irradiated (lanes 1, 4, 7, 10). Samples containing $6 \mu\text{g}$ (lanes 1-3) or $9 \mu\text{g}$ (lanes 4-12) of total protein were separated by 15% SDS-PAGE, electrotransferred to nitrocellulose and blotted with antibodies against factor B (lanes 1-3, 1:10,000 dilution) and Fo subunits e (lanes 4-6), f (lanes 7-9) and g (lanes 10-12), all at a 1:100 dilution. Arrowheads indicate the UV-generated $\sim 30 \text{ kDa}$ cross-linked products. The positions of low molecular weight standards are indicated on the left.

Fig. 11.

Identification of endogenous bovine factor B in the DDM extract of heavy bovine heart mitochondria. **A**, Heavy bovine heart mitochondria (10 mg/ml), solubilized with DDM at protein: detergent ratio 1:1.6, w/w, were fractionated in a 15-35% sucrose density gradient. Following centrifugation, fractions were collected from top (lane 1) to bottom (lane 9), separated by 15% SDS-PAGE and stained with Coomassie Brilliant Blue. **B**, Western blot analysis of the fractions shown in panel **A** with antibodies against FoF₁ subunits: F₁ α , *b*, *a*, *e*, *f*, *g* and *c*. FB, factor B; AAC, ADP/ATP carrier; AIF, apoptosis-inducing factor. **C**, The region of the gel, enclosed by a rectangle in lane 3 of panel **A**, was excised, digested with trypsin, and the eluted peptides were subjected to MS/MS analysis. Five identified peptides, whose sequences were identical to the sequences derived from the mature bovine factor B (GenBank Accession No. AAV52866), are shown in *bold*. The sequence coverage was 29% with a score of 554.