Articles

The ATPase Inhibitor Protein from Bovine Heart Mitochondria: The Minimal Inhibitory Sequence†

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Received March 14, 1996; Revised Manuscript Received May 6, 1996⊗

ABSTRACT: The mitochondrial ATPase inhibitor subunit is a basic protein of 84 amino acids that helps to regulate the activity of F_1F_o-ATPase. In order to obtain structural information on the mechanism of inhibition, the bovine inhibitor subunit has been expressed in *Escherichia coli* and purified in high yield. The recombinant protein has a similar inhibitory activity to the inhibitor subunit isolated from bovine mitochondria. Progressive N-terminal and C-terminal deletion mutants of the inhibitor subunit have been produced either by overexpression and purification, or by chemical synthesis. By assaying the truncated proteins for inhibitory activity, the minimal inhibitory sequence of the inhibitor subunit has been defined as consisting of residues 14−47. The immediately adjacent sequences 10−13 and 48−56 help to stabilize the complex between F_1F_o-ATPase and the inhibitor protein, and residues 1−9 and 57−84 appear to be dispensable. At physiological pH values, the inhibitor subunit is mainly α-helical and forms monodisperse aggregates in solution. Smaller inhibitory fragments of the inhibitor protein, such as residues 10−50, seem to have a mainly random coil structure in solution, but they can adopt the correct inhibitory conformation when they form a complex with the ATPase. However, these latter fragments are mainly monomeric in solution, suggesting that the aggregation of the inhibitor subunit in solution may be due to intermolecular α-helical coiled-coil formation via the C-terminal region. The noninhibitory peptides consisting of residues 10−40 and 23−84 of the inhibitor protein can bind to F_1F_o-ATPase, and interfere with inhibition by the intact inhibitor subunit. The noninhibitory fragments of the inhibitor protein consisting of residues 22−46 and 44−84 do not compete with the inhibitor subunit for its binding site on F_1F_o-ATPase.

The mitochondrial ATPase inhibitor protein, IF_1, is a small basic protein that helps to regulate the activity of the ATP synthase by inhibiting its ATP hydrolase activity at a pH of 6.7 or less by forming a 1:1 complex (for reviews, see Harris & Das, 1991; Walker, 1994). Binding of IF_1 requires hydrolysis of Mg·ATP, and the K_d of the bovine IF_1 is 96 nM (Power et al., 1983). Restoration of a mitochondrial membrane potential favoring ATP synthesis displaces IF_1 from its inhibitory site and releases bound ATP, indicating that IF_1 prevents product release (Pedersen et al., 1981; Penin et al., 1988). By cross-linking yeast IF_1 to ATP synthase, the binding site was shown to encompass both α- and β-subunits of the F_1 domain, suggesting that the site could be at an α−β interface (Mimura et al., 1993). The binding site probably includes the sequence DELSEED (Jackson & Harris, 1988) found at residues β394−400 (Runswick & Walker, 1983) in the C-terminal domain of the β-subunit. This domain consists of a bundle of six α-helices and makes a major intersubunit contact via a loop containing residues 394−400 with an α-helical coiled-coil structure in the γ-subunit (Abrahams et al., 1994). This interaction could be a key element in a mechanism for cyclic interconversion of the three catalytic sites present in each enzyme which are proposed to pass consecutively through three different conformational states associated with the binding of substrates, the synthesis of ATP, and the release of bound ATP (Boyer, 1993). Therefore, binding of IF_1 to this region of the β-subunit could stabilize or impede this interaction, and so inhibit the enzyme.

So far, little is known about the three-dimensional structure of IF_1. Circular dichroism (CD) spectra indicate that the protein is mainly α-helical above pH 7.0, but below this pH, conflicting proposals have been made. No large structural change in bovine IF_1 was detected at pH 6.7 (Van Heeke et al., 1993), whereas at pH 6.0 buffalo IF_1 was said to adopt a predominantly β-structure (Sah et al., 1993), and rat IF_1's α-helical content diminished with decreasing pH with concomitant increase in random structure (Lebowitz & Pedersen, 1993). It has been proposed from the CD spectra of bovine IF_1 that its C-terminal region may form an intermolecular α-helical coiled-coil, possibly leading to the formation of dimers and higher oligomers. Preliminary nuclear magnetic resonance (NMR) data support dimer formation (Van Heeke et al., 1993), and the tendency of IF_1 to oligomerize, particularly at the high protein concentrations required for high resolution structural analysis by NMR

† M.J.v.R. and G.L.O. are supported by MRC Research Studentships.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

S0006-2960(96)00628-9 CCC: $12.00

Table 1: Synthetic Oligonucleotides for Amplification of Coding Sequences of the ATPase Inhibitor and Related Proteins

Protein	Oligonucleotide sequence (5' to 3')	Plasmid
Inhibitor	TAG GAA TTC ATA TGG GCT CGG AAT CGG GAG ATA ATG TTA GG	pRKIF1
"	CGA AAG CTT TTA TTA GTC GTC ATC CTC ACT CTG TTT TAG	pRKIF1
IΔ1-9	TAG GAA TTC ATA TGT CCA GTG CGG GCG CGG TCC GGG ACG CC	pRKI10-84
IΔ1-13	TAG GAA TTC CAT ATG GCG GTC CGG GAC GCC GGT GGG GCC TTC	pRK14-84
IΔ1-17	TAG GAA TTC CAT ATG GCC GGT GGG GCC TTC GGA AAA AGA GAG	pRKI18-84
IΔ1-22	TAG GAA TTC CAT ATG GGA AAA AGA GAG CAG GCC GAA GAG GAG	pRKI23-84
IΔ1-43	TAG GAA TTC CAT ATG GCC TTG AAG AAA CAC CAT GAA AAT GAG	pRKI44-84
I1-60	CGA AAG CTT TTA TTA AAT CTC CTT TGC ATG ATG AGA GAT CTC	pRKI1-60
I1-64	CGA AAG CTT TTA TTA CTG CAG GCG CTC AAT CTC CTT TGC ATC	pRKI1-64
I1-69	CGA AAG CTT TTA TTA CCG CTC AAT TTC TTT CTG CAG GCG CTC	pRKI1-69
I1-74	CGA AAG CTT TTA TTA GAT CGA CTG CTT ATG CCG CTC AAT TTC	pRKI1-74
I1-78	CGA AAG CTT TTA TTA TTT TAG TTT CTT GAT CGA CTG CTT ATG	pRKI1-78

techniques, has thwarted structural analysis. This tendency to oligomerize is also likely to impede preparation of crystals of IF_1 that are suitable for X-ray crystallography. Therefore, in an attempt to produce an active form of IF_1 suitable for structural analysis, we have prepared forms that are progressively truncated from both N- and C-terminals, and thereby we have defined a minimal bovine inhibitory sequence as amino acids 14−47. Preliminary results from this work have been quoted by Harris (1995).

MATERIALS AND METHODS

Nomenclature. Forms of bovine IF_1 truncated at the N-terminus by 9, 13, 17, 22 and 43 residues were named IΔ1−9, IΔ1−13, IΔ1−17, IΔ1−22, and IΔ1−43, respectively. Internal fragments and forms with C-terminal truncations are designated as I followed by the residue numbers in the sequence of the intact mature IF_1, which has 84 amino acids.

Materials. Rabbit muscle pyruvate kinase and lactate dehydrogenase were purchased from Boehringer Mannheim (Lewes, U.K.). Prepacked columns of Q-Sepharose HP, S-Sepharose HP, and Sephacryl S-100 HR and loose samples of DEAE-Sepharose CL-6B and S-Sepharose HP were obtained from Pharmacia Biotech, Ltd. (Milton Keynes, U.K.). Reverse phase HPLC columns were supplied by Applied Biosystems (Warrington, U.K.) and by The Separations Group, Hesperia, CA. Oligonucleotides were synthesized with an Applied Biosystems 380B oligonucleotide synthesizer. I1−22, I22−46, I1−45, and I10−48 were synthesized by Dr. A. Johnson and colleagues in this laboratory, I10−40 and I10−50 by Peptide Products Ltd. (Salisbury, U.K.), and I10−45, I10−46, and I10−47 by Genosys, Cambridge, U.K.

Analytical Methods. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL) or by amino acid analysis. Denaturing polyacrylamide gels containing a 12−22% (w/v) acrylamide gradient separating gel and 4% (w/v) stacking gel (acrylamide:*N*,*N*′-methylenebis(acrylamide), 30:0.8 w/w) were cast in 10 cm × 10 cm × 0.6 mm format and run in the buffer system of Laemmli (1970). Alternatively, gels were cast and run using the method of Schägger and von Jagow (1987). Proteins were transferred from unstained gels onto poly(vinylidene difluoride) (PVDF) membranes using a Milliblot Graphite Electroblotter II semi-dry blotting apparatus (Millipore, Watford, U.K.). After detection of the proteins with PAGE[1] blue 83 dye, their N-terminal sequences were determined by automated Edman degradation in a modified Applied Biosystems 477A protein sequencer (Fearnley et al., 1989). Samples of proteins for electrospray mass spectrometric analysis were either dialyzed against 0.1%(v/v) trifluoroacetic acid or purified by reverse phase HPLC on a C_8 Aquapore RP-300 column (7 μm particles, 300 Å pore size; 10 cm × 2.1 mm i.d.) equilibrated in 0.1% (v/v) trifluoroacetic acid. Proteins were applied to the column in 3 M guanidine-HCl made in 17 mM sodium acetate buffer (pH 4.0) and eluted in 0.1% trifluoroacetic acid with a gradient of acetonitrile at a flow rate of 0.1 mL/min. The absorbance of the effluent was monitored at 225 nm. Samples of the effluent were introduced into either a VG BIO-Q triple quadrupole mass spectrometer with electrospray ionization or a Perkin-Elmer Sciex API III$^+$ instrument, and spectra were measured as described previously (Walker et al., 1992).

Construction of Bacterial Expression Plasmids. The coding sequence of the bovine ATPase IF_1 was amplified by PCR from an M13 clone derived from plasmid pBovIn.1 (Walker et al., 1987) with the primers shown in Table 1. The sequence of the product was verified by DNA sequencing (Sanger et al., 1977; Biggin et al., 1983), and then the fragment was cloned, as described previously (Collinson et al., 1994), into the *Nde*I/*Hin*dIII site of the expression vector pMW172 (Way et al., 1990). The resulting expression plasmid, containing the entire bovine IF_1, is known as $pRKIF_1$. Deletion mutants of IF_1 were made from $pRKIF_1$ by PCR using the oligonucleotide primers listed in Table 1.

Overexpression of the Bovine IF_1 and Related Proteins in Escherichia coli. A colony of recently transformed cells of *E. coli* BL21 (DE3) was inoculated into 1 L of 2× TY broth (16 g/L Bacto tryptone, 10 g/L Bacto yeast extract, 5 g/L sodium chloride, pH 7.4) containing 100 μg/mL ampicillin. The culture was grown with shaking in a 2 L flask at

[1] Abbreviations: AMP-PNP, adenylyl imidodiphosphate; DEAE, (diethylamino)ethyl; EDTA, ethylenediaminetetraacetic acid; HPLC, high pressure liquid chromatography; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

37 °C until the optical density at 600 nm had reached 0.6. Expression of the protein was induced by addition of isopropyl β-D-thiogalactopyranoside (150 mg), and growth was continued for a further 3−4 h. The bacteria were centrifuged (7000*g*, 15 min, 4 °C) and resuspended in TEP buffer (150 mL), consisting of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.001% (w/v) PMSF.

Purification of Bacterially Expressed Proteins. All of the procedures were carried out at 4 °C. Bacterial cells containing the various recombinant proteins were broken by being passed twice through a French pressure cell. Fragment IΔ1−22 formed inclusion bodies which were harvested by low speed centrifugation (20000*g*, 30 min). All of the other recombinant proteins were soluble in the cytoplasmic fraction which was clarified by high speed centrifugation (142000*g*, 1 h). The broken cell supernatants containing IF_1, IΔ1−9, IΔ1−43, I1−69, I1−64, I1−60, and I1−56 (each from 10 L of bacterial culture) were dialyzed twice against TEP buffer (4 L) using a membrane with a molecular weight cutoff of 3.5 kDa. To the supernatants containing I1−74, I1−78, IΔ1−13, and IΔ1−17, sodium chloride was added to give a final concentration of 0.5 M, and then a solution of streptomycin sulfate (10% w/v) was added dropwise to give a final concentration of 1%. After 30 min, precipitated DNA was removed by centrifugation (45000*g*, 20 min), producing supernatants referred to as "post streptomycin sulfate supernatants".

(i) The Inhibitor Protein, IF_1. The dialyzed broken cell supernatant was applied to a column of DEAE-Sepharose CL-6B (150 mL) equilibrated in TEP buffer. The IF_1 eluted from the column at 0.2 M on a linear gradient of 0−0.5 M sodium chloride in TEP buffer (total volume 500 mL). Appropriate fractions were concentrated by ultrafiltration through an Amicon YM3 membrane, and the concentrate (5 mL) was applied to a Sephacryl S-100 HR column (HiLoad 26/60) equilibrated in a buffer containing 10 mM Tris-HCl, pH 8.0, 1 M sodium chloride, and 0.001% (w/v) PMSF. Pooled fractions containing IF_1 were dialyzed against 10 mM sodium citrate, pH 6.0, and loaded onto an S-Sepharose column (HiLoad 26/10) equilibrated with the same buffer. Pure IF_1 emerged on a linear gradient of 0−1 M sodium chloride at 0.45 M. The recovery of bovine IF_1 from 10 L of culture was 50 mg.

(ii) IΔ1−9. The dialyzed broken cell supernatant was applied to a column of DEAE-Sepharose CL-6B (150 mL) equilibrated in TEP buffer. IΔ1−9 eluted at a salt concentration of 0.3 M on a linear gradient of 0−1 M sodium chloride in TEP buffer (total volume of 500 mL). Pooled fractions containing IΔ1−9 were dialyzed against TEP buffer and loaded onto an S-Sepharose HP column (HiLoad 26/10) equilibrated with the same buffer. IΔ1−9 eluted at a salt concentration of 0.3 M on a linear gradient of 0−1 M sodium chloride. The pooled fractions were concentrated to 10 mL by ultrafiltration through an Amicon YM3 membrane and then applied to a Vydac C_8 reverse phase column (25 cm × 2.2 cm i.d.). Pure IΔ1−9 eluted at 25% (v/v) acetonitrile on a linear gradient of 10−90% (v/v) acetonitrile in 0.1% trifluoroacetic acid. The pH of appropriate fractions was raised to 8.0 by addition of 1 M Tris, and they were dialyzed twice against TEPG buffer [4 L; 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, and 0.001% (w/v) PMSF]. The recovery of pure IΔ1−9 from 10 L of culture was 10 mg.

(iii) IΔ1−13 and IΔ1−17. The post streptomycin sulfate supernatant containing IΔ1−13 was dialyzed against TEP buffer (2 × 4 L), thereby precipitating the protein. The suspension was centrifuged (15000*g*, 10 min), and the pellet was dissolved in TEP buffer (50 mL) containing 6 M guanidine-HCl. This solution was dialyzed twice against MEP buffer [4 L; 10 mM MOPS-NaOH, pH 7.0, 1 mM EDTA, and 0.001% (w/v) PMSF] containing 0.2 M sodium chloride and was loaded onto an S-Sepharose HP column (HiLoad 26/10) equilibrated in the same buffer. IΔ1−13 eluted at about 0.35 mM on a linear gradient of 0.2−1.0 M sodium chloride in MEP buffer. Pooled fractions containing IΔ1−13 were dialyzed against TEP buffer, pH 8.5 (4 L), and loaded onto a Q-Sepharose HP column (HiLoad 26/10). The purified protein eluted at 0.2 M on a linear sodium chloride gradient of 0−1.0 M in the same buffer. The recovery of pure IΔ1−13 peptide from 5 L of culture was 1 mg. IΔ1−17 (2 mg) was purified from 5 L of bacterial culture in a similar way to IΔ1−13.

(iv) IΔ1−22. The inclusion bodies containing IΔ1−22 were washed with TEP buffer (100 mL), dissolved in MEP buffer (500 mL) containing 6 M guanidine-HCl and 0.2 M sodium chloride, and dialyzed twice against MEP buffer (4 L) containing 0.2 M sodium chloride. The sample was centrifuged (25000*g*, 30 min), and the supernatant was loaded onto an S-Sepharose HP column (HiLoad 26/10) equilibrated in the same buffer. IΔ1−22 eluted at about 0.4 M on a linear gradient of 0.2−1.0 M sodium chloride in MEP buffer. Appropriate fractions were dialyzed against TEP buffer (4 L, pH 8.5) containing 100 mM sodium chloride and loaded onto a Q-Sepharose column HP (HiLoad 26/10). The protein eluted at about 0.2 M on a linear sodium chloride gradient from 0.1 to 1 M in the same buffer. The recovery of pure IΔ1−22 from 10 L of culture was 40 mg.

(v) IΔ1−43. The dialyzed broken cell supernatant was purified on DEAE-Sepharose CL-6B as described for IF_1 and then purified by reverse phase HPLC on a Vydac C_8 reverse phase column (25 cm × 2.2 cm i.d.). The recovery was 1 mg from 1 L of bacterial culture.

(vi) I1−78. The post streptomycin sulfate supernatant containing I1−78 was dialyzed twice against TEP buffer (4 L) using a membrane with a molecular weight cutoff of 3.5 kDa. The resulting precipitate containing I1−78 was centrifuged (45000*g*, 20 min), and the pellet was dissolved in 6 M guanidine-HCl (50 mL). This solution was dialyzed twice against MEP buffer (4 L) containing 0.2 M sodium chloride. The refolded I1−78 was then loaded onto an S-Sepharose HP column (400 mL) equilibrated in the same buffer. I1−78 eluted at 0.6 M on a linear gradient of 0.2−1.0 M sodium chloride in MEP buffer. The pooled fractions were dialyzed against MEP buffer (4 L) and applied to a Q-Sepharose HP column (HiLoad 26/10) equilibrated in the same buffer. I1−78 was in the breakthrough fractions. They were pooled and loaded onto a DEAE-Sepharose CL-6B column (150 mL) equilibrated in MEP buffer. The breakthrough fractions contained pure I1−78. The recovery of pure I1−78 from 10 L of culture was 10 mg.

(vii) I1−74. The post streptomycin sulfate supernatant was dialyzed twice against TEP buffer (4 L) using a membrane with a molecular weight cutoff of 3.5 kDa. A precipitate was removed by centrifugation (45000*g*, 20 min), and the supernatant containing I1−74 was applied to a column of DEAE-Sepharose CL-6B (500 mL) equilibrated

in TEP buffer. I1−74 was in the breakthrough which was loaded onto an S-Sepharose column HP (400 mL) equilibrated in TEP buffer. It eluted at 0.65 M on a linear gradient of 0−1 M sodium chloride in TEP buffer. Pooled fractions were dialyzed against TEP buffer (4 L) and loaded onto a Q-Sepharose HP column (HiLoad 26/10) equilibrated in the same buffer. The breakthrough fractions containing pure I1−74 were pooled. The recovery of I1−74 from 10 L of culture was 40 mg.

(viii) I1−69, I1−64, I1−60, and I1−56. I1−69 was purified on DEAE-Sepharose from the dialyzed broken cell supernatant from a 1 L culture, as described for IF_1. It eluted at 0.15 M sodium chloride. Fractions containing I1−69 were pooled, dialyzed against 4 L of 10 mM sodium citrate, pH 6.0, and loaded onto an S-Sepharose HP column (HiLoad 26/10) equilibrated with the same buffer. The protein eluted at about 0.3 M on a linear gradient of 0−1 M sodium chloride in the same buffer. The recovery of pure I1−69 from 1 L of culture was 2.5 mg. I1−64 and I1-60 were purified in a similar manner, yielding 8 and 18 mg of pure protein from 1 and 10 L of bacterial cultures, respectively. The latter preparation contained between 10% and 20% of I1−56, presumably formed by proteolytic degradation of I1−60. I1−60 and I1−56 were separated by reverse phase HPLC on a Vydac C_8 column (25 cm × 2.2 cm i.d.) using a gradient of 25−30% (v/v) acetonitrile in 0.1% trifluoroacetic acid. The products were dried under vacuum, redissolved in 0.4 mL of 6 M guanidine-HCl, pH 8.0, and dialyzed twice against TEP buffer (1 L).

Measurement of Circular Dichroism Spectra. CD measurements of IF_1 and of peptides derived from it were performed in a buffer consisting of 10 mM MOPS−sodium hydroxide, pH 7.0, 200 mM sodium chloride, 1 mM EDTA, and 0.001% (w/v) PMSF, at protein concentrations of approximately 0.1 mg/mL. The protein concentration of each sample was determined by amino acid analysis. Spectra from 200 to 250 nm were measured on a Jasco J-720 spectropolarimeter at 37 °C using a cell with a path length of 0.1 cm. The α-helical content was calculated from the relationship: $f_H = -([\theta]_{222} + 2340)/30300$, where f_H is the fractional α-helical content and $[\theta]_{222}$ is the mean residue ellipticity in deg·cm^2·dmol^{-1} (Chen et al., 1972).

Examination of Polydispersity by Dynamic Light Scattering. Dyamic light scattering measurements were performed with a DynaPro-801 dynamic light scattering/molecular sizing instrument (Protein Solutions Ltd., High Wycombe, Buckinghamshire, U.K.).

Assay of IF_1 and Deleted Forms. One unit of IF_1 is defined as the amount that inhibits 0.2 U of ATPase by 50%, where 1 U of ATPase hydrolyzes 1 μmol of ATP/min. Activities of the recombinant bovine IF_1 and fragments thereof were assayed for the inhibition of ATPase of either bovine submitochondrial particles that had been depleted of endogenous IF_1 (Horstman & Racker, 1970; Gomez-Fernandez & Harris, 1978), or of isolated, nucleotide-stripped and inhibitor-free, F_1-ATPase (Lutter et al., 1993). The depleted membranes were a generous gift from Dr. D. A. Harris (Oxford University, U.K.). Between 0 and 2 μg of IF_1 (or fragments) were mixed with IF_1-depleted membranes (100 μg of protein) or F_1-ATPase (12 μg, 32 pmol) to give a total volume of 200 μL in 250 mM sucrose, 10 mM MOPS-NaOH, pH 6.7. The substrate Mg·ATP (1 μL of a neutralized 200 mM solution) was added to this mixture. When the assay was conducted with isolated F_1-ATPase, sucrose was omitted from the buffer. The mixture was incubated at 37 °C for 5 min, and then the ATPase activity was measured by transferring 10 μL of the mixture to 1 mL of ATPase assay mixture at 37 °C, and by measuring the decrease in the absorbance of NADH at 340 nm for 5 min. The ATPase assay mixture contained 60 mM sucrose, 50 mM Tris−sulfate, pH 8.0, 50 mM potassium chloride, 2 mM magnesium chloride, 1 mM EDTA, pyruvate kinase (20 μg/mL), lactate dehydrogenase (10 μg/mL), 0.2 mM NADH, 1 mM phosphoenolpyruvate, and 1 mM Mg·ATP.

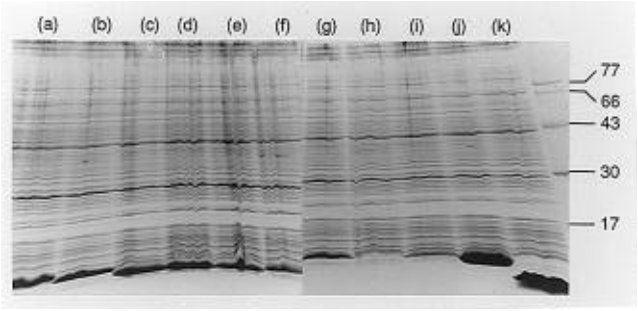

FIGURE 1: Bacterial expression of bovine IF_1 and fragments derived from it. Samples of bacterial cultures were withdrawn 3 h after induction of expression of the recombinant proteins. They were analyzed by SDS−PAGE and stained with PAGE blue 83 dye. Lanes (a) bovine IF_1; (b) IΔ1−9; (c) IΔ1−13; (d) IΔ1−17; (e) IΔ1−22; (f), IΔ1−43; (g) I1−78; (h) I1−74; (i) I1−69; (j) I1−64; (k) I1−60. The positions of marker proteins are indicated on the right. Lanes a−f and g−k were run on separate gels.

For competition assays, 2 μg of competitor was mixed with the IF_1-depleted membranes (100 μg of protein) in the assay buffer, Mg·ATP (1 μL of a neutralized 200 mM solution) was added, and the mixture was preincubated at 37 °C for 5 min. IF_1 was then added, followed by another addition of Mg·ATP, and the assay was performed as before.

RESULTS AND DISCUSSION

Bacterial Expression of the Bovine IF_1 and Truncated Fragments. The IF_1 has been purified previously from mitochondria (Horstman & Racker, 1970), but the yield is rather low, and the procedure involves a heat denaturation step, which could conceivably damage the IF_1. So, in order to obtain a plentiful supply of IF_1 purified by a mild procedure, and to have the possibility to make mutants, the bovine IF_1 was expressed in *E. coli* using the expression system of Studier et al. (1990), as modified by Way et al. (1990). The bovine protein has been highly expressed in *E. coli* from a synthetic gene and secreted into the bacterial periplasm (van Heeke et al., 1993). As shown in Figure 1 (lane a), the bovine IF_1 was also highly expressed in the system employed in the present work, as were most of the various truncated forms (Figure 1). With the exception of IΔ1−22, the bovine proteins were soluble in the bacterial cytoplasm. Fragment IΔ1−22 formed inclusion bodies and was converted into a soluble form by dissolving the inclusion bodies in 6 M guanidine hydrochloride and by subsequently removing the denaturant by dialysis. The intact bovine IF_1 and the truncated forms were all purified by chromatography, as described in Materials and Methods (Figure 2). Fragment I1−56 was obtained as a byproduct of the purification of fragment I1−60, presumably having arisen by proteolytic removal of amino acids 57−60, either in the bacterium, or

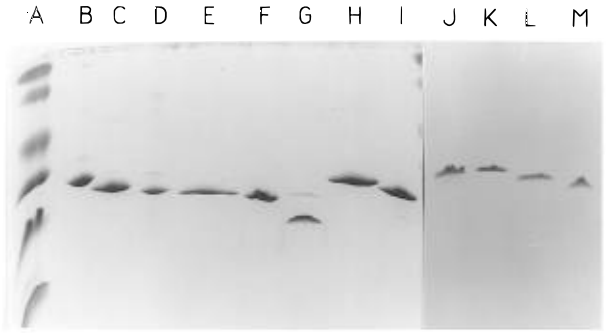

FIGURE 2: Analysis of purified recombinant bovine IF_1 and fragments of it. Approximately 1 μg of each peptide was analyzed by SDS−PAGE (Schägger & von Jagow, 1987). Lanes: (A) marker proteins, molecular weights 42, 30, 17, 13, 8, and 3 kDa; (B) IF_1; (C) IΔ1−9; (D) IΔ1−13; (E) IΔ1−17; (F) IΔ1−22; (G) IΔ1−43; (H) I1−78; (I) I1−74; (J) I1−69; (K) I1−64; (L) I1−60; (M) I1−56. Lanes A−I and J−M are from separate gels.

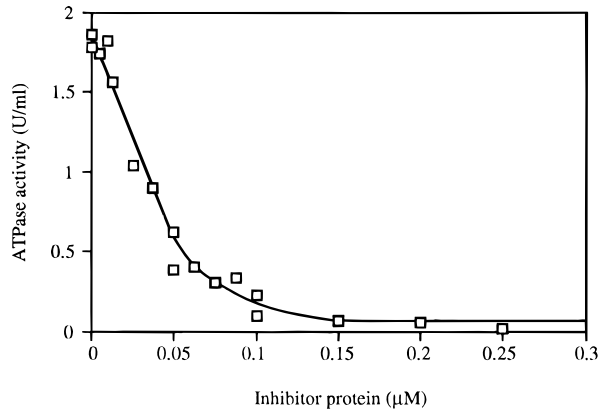

FIGURE 3: Inhibition assay of IF_1. Inhibitor-free particles were incubated at 37 °C and pH 6.5 with IF_1 at the indicated concentrations and Mg·ATP. A portion was withdrawn, and its ATPase activity (1 U of ATPase activity hydrolyzes 1 μmol of ATP/min) was determined in the presence of an ATP regenerating system. For further details see Materials and Methods.

during isolation. Expression vectors for fragments I1−45, I1−50, and I1−55 failed to yield the desired proteins, possibly because of proteolytic degradation in the bacteria. Therefore, the fragments I1−45, I10−40, I10−45, I10−46, I10−47, I10−48, I10−50, I22−46, and I1−22 were all obtained by chemical synthesis.

The N-terminal sequences of all of the proteins produced by bacterial expression lacked the translational initiatior methionine, and in all cases, the expected sequences were observed. The masses of the expressed proteins and of the chemically synthesized peptides determined by ESI-MS were the same as the calculated values.

Inhibitory Activities of the Recombinant IF_1 and Truncated Forms. The bovine IF_1 and truncated forms were assayed for inhibition of ATP synthase using IF_1-depleted submitochondrial particles, as this assay approximates more closely the natural situation than the alternative assay using F_1-ATPase. The recombinant protein had a specific activity of 26 000 U/mg (see Figure 3) which can be compared with values of 2610 and 41 000 U/mg determined with samples of IF_1 isolated from bovine mitochondria (Pullman & Monroy, 1963; Horstman & Racker, 1970). The activities of IF_1 purified from rat mitochondria and buffalo hearts were both 5000 U/mg (Cintrón & Pedersen, 1979; Sah et al., 1993), and recombinant rat IF_1 had a similar activity to the material isolated from rat liver (Lebowitz & Pedersen, 1993). The recombinant rat IF_1 was expressed as a fusion protein with maltose binding protein and then cleaved with factor Xa at the fusion junction.

Table 2: Inhibitory Activities, Estimated α-Helical Contents, Hydrodynamic Radii, Polydispersities, and Estimated Molecular Weights of IF_1 from Bovine Mitochondria and of Fragments Derived from It[a]

peptide	$C_{50\%}$[b] (μM)	f_H[c]	R_H[d] (nm)	MW[e] (kDa)	polydispersity[f] (%)
IF_1	0.034	1.37	4.2	100	M
IΔ1−9	0.035	1.62	4.9	137	M
IΔ1−13	0.018[g]	0.82	3.6	66	26.3
IΔ1−17	1.3[g]	0.60	4.4	106	M
IΔ1−22	>100	1.68	3.3	56	25.1
IΔ1−43	>100	1.58	2.3	22	15.4
I1−22	>100	0.12	0.78	2.0	39.7
I1−45	4.5[g]	0.22	ND[h]		
I1−56	0.032	0.27	2.1	18	B
I1−60	0.019	0.53	ND[h]		
I1−64	0.024	0.79	4.3	106	39.5
I1−69	0.022	0.38	4.0	91	37.5
I1−74	0.030	0.99	3.8	78	M
I1−78	0.028	1.32	3.5	61	M
I10−40	>100	0.16	1.2	4.2	B
I10−45	38[g]	0.25	1.2	4.8	M
I10−46	0.35[g]	0.35	1.1	4.3	50.0
I10−47	0.045[g]	0.51	1.1	3.8	B
I10−48	0.050[g]	0.15	1.6	9.0	M
I10−50	0.055[g]	0.37	1.3	6.2	53.8
I22−46	>100[g]	0.21	1.4	7.3	54.3

[a] The inhibitory activities were determined from ATPase assays; α-helical contents were calculated from CD spectra; hydrodynamic radii, molecular weights and polydispersities were determined by dynamic light scattering (see Materials and Methods). [b] Concentration at which 50% inhibition was observed. [c] Fractional α-helical content. [d] Hydrodynamic radius. [e] Estimated molecular weight. [f] M: sample has a polydispersity smaller than 15% of hydrodynamic radius and can be considered monodisperse. B: sample also contains large aggregates (>500 kDa), which could be resolved from the monomeric species only by bimodal regression. When bimodal regression is used, the polydispersity of the two components cannot be determined. [g] ATPase activity deviates from linearity and increases with time. [h] ND, not determined.

Of the N-terminally deleted mutants of IF_1 that were investigated, IΔ1−9 was fully active (see Table 2). Fragment IΔ1−13 also had activity similar to that of the complete IF_1. However, the course of ATPase inhibition by this fragment was nonlinear, and the ATPase activity increased during the assay period, presumably because the peptide was dissociating from F_1F_o-ATPase. Fragment IΔ1−17 had some residual inhibitory activity, but less than fragment IΔ1−13, and fragment IΔ1−22 was completely inactive. Therefore, it appears that only N-terminal amino acids 1−9 of the bovine IF_1 can be deleted without effect on inhibitory activity and that progressive loss of activity ensues in more extensive N-terminal deletions up to residue 22, when no activity remains. The activity of IΔ1−22 could not be restored by adding the deleted fragment, I1−22, as a separate peptide (data not shown). A previous study, in which parts of the N-terminus of IF_1 were deleted by proteolytic digestion, demonstrated inhibitory activity for peptides corresponding to IΔ1−9 and IΔ1−16 and found that IΔ1−22 and IΔ1−33 were inactive (Dianoux et al., 1982). These results are consistent with those reported here.

Assay of a series of C-terminally deleted IF_1 molecules showed that forms lacking 6, 10, 15, 20, 24, and 28 amino

acids from the C-terminus (I1−78, I1−74, I1−69, I1−64, I1−60, and I1−56, respectively) were all fully active (Table 2), whereas a form lacking the C-terminal 39 amino acids (I1−45) was only slightly active. A second series lacking amino acids 1−9, which had already been shown to be dispensable, and additionally lacking 34, 36, 37, 38, 39, and 44 amino acids from the C-terminus (I10−50, I10−48, I10−47, I10−46, I10−45, and I10−40, respectively) was performed to investigate the necessity for inhibition of the region between residues 45 and 56. The peptides I10−47, I10-48, and I10−50 still had inhibitory activity, although around 5-fold less than the complete IF_1. With these peptides, the course of the ATPase activity deviated from nonlinearity and increased with time, in a similar way as observed for IΔ1−13. Fragment I1−56 contains at positions 47, 48, 55, and 56 four of the five histidine residues that are present in the intact IF_1. These residues are not absolutely necessary for the inhibitory activity, but appear to increase the stability of the IF_1•F_1F_o-ATPase complex at higher pH. The peptides I10−46 and I10−45 showed a further decrease in inhibitory activity, with I10−45 being less active than I10−46. No inhibitory activity was detected with I10−40. Therefore, the minimal inhibitory peptide determined with the assay used in this work consists of amino acids 14−47 of the bovine IF_1. Some residues directly around this region (10−13 and 48−56) appear to stabilize the IF_1•F_1F_o-ATPase complex at higher pH. The sequence I14−47 contains all but four of the conserved residues in ATPase inhibitor proteins of known sequence (see Figure 5). Of these inhibitors, it is known that both the rat (Cintrón & Pedersen, 1979) and *Candida utilis* (Satre et al., 1975) inhibitors inhibit bovine F_1F_o-ATPase. In view of the high conservation of sequence between the two yeast inhibitors, it is likely that the *Saccharomyces cerevisiae* protein also inhibits the bovine ATPase. The ATPase inhibitor from potato mitochondria does not inhibit the bovine ATPase (Polgreen et al., 1995), and therefore it has been omitted from the comparison.

Recently, a proteolytic fragment of bovine IF_1 containing residues 1−51 was reported to be an active inhibitor of bovine F_1-ATPase (Hashimoto et al., 1995). This report is consistent with our results. It has also been claimed that a peptide consisting of residues 22−46 of the bovine protein is a potent inhibitor of F_1-ATPase (Stout et al., 1993), but in the assays employed in the present work, no significant inhibitory activity was detected with this peptide (Table 2). It was noted that the ATPase activity was not linear and increased with time. It could be that the 22−46 peptide has some inhibitory activity at pH 6.5, but that it dissociates at the higher pH (8.0) of the assay.

Competition Experiments. Although some of the fragments of bovine IF_1 do not inhibit particulate ATPase, it is possible that they could still bind at the inhibitor site. Therefore, experiments were performed in which the ability of these peptides to compete with the full-length IF_1 was investigated. The N-terminal deletion mutant IΔ1−22 competed effectively (Figure 4), but when a further 21 amino acids were deleted, the resulting peptide IΔ1−43 did not compete. According to Hashimoto et al. (1995), peptides produced by proteolytic digestion lacking the N-terminal 45 or 51 residues do compete with the full-length IF_1 for its binding site on F_1-ATPase. The peptide I10−40 did compete, although not as strongly as fragment IΔ1−22. Fragment I22−46 did not compete with intact IF_1. These

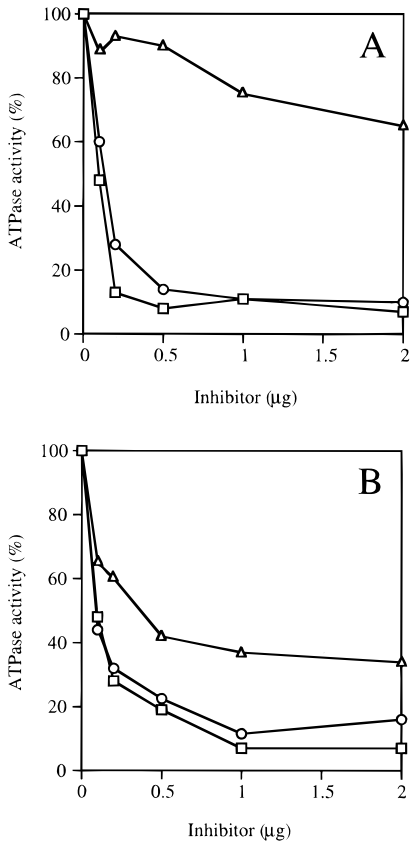

FIGURE 4: Competition of noninhibitory peptides with IF_1. The noninhibitory peptides IΔ1−22 and I10−40 compete with IF_1 for binding to F_1F_o-ATPase, but IΔ1−43 and I22−46 do not. (Part A) Squares, control experiment without competitor; triangles, 2 μg of IΔ1−22 added; circles, 2 μg of IΔ1−43 added. (Part B) Squares, control experiment without competitor; triangles, 2 μg of I10−40 added; circles, 2 μg of I22−46 added. Inhibitor-free submitochondrial particles were preincubated with competitor peptides and Mg•ATP; then IF_1 and another portion of Mg•ATP were added. The ATPase assay was performed as described in Materials and Methods.

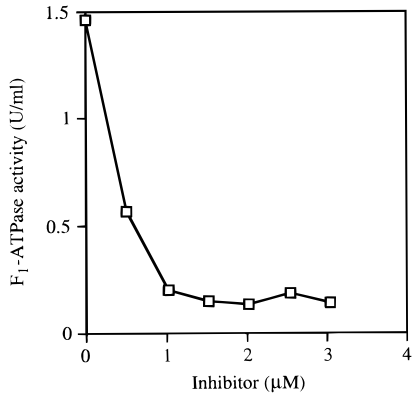

FIGURE 5: Inhibition of F_1-ATPase by IF_1. For assay conditions, see Materials and Methods. Instead of inhibitor-free submitochondrial particles, 12 μg (32 pmol) of F_1-ATPase was added to the reaction mixture.

experiments suggest that the C-terminal region of the minimal inhibitory sequence (23−47) is involved in binding the ATPase and that the region 10−22 contains other residues that are important for inhibition.

Inhibition of Soluble F_1-ATPase. The IF_1 also inhibits isolated F_1-ATPase (see Figure 5), but at higher concentrations than are necessary to inhibit the same amount of ATPase activity in the membrane bound enzyme. This is

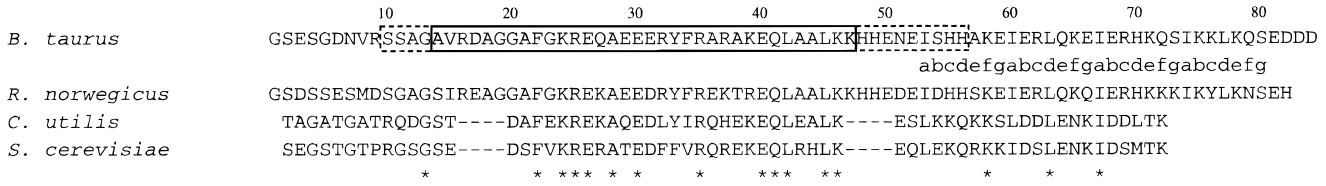

FIGURE 6: Alignment of the sequences of ATPase inhibitor proteins from various species. Asterisks denote amino acids that are identical in all sequences. Boxes contain the minimal bovine inhibitory sequence (residues 14−47, solid lines) and flanking residues which appear to be involved in stabilizing the complex formed between IF_1 and F_1F_o-ATPase (residues 10−13 and 48−56, dashed lines). Beneath the bovine sequence are shown positions a−g in heptad repeats that are predicted to be involved in formation of α-helical coiled-coils (see text). References: *Bos taurus*, Frangione et al. (1981); *Rattus norwegicus*, Lebowitz and Pedersen (1993); *Saccharomyces cerevisiae*, Matsubara et al. (1981); *Candida utilis*, Dianoux and Hoppe (1987).

partly due to the fact that soluble F_1-ATPase hydrolyzes ATP less efficiently than particulate F_1F_o-ATPase, but IF_1 also appears to bind to F_1-ATPase less effectively than to particulate F_1F_o-ATPase.

Secondary Structure of the Bovine IF_1 and Truncated Forms. The circular dichroism spectra of bovine IF_1 show that the protein is α-helical at a pH of 7.0. Its estimated helical fraction exceeds unity, which could be attributed to α-helical coiled-coil formation (Peters et al., 1995). It is unlikely to be due to nonspecific aggregation, because a similar value was observed at a 10-fold lower protein concentration (results not shown). Some of the fragments also show α-helicity to a varying degree (Table 2), and others appear to form random coils. The α-helicity and coiled-coil formation appears to be associated with the C-terminal part of IF_1, since deletion of the N-terminal 43 residues yields a peptide that has an apparent α-helical fraction greater than unity, but deletion of 10 or more residues from the C-terminus leads to fragments in which the estimated α-helical fraction is less than unity. The computer programs COILS (Lupas et al., 1991) and PAIRCOIL (Berger et al., 1995) both predict that amino acids 48−84 of the bovine inhibitor protein have a high probability (99% and 94%, respectively) of forming an α-helical coiled-coil. With the exceptions of amino acids 56 and 70, which are both histidine residues, hydrophobic amino acids are found in positions a and d of the heptad repeats that are predicted to be involved in coiled-coil formation (see Figure 6).

Fragment I10−50 has little α-helical structure in free solution, but it inhibits the ATPase effectively. Fragment I10−48 is also an active inhibitor, and it seems from NMR studies to be mainly random coil (D. Neuhaus, personal communication). Therefore, the inhibitory activities of the free IF_1 and fragments are not directly correlated with α-helical structure; some inactive mutants, such as IΔ1−22, are α-helical, whereas other peptides (I10−48 and I10−50) are active, but have mainly random coil structure. Presumably, they can still adopt the correct conformation when they bind to the ATPase and inhibit it.

Aggregation State of IF_1 and Related Peptides. Light scattering measurements show that IF_1 forms a monodisperse aggregate in solution (Table 2), as do gel filtration experiments (the molecular weight estimated by gel filtration at pH 6.7 was 66 kDa; results not shown). In addition, crystals of IF_1 have a large unit cell (dimensions $235 \times 92 \times 72$ Å^3, $\alpha = \beta = \gamma = 90°$, M.J.v.R. and J.E.W., unpublished results). The light scattering measurements suggest that the number of copies of IF_1 in this aggregate could be as high as 12, although an elongated α-helical coiled-coil of as few as 4 copies (Peters et al., 1995) could explain both the dynamic light scattering and gel filtration results. Except for I10−40, I10−46, I10−47, I10−48, and I10−50, most truncated forms are also aggregated; fragment I1−56 has intermediate behavior. Aggregation does not correlate with inhibitory activity, because aggregation occurs in both active (for instance, complete IF_1) and inactive peptides (IΔ1−22), and nonaggregated peptides can be both active (I10−50) or inactive (I10−40).

Perspectives for Solving the Atomic Structure of IF_1. Although crystals of bovine IF_1 have been grown (see above), they diffract X-rays anisotropically and to a resolution of only 3−4 Å. Furthermore, their large unit cell introduces additional complications for solving the crystal structure. Another crystal form has been obtained for the inactive peptide IΔ1−22, which in the present work has been shown to be able to bind to the F_1F_o-ATPase, but they also diffract weakly and only to 3.5 Å resolution (M.J.v.R. and J.E.W., unpublished results). Therefore, if the structure of IF_1 is to be determined by X-ray crystallography, it will be necessary either to improve the existing crystals or to grow a new crystal form. Because of their aggregation properties, neither the intact bovine IF_1 nor many of the active fragments appear to be suitable for structural analysis by NMR, and shorter peptides with inhibitory activity seem to adopt a predominantly random coil structure in solution. It may be possible to abolish the tendency of the intact IF_1 to form coiled-coils by the introduction of suitable mutations in the C-terminal region.

An alternative approach of determining the structure of IF_1 is to try to solve the structure of the F_1-ATPase·IF_1 complex. The structures of the bovine F_1-ATPase inhibited by aurovertin and by efrapeptin have been solved recently (van Raaij et al., 1996; Abrahams et al., 1996). They show that, as anticipated (Lardy et al., 1975), the two antibiotics bind at independent sites in the catalytic β-subunits. Neither site involves directly the region in the C-terminal part of the β-subunit where IF_1 is thought to interact, and therefore, the inhibitor protein appears to act by binding at a third inhibitory site.

ACKNOWLEDGMENT

We thank Drs. D. A. Harris and J. Featherstone for help and advice on the assay of IF_1, Mr. T. V. Smith, Mr. R. Grenfell, and Mrs. J. Fogg for synthesizing oligonucleotides, Drs. A. Johnson and D. Owen for synthesizing peptides, and Mrs. C. Sparks for performing amino acid analyses.

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BI960628F