Expression, Purification, and Mechanistic Studies of Bovine Mitochondrial Translational Initiation Factor 2*

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A complete cDNA clone encoding bovine mitochondrial translational initiation factor 2 (IF-2mt) has been obtained. The regions of the cDNA corresponding to mature IF-2mt and several of its functional domains have been expressed in Escherichia coli as histidine-tagged proteins. The precursor (~90 kDa) and mature (~85 kDa) forms of IF-2mt are toxic to E. coli and can only be expressed at low levels. Shorter forms of this factor (~80 and ~72 kDa) are also found during the expression of mature IF-2mt. The various forms of IF-2mt can be separated by high performance liquid chromatography. All of these forms are active in promoting the GTP-dependent binding of formyl-Met-tRNA to the small subunit of either E. coli or bovine mitochondrial ribosomes. IF-2mt can bind to mitochondrial ribosomes in the absence of GTP, initiator tRNA, or messenger RNA. The presence of GTP stimulates IF-2mt binding to ribosomes about 3-fold. IF-2mt interacts only weakly with GTP or with the initiator tRNA in the absence of ribosomes. Molecular dissection of IF-2mt shows that a long deletion (~150 amino acid residues) from the NH2-terminal region does not affect its activity in vitro. The COOH domain of IF-2mt (amino acid residues 332–727) can bind to ribosomes even though it does not promote initiator-tRNA binding.

The initiation of protein biosynthesis has been widely studied in the prokaryotic and eukaryotic cytoplasmic systems. But the process of translational initiation in mitochondria is poorly understood. The only initiation factor that has been identified to date in animal mitochondria is translational initiation factor 2 (IF-2mt) and many questions remain about how the initiation of protein biosynthesis occurs in this organelle (1). IF-2mt promotes the binding of the initiator tRNA to the 28 S ribosomal subunit in the presence of GTP and a mRNA (1). This factor belongs to the family of GTPases which are molecular switches capable of alternating between an active (IF-2mt-GTP) and an inactive (IF-2mt-GDP) conformation (2).

We have obtained a complete cDNA clone encoding bovine IF-2mt (3). The mature form of this protein is predicted to have 698 amino acid residues proceeded by a 29-amino acid mitochondrial import signal at the NH2 terminus. The mature form of bovine IF-2mt can be divided into three regions. The NH2-terminal region (Leu-30–Ser-180) is rich in charged amino acids. The function of this domain is unclear even for prokaryotic IF-2s. The middle region (Pro-181–Asn-330) encompasses the nucleotide binding domain (G-domain) and is quite homologous to the G-domain of analogous factors. The similarity between bovine IF-2mt and prokaryotic IF-2s decreases in the COOH-terminal region. Two unusual features are observed in the COOH-terminal half of bovine IF-2mt. First, the sequence between Asp-429 and Glu-512 has 60% charged residues and has the highest surface probability in the whole molecule. Second, there are 37 extra amino acid residues present in two clusters not found in any prokaryotic IF-2 (3). The DNAs encoding IF-2mt from yeast and humans have also been cloned and sequenced, although neither of these factors has been purified and no studies on their properties have been carried out (4, 5).

The identification of the important sites in IF-2mt responsible for the interaction with GTP, fMet-tRNA, and ribosomes is of particular interest. Studies with the IF-2s from prokaryotes have led to the identification of the GTP-binding site. However, little is known concerning other important regions of this protein. A six-domain model for the α form of Escherichia coli IF-2 (97 kDa) has been proposed (6). This model is based on data from DNA sequence analysis, protein sequence comparisons, patterns of proteolysis, and secondary structure predictions. The first three domains (residues 1–103, 104–155, and 156–391 of IF-2α) are in the NH2 terminus proceeding the GTP binding domain (domain IV, residues 392–540). Domain V (residues 541–671) and VI (residues 672–890) are defined by proteolytic cleavage patterns. Two structurally compact and functional domains have been observed in Bacillus steaetherophilus IF-2 (7). The G-domain corresponding to domains III–V in E. coli IF-2α (~41 kDa) contains the GTP binding site, the catalytic center for the GTP hydrolysis, and probably also has a site that interacts with the 30 S ribosomal subunit. The COOH-terminal domain (~24 kDa) corresponding to domain VI in the six-domain model of E. coli IF-2α (6) probably contains the fMet-tRNA binding site.

During the past several years, studies on structure/function relationships in IF-2mt have been hampered by the limited amount of the native protein that can be obtained. To circumvent this problem, the regions of the cDNA corresponding to mature IF-2mt and several of its functional domains have been subcloned and expressed in E. coli, and their properties have been investigated.

EXPERIMENTAL PROCEDURES

Materials

Oligonucleotide primers were made in the Lineberger Comprehensive Cancer Center at the University of North Carolina. Restriction endonucleases, the Klenow fragment of DNA polymerase I, T4 DNA

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¶The abbreviations used are: IF, initiation factor; IF-2mt, mitochondrial translational initiation factor 2; fMet, formylmethionine; IPTG, isopropyl-β-D-thiogalactopyranoside; BME, β-mercaptoethanol; NTA, nitritolactiactric acid, ECL, enhanced chemiluminescent; GMP-PNP, 5′-guanylylimidodiphosphate; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
ligase, and Taq DNA polymerase were obtained from Promega. Mini- and Midi-plasmid preparation kits, the expression vector pQE60, E. coli M15(pREP4), and Ni-iron charged nitriotratic acid affinity resin (Ni-NTA) were obtained from Qiagen Inc. GeneClean was from Bio101. β-Mercaptoethanol (BME), bovine serum albumin, pyruvate kinase, phosphoenolpyruvate, poly(A, U, G), isopropyl-β-D-thiogalactopyranoside (IPTG), and 25% glutaraldehyde were purchased from Sigma. [3H]GDP and [35S]methionine were obtained from DuPont NEN. Pure nitricellulose blotting membranes BA85 were purchased from Schleicher & Schuell. The enhanced chemiluminescent kit (ECL), Hyperfilm-ECL, and goat anti-rabbit IgG (heavy + light) antibodies were obtained from Amersham Corp. Yeast tRNA and GMP-PNP were from Boehringer Mannheim. [35S]Met-tRNA<sup>Met</sup> was prepared from yeast tRNA<sup>Met</sup> as described previously (8). High performance liquid chromatography (HPLC) columns TSKgel DEAE-5PW and TSKgel SP-5PW were obtained from Beckman. Bovine mitochondrial 28 S ribosomal subunits were kindly provided by Huanshu Yang (Department of Chemistry, University of North Carolina). Digo- nitron-treated bovine mitochondrial ribosomes were prepared from 4 kg of fresh liver basically as described by Matthews et al. (9) with modifications as described previously (10). A partially purified preparation of IF-2<sub>m</sub> was prepared as described elsewhere (11).

Buffers

The buffers used are as follows: buffer JM1, 20 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM BME, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF); buffer JM2, 20 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM BME, 0.1 mM PMSF, 1 mM KCl, and 10 mM imidazole, pH 7.6; buffer JM3, 20 mM HEPES-KOH, pH 7.6, 10 mM MgCl<sub>2</sub>, 6 mM BME, 50 mM KCl, and 10% glycerol; buffer TGM, 25 mM Tris-Base, 192 mM glycine, and 20% methanol; buffer TBST, 10 mM Tris-HCl, pH 8.0, 0.17 M NaCl, and 0.04% Tween-20; buffer LD, 12.5 mM Tris-HCl, pH 6.8, 0.4% SDS, 2% glycerol, 1% BME, and 0.1% bromophenol blue.

Construction of Expression Clones

The Qiagen pQE60 vector was used to express His-tagged forms of bovine IF-2<sub>m</sub>. Before subcloning bovine IF-2<sub>m</sub> cDNA into pQE60, the BglII site within bovine IF-2<sub>m</sub> cDNA was eliminated by polymerase chain reaction-directed mutagenesis without changing the encoded amino acid residues (12). The mutated clone (pTZBIF2<sub>m</sub>) carrying the complete bovine IF-2<sub>m</sub> coding sequence was cloned into Ncol/NtII cut pQE60. This DNA was used as the template to prepare the expression constructs of bovine IF-2<sub>m</sub>. The regions of bovine IF-2<sub>m</sub> to be expressed were amplified by polymerase chain reaction and subcloned into pQE60 using Ncol and BglII restriction sites. The recombinant plasmids were transformed into E. coli M15(pREP4). Positive clones were identified by hybridization using the full-length bovine IF-2<sub>m</sub> as the probe and DNA sequencing.

Expression of Mature Bovine IF-2<sub>m</sub> and Its Functional Domains in E. coli

Cells were grown at 37 °C overnight in 5 ml of Luria-Bertani broth for small scale experiments or in 50 ml of Luria-Bertani broth for large scale experiments. The overnight culture (0.125 volume for small scale or 0.02 volume for large scale experiments) was transferred into fresh medium and incubated at 37 °C with vigorous shaking (around 250 rpm) until late-log phase (OD<sub>600</sub> was 1.0–1.2). For large scale preparations (1–6 liters), cells were harvested by centrifugation at 10,000 rpm in a H-6000A rotor at 4°C. The cell pellets were then fast frozen and stored at −70°C.

The level of expression following induction was evaluated by one-step purification procedure under denaturing conditions according to the protocol provided by Qiagen, Inc. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5, 10, or 12% gels, and proteins present in the gel were visualized by silver staining (13).

Purification of the Expressed Forms of Bovine IF-2<sub>m</sub> and the GC-domain

Ni-NTA Affinity Chromatography—Cells were ground with twice the cell weight of Alumina type A-5 for 10 min at 0 °C. The cell paste was resuspended in 2 volumes of buffer JM1 and subjected to centrifugation at 10,000 rpm for 15 min at 4°C in a SS34 rotor. DNA was added to a final concentration of 5 μg/ml, and the sample was subjected to centrifugation at 30,000 × g for 40 min at 4°C in a SS34 rotor. NH<sub>4</sub>Cl was added to a final concentration of 1 M to the supernatant (S30, supernatant obtained following the 30,000 × g centrifugation step), and the sample was incubated for 15 min at 4°C. The supernatant was mixed for 40 min at 4°C with a 50% slurry of Ni-NTA agarose resin which had been equilibrated in buffer JM1 (0.5 ml of resin for each liter of cell culture). The mixture was subjected to centrifugation at 30,000 × g for 4 min. The supernatant was removed, and the resin was resuspended in 30 ml of buffer JM2. The resin was transferred to a column and washed by buffer JM2 at a flow rate 5 ml/min until the A<sub>280</sub> was less than 0.05. The retained protein was then eluted with Buffer JM3 until the A<sub>280</sub> was less than 0.05. The sample was dialyzed immediately against a 100-fold excess of buffer JM4 for 1.5 h. The samples were fast frozen and stored at −70 °C.

TSKgel DEAE-5PW Chromatography—The dialyzed sample (6.7 mg for mature bovine IF-2<sub>m</sub> in 8 ml or 27.5 mg for the GC-domain in 14 ml) was applied to a TSKgel DEAE-5PW HPLC column (7.5 × 75 mm) equilibrated with buffer JM4 at a flow rate of 1.0 ml/min. The column was washed until the A<sub>280</sub> returned to baseline and then developed with a linear gradient (60 ml) from 0.05 to 0.25 M KCl in buffer JM4 at a flow rate 0.5 ml/min. Fractions (0.6 ml) were collected in Eppendorf tubes, fast frozen, and stored at −70 °C.

TSKgel SP-5PW Chromatography—The TSKgel DEAE-5PW-purified material was dialyzed against 50–100-fold excess of buffer JM4 for 1.5 h. The dialyzed sample (1.7 mg in the IF-2<sub>m</sub> preparation or 7.7 mg in the GC-domain preparation) was applied to a TSKgel DEAE-5PW HPLC column (7.5 × 75 mm) equilibrated with buffer JM4 at a flow rate of 1.0 ml/min. The column was washed until the A<sub>280</sub> returned to baseline and then developed with a linear gradient (80 ml) from 0.05 M to 0.32 M KCl for mature IF-2<sub>α</sub>, or with a gradient (40 ml) from 0.08 to 0.2 M KCl for the GC-domain in buffer JM4 at a flow rate 0.5 ml/min. Fractions (0.5 ml) were collected in Eppendorf tubes. Aliquots were removed for assays and analysis by SDS-PAGE. The remainder of each fraction was fast frozen and stored at −70 °C.

During purification, protein concentrations were determined by the Bio-Rad protein assay kit using bovine serum albumin as a standard. The NH<sub>4</sub>-terminal sequence of several purified forms of expressed IF-2<sub>m</sub> was determined using the Applied Biosystems 477A protein sequencer with the 120A parathyroid hormone analyzer by Mary Moyer and William Burkhart (Glaxo Research Institute, Research Triangle Park, NC). About 100 pmol of the purified protein was provided for protein sequencing.

Production of Polyclonal Antibody against the GC-domain of Bovine IF-2<sub>m</sub>

Antibodies were prepared by immunizing two New Zealand rabbits with 200 μg of the GC-domain using the adjuvant RIBI. After 3 weeks, an additional 200 μg of the GC-domain was injected, and blood was collected approximately 1 month after the second injection. The serum was aliquoted and stored at −20 °C.

Western Blotting

Protein samples were subjected to SDS-PAGE according to Laemmli (14) on mini slab gels (80 × 75 × 0.75) containing either 7.5, 10, or 12% acrylamide and 0.4% bisacrylamide. Upon completion of electrophoresis, the proteins were electrothermally transferred to nitrocellulose filters in prechilled buffer TGM using a Bio-Rad Trans-Blot transfer cell according to the manufacturer's suggested protocol at 50 V/0.35 A for 2–3 h. Filters were cut with buffer TGS and washed with dry milk. The blots were washed three times with buffer TBST and incubated with a 1:20,000 or 1:40,000 dilution of polyclonal antibody to the GC-domain for 1 h at room temperature. The blots were washed three times with buffer TBST and incubated with a 1:5,000 dilution of goat anti-rabbit IgG (H + L) antibodies coupled to horseradish peroxidase for 45 min. Filters were washed again with three changes of fresh TBST, developed with the enhanced chemiluminescent (ECL) detection system (Amersham Corp.) according the manufacturer's instruction, and exposed to film for various time intervals.
Amounts of bovine IF-2mt. One unit is defined as the amount of IF-2mt using 1 unit of precursor and mature forms of IF-2mt, the NG-, G-, GC-, and C-domains of bovine IF-2mt as indicated. The numbers indicate amino acid residues.

**Airfuge Centrifugation**

Airfuge analysis was performed to measure the binding of IF-2mt and its domains to bovine mitochondrial ribosomes. Ribosome binding reactions (400 μl), unless otherwise indicated, contained 50 mM Tris-HCl, pH 7.6, 0.1 mM spermine, 1 mM dithiothreitol, 35 mM KCl, 7.5 mM MgCl₂, 40 μg of poly(A,U,G), 1.5 A₅₅₀ (1 A₅₅₀ = 32 pmol) mitochondrial ribosomes, 24 pmol of [³⁵S]Met-tRNA, 0.4 mM GMP-PNP, and the indicated amount of IF-2mt, the GC- or the C-domain. Samples were incubated at 27 °C for 10 min. A portion of the binding reaction (350 μl) was subjected to centrifugation at 30 p.s.i. (134,000 g) for 70 min in a Beckman air-driven Ultrafuge using an A-95 rotor at room temperature. The remainder of the reaction (50 μl) was incubated at room temperature for the duration of the Airfuge centrifugation. Reaction mixtures were then filtered through nitrocellulose membranes, dried, and counted (8). Following Airfuge centrifugation, the supernatant was completely removed, and the resultant pellets were resuspended in 30 μl of buffer LD. An aliquot of 5 μl was removed to determine the amount of radioactivity present (when applicable), and 12.5 μl of the remaining sample was analyzed by Western blotting as described above.

**Assays of Bovine IF-2mt Activity**

The activity of IF-2mt was determined by measuring the ability of this factor to promote the binding of Met-tRNA to mitochondrial ribosomes basically as described previously (1). Assays of the activity of IF-2mt on E. coli ribosomes were carried out as described previously (8) using 1 unit of E. coli IF-1, 2 units of E. coli IF-3, and the indicated amounts of bovine IF-2mt. One unit is defined as the amount of IF-2mt required to promote the binding of 1 pmol of [³⁵S]Met-tRNA to ribosomes (1).

**RESULTS AND DISCUSSION**

**Expression of Bovine IF-2mt and Its Domains**—The Qiagen pQE60 vector was used to express bovine IF-2mt, and its domains as histidine-tagged proteins. All of the expressed proteins have an extra Met at their NH₂ terminals and Arg-Ser-Numbers. The regions of IF-2mt corresponding to the six domains of E. coli IF-2α have been labeled from II to VI. Constructs were prepared including the precursor and mature forms of IF-2mt, the NG-, G-, GC-, and C-domains of bovine IF-2mt as indicated. The numbers indicate amino acid residues.

In E. coli was determined by comparing the time courses of cell growth with and without IPTG induction. Both the precursor and mature forms of IF-2mt are toxic to E. coli and cells stop growing within 30 min following induction (data not shown). The expression of the NG-, G-, GC-, and C-domains was considerably less toxic to E. coli and cells continued to grow following induction (data not shown). The level of expression of IF-2mt or its domains following induction was evaluated by a one-step purification procedure on a Ni-NTA affinity resin following the preparation of cell extracts under denaturing conditions. The effect of the time of induction on the yield of the expressed proteins was determined. Overall, the yields of the expressed proteins increase with time (data not shown). Low concentrations of IPTG were found to give higher yields of mature IF-2mt (data not shown). The yield of the GC-domain of IF-2mt was about 3–4-fold higher than that of IF-2mt. Thus, the GC-domain was purified and used to prepare antibodies to facilitate the identification of the expressed products. These antibodies do not recognize E. coli IF-2 (data not shown).

When the precursor of IF-2mt is expressed, a faint band designated by a small arrow with the expected molecular mass (90 kDa) is observed on SDS-PAGE (Fig. 2A). This band reacts strongly with antibodies raised against the GC-domain of IF-2mt (Fig. 2B, lane 1). However, the major form seen following induction of the precursor of IF-2mt migrates at 80 kDa. This band reacts with antibodies prepared against the GC-domain of IF-2mt (Fig. 2B, lane 1). A significant amount of a form migrat-
ing at 72 kDa can be observed especially at longer times of induction. These observations suggest that the precursor form of IF-2mt is subjected to proteolysis in the cell or that an internal start site in the gene is being used.

When the mature form of IF-2mt is expressed, three major bands of protein are observed (Fig. 2A). The highest molecular mass form (indicated by a small arrow) migrates at 85 kDa, the same apparent molecular mass as IF-2mt purified from liver. Shorter forms of IF-2mt migrate at 80 kDa and 72 kDa on SDS-PAGE. These bands react with the antibody prepared against the GC-domain of IF-2mt (Fig. 2B, lane 2). When the clone encoding the mature form was expressed for 1 h, two major bands were observed corresponding to the mature form (85 kDa) and to the 80-kDa form observed in cell extracts prepared under denaturing conditions. The 72-kDa form of IF-2mt was present only in small amounts after a 1-h induction. When IF-2mt was purified by Ni-NTA affinity chromatography under native conditions, most of the mature form of this factor was degraded into the species migrating at 72 kDa or into even shorter fragments (Fig. 2A, lane 3), suggesting that it was being degraded by proteases in the cell extract.

The Ng-, G-, and C-domains were also expressed in E. coli. Their apparent sizes on a 12% SDS-PAGE gel are 38, 25, 69, and 52 kDa, respectively (Fig. 2A). The apparent molecular masses of these expressed proteins on SDS-PAGE appear to be ~5 kDa larger than the calculated value presumably partially due to the His tag. A shorter form of the Ng-domain (33 kDa) was also observed (Fig. 2A). Some degradation products were also found when the G-, GC-, and C-domain were expressed.

The antisera prepared against the GC-domain of IF-2mt reacted with the precursor and mature forms of IF-2mt (Fig. 2B), the GC-domain and the C-domain (Fig. 2C). However, the antibodies did not react with the Ng-domain or the G-domain (Fig. 2C). Therefore, the epitopes of IF-2mt appear to be concentrated in the COOH-terminal region of this protein. This observation is not surprising since the sequence of bovine IF-2mt between Asp-429 and Glu-512 has 60% charged residues and has the highest surface probability in the whole molecule.

**Purification of the Expressed Forms of IF-2mt and the GC-domain**—In order to obtain active IF-2mt, strategies for the purification of IF-2mt and the GC-domain under native conditions were designed using a combination of Ni-NTA affinity chromatography and HPLC. Cell extracts were prepared and adjusted to 1 M NH4Cl to release any IF-2mt associated with the ribosomes. This step was important since IF-2mt was found both in the postribosomal supernatant and associated with ribosomes when extracts were prepared under native conditions in low salt. A similar observation has been reported with *E. coli* IF-2 (15). The expressed IF-2mt was purified by Ni-NTA affinity chromatography. The recovery from the Ni-NTA column was set at 100% for convenience since the activity of

| Step       | Protein mg | Total units* | Specific activity units/mg | Overall recovery % | Purification fold |
|------------|------------|--------------|---------------------------|--------------------|------------------|
| S30        | 740        |              |                           |                    |                  |
| Ni-NTA     | 6.66       | 9400         | 1,410                     | 100b               | 120              |
| DEAE-5PW   | 1.72       | 5000         | 2,990                     | 53                 | 250              |
| SP-5PW:    |            |              |                           |                    |                  |
| IF-2mt_S1 (peak 1) | 0.15 | 1260         | 8,300                     | Total              | 1000             |
| IF-2mt_S2 (peak 2) | 0.15 | 970          | 6,500                     |                    |                  |
| IF-2mt_S3 (peak 4) | 0.024 | 230          | 9,600                     |                    |                  |
| IF-2mt_S4 (peak 8) | 0.026 | 530          | 20,000                    |                    |                  |

* 1 unit = the binding of 1 pmol of fMet-tRNA to bovine mitochondrial ribosomes.  
  b This value is set at 100% since input activity cannot be measured directly. The estimated fold purification in this step is indicated as 120-fold based on the amount of protein recovered.

**Table 1. Purification of expressed bovine IF-2mt**

**Fig. 3. Purification of various forms of bovine IF-2mt.** A, elution profile of bovine IF-2mt on Tskgel DEAE-5PW HPLC. The mature form of bovine IF-2mt, initially purified by Ni-NTA affinity chromatography was subjected to chromatography on a Tskgel DEAE-5PW column as described under “Experimental Procedures.” Aliquots (20 μl) of various fractions were tested for IF-2mt activity (■). The absorbance at 280 nm was monitored (solid line) with an ISCO UA-5 absorbance monitor on a 0.5 scale, and the column was developed with a salt gradient (dashed line). B, elution profile of bovine IF-2mt on Tskgel SP-5PW HPLC. Bovine IF-2mt purified by Tskgel DEAE-5PW chromatography was subjected to chromatography on a Tskgel SP-5PW column. Aliquots (20 μl) of various fractions were tested for IF-2mt activity (■). The absorbance at 280 nm was monitored (solid line) with an ISCO UA-5 absorbance monitor on a 0.1 scale and the column was developed with a salt gradient (dashed line). C, the purity of mature bovine IF-2mt and the GC-domain was analyzed by 10% SDS-PAGE. Lane 1, sample of the preparation obtained from Ni-NTA affinity chromatography (4.4 μg). Lane 2, sample from the Tskgel DEAE-5PW preparation (1.2 μg). Lanes 3–6, sample from peaks 1–4 of bovine IF-2mt obtained from the Tskgel SP-5PW column. Lane 3, IF-2mt_S1 (~0.4 μg); lane 4, IF-2mt_L (~0.4 μg); lane 5, IF-2mt_S2 (~0.1 μg); lane 6, IF-2mt_A (~0.2 μg). Lane 7, the purified GC-domain of IF-2mt (0.72 μg).
separated by this procedure (Fig. 3B). Analysis of the material in these peaks by SDS-PAGE indicated that peaks 1, 3, and 4 all contain the 72-kDa form of IF-2mt. These forms (designated IF-2mtS1, S2, and S3, respectively) have almost identical molecular masses on SDS-PAGE. The second peak contains the 80-kDa form of IF-2mt (IF-2mtL). The purity of all of these forms was estimated to be >85% (Fig. 3C, lanes 3–6). The overall purification procedure resulted in about a 1000-fold purification with a 32% yield of the initial IF-2mt activity. All of the purification procedure resulted in about a 1000-fold purification.

AUG codon and the putative Shine-Dalgarno sequence is long-stream of an AUG codon. The distance between the Met-106 has a potential Shine-Dalgarno sequence 12 nucleotides upstream of an AUG codon. The specific activity of IF-2mtS3 was estimated to be 80-kDa form of IF-2mt (IF-2mtL). The purity of all of these forms seemed to be higher than that of the other purified forms. However, since the amount of IF-2mtS3 was very small, the protein concentration in the sample was difficult to determine accurately.

To clarify the relationship between the various forms of IF-2mt, purified proteins were subjected NH2-terminal sequence analysis (Table I). NH2-terminal analysis of the 80-kDa form (IF-2mtL) suggested that it arose from the use of the Met-106 codon as an internal start site (Table II). This codon has a potential Shine-Dalgarno sequence 12 nucleotides upstream of an AUG codon. The distance between the Met-106 AUG codon and the putative Shine-Dalgarno sequence is longer than usual (12 nucleotides as opposed to an average of 7 nucleotides 5’ to the start codon). However, the mRNA may lack secondary structure in this region allowing the 30 S subunit to bind and initiate. It has been proposed that efficient initiation in unstructured regions of mRNAs does not require a strong Shine-Dalgarno sequence (16).

It is also possible that IF-2mtL could arise from proteolysis since a form of E. coli IF-2, IF-2y (65 kDa), is found to be the result of cleavage of IF-2oa by the outer membrane protease OmpT (17). OmpT is an endoprotease associated with the outer membrane in E. coli K-12, which specifically hydrolyses peptide bonds between consecutive basic residues (Lys-Lys, Lys-Arg, Arg-Lys, and Arg-Arg) (18, 19). However, IF-2mtL was produced in similar amounts when expressed in either E. coli M15 or in E. coli BL-21(DE-3) (data not shown). The latter strain is deficient in the lon and OmpT proteases. These observations suggest that IF-2mtL is not the product of proteolysis in the cell.

The major species of the shorter forms of IF-2mt (IF-2mtS1) begins with the lysine at position 154 of the full-length amino acid sequence (Table II). The minor species, IF-2mtS2, begins with a methionine inserted in response to the Met-147 codon while IF-2mtS3 begins with a Met inserted in response to the Val-141 codon (Table II). IF-2mtS1 and IF-2mtS2 most likely result from proteolysis during purification or in the cell itself. However, since the amount of IF-2mtS3 was very small, the protein concentration in the sample was difficult to determine accurately.

The GC-domain was also purified under conditions similar to those used for IF-2mt. This derivative of IF-2mt was active in promoting fMet-tRNA binding to mitochondrial and E. coli ribosomes. Two mg of the GC-domain with a purity of >95% were obtained from about 24 g of induced E. coli cells after purification by the Ni-NTA affinity chromatography followed by anion and cation exchange chromatography on HPLC (Fig. 3C, lane 7).

Molecular Dissection of Bovine IF-2mt—According to the six-domain model of E. coli IF-2 (6), the native form of IF-2mt from bovine liver contains domains II–VI of E. coli IF-2a. The purified IF-2mtL (Met-106–Phe-727), IF-2mtS1 (Leu-154–Phe-727), IF-2mtS2 (Met-147–Phe-727) and IF-2mtS3 (Met-141–Phe-727) contain parts of domain III and complete copies of domains IV, V, and VI. The N-domain of IF-2mt contains domain II (Ala-30–Lys-72 in IF-2mt) and III (Lys-73–Arg-179 in IF-2mt) (Fig. 1). The G-domain corresponds to domain IV (Ser-180–Met-330) and the C-domain contains domains V (Met-331–Lys-461) and VI (Leu-462–Phe-727). The function of the NH2-terminal region of IF-2mt remains a mystery as does the role of this region in prokaryotic IF-2.

The activities of IF-2mtL, IF-2mtS1, and the GC-domain have been compared in more detail (Fig. 4). All of these proteins are active in promoting fMet-tRNA binding to mitochondrial 28 S ribosomal subunits, mitochondrial 55 S ribosomes and E. coli 70 S ribosomes. IF-2mtS1 has about the same activity as IF-2mtL. The GC-domain is 50% as active as IF-2mtS1. These results suggest that the 20 amino acid residues of IF-2mtS1

### Table II

| Name of protein | Amino Acid sequences | Possible origin |
|-----------------|----------------------|----------------|
| IF-2mtS1        | LKQDKVDRNKDA         | Proteolysis    |
| IF-2mtL         | MEKIDICVYVESL        | Internal start |
| IF-2mtS2        | MKLKWSKLQKQDKYR      | Proteolysis    |
| IF-2mtS3        | MIKKSMKLLKWSKL       | Internal start |
| GC-domain       | ALLIPRSVPVTIMGHDHGK   | Expressed as expected |
proceeding the GC-domain are somewhat important for the function of IF-2mt although the 20 amino acid residues are not conserved among different IF-2 s. The activities of IF-2mtS1, IF-2mtL, and the GC-domain have also been determined in the presence of either GTP or its nonhydrolyzable analog GMP-PNP. The activities of these factors decrease 2–3-fold in the presence of GMP-PNP, suggesting that all of these forms of IF-2mt can be recycled under the assay conditions used (data not shown).

Partially purified NG-, G-, and C-domains were not active in promoting fMet-tRNA binding to ribosomes indicating that both the G- and C-domains are essential for IF-2mt activity.

Binding of IF-2mt to Bovine Mitochondrial 55 S Ribosomes—To examine which regions of IF-2mt are responsible for the binding of this factor to mitochondrial 55 S ribosomes, initiation complexes were formed using IF-2mtS1, IF-2mtL, the GC-domain, and the partially purified C-domain in the presence of GMP-PNP. The initiation complexes were separated from unbound IF-2mt or its domains by Airfuge centrifugation. About 40% of the preformed initiation complexes could be recovered in the ribosomal pellets following this centrifugation step. The polyclonal antibodies against the GC-domain were then used to test for the presence of IF-2mt in ribosomal complexes using Western blots. As a control, reaction mixtures were incubated in the absence of IF-2mt and the 55 S ribosomes pelleted by Airfuge centrifugation. As indicated in Fig. 5 (lane 1), no cross-reaction was observed between the antibodies and ribosomal proteins indicating that the procedure can be used to detect IF-2mt in appropriate ribosomal complexes. Various derivatives of IF-2mt were then examined. As indicated in Fig. 5, IF-2mtS1 (lane 2), IF-2mtL (lane 3), the GC-domain (lane 4) were all capable of binding to the ribosome. This observation is not surprising since all of these forms of IF-2mt can promote fMet-tRNA binding to ribosomes.

Interestingly the C-domain of IF-2mt clearly bound to 55 S ribosomes (Fig. 5, lane 5), although it did not promote fMet-tRNA binding to the ribosomes. Gualerzi et al. (20) have proposed that a portion of the C-domain (24 kDa) of B. stearothermophilus IF-2 corresponding to domain VI of E. coli IF-2mt contains the fMet-tRNA binding site, but has negligible binding to ribosomes. The C-domain of IF-2mt under study here contains both domains V and VI of E. coli IF-2mt. Therefore, the region corresponding to domain V (Met-331–Lys-461) may contain the ribosome binding site.

The roles of guanine nucleotides, fMet-tRNA, and poly(A,U,G) in the binding of IF-2mt to ribosomes was examined again using Airfuge centrifugation. As indicated in Fig. 6A (lane 1), a significant amount of IF-2mtL could be detected bound to ribosomes in the absence of other components of the initiation machinery. The presence of the GTP analog, GMP-PNP, enhanced the binding of IF-2mt to the ribosomes about 3-fold (Fig. 6A, lane 2). No further enhancement of binding was observed upon the addition of fMet-tRNA and poly(A,U,G) (compare lanes 2 and 3). These observations suggest that IF-
2\textsubscript{mt} has an intrinsic affinity for the ribosome and that this affinity is enhanced by the presence of the GTP analog.

The effects of GDP, GTP, and GMP-PNP on the binding of IF-2\textsubscript{mt} to 55 S ribosomes were then determined. Again, the binding of IF-2\textsubscript{mt} could be detected in the presence of added nucleotide (Fig. 6B, lane 1). A substantial enhancement of IF-2\textsubscript{mt} binding to ribosomes was, once again, observed with GMP-PNP (Fig. 6B, lane 2). GTP also strongly enhanced the binding of this factor to ribosomes (lane 3). Surprisingly, enhanced binding of IF-2\textsubscript{mt} to ribosomes was also observed in the presence of GDP although less binding was observed in the presence of GDP than in the presence of GTP (Fig. 6B, lane 4). GTP hydrolysis is thought to facilitate the release of IF-2 from the ribosome (21). The formation of the IF-2\textsubscript{mt}-GDP complex on the ribosome following GTP hydrolysis may weaken the interaction of IF-2\textsubscript{mt} with the ribosome sufficiently to result in the dissociation of the IF-2\textsubscript{mt}-GDP complex. Alternatively, the dissociation of GDP from the IF-2\textsubscript{mt}-GDP complex while still on the ribosome could result in a conformational change on IF-2\textsubscript{mt} that further weakens its interaction with the ribosome and IF-2\textsubscript{mt} would then dissociate.

It has been reported that E. coli IF-2 binds to ribosomal particles with decreasing affinity: 30 > 70 > 50 S (22). GTP and GDP have no effect on the binding of E. coli IF-2 to 70 S ribosomes (23). GTP stimulates the binding of this factor to the 30 S subunit and somewhat decreases its binding to 50 S subunits; GDP has the opposite effect. These results, and the data presented here, suggest that the dissociation of GDP from IF-2, while the factor is still on the ribosome, results in a conformation with a lower affinity for the ribosome, thus, promoting the release of the factor.

The Interaction of IF-2\textsubscript{mt} with GTP and fMet-tRNA—In order to understand the role of IF-2\textsubscript{mt} in the initiation cycle of protein synthesis, an effort was made to determine the binding constants of IF-2\textsubscript{mt} for guanine nucleotides and fMet-tRNA in the absence of ribosomes. The binding of GDP and fMet-tRNA to IF-2\textsubscript{mt} has been tested using a direct nitrocellulose binding assay based on methods used with E. coli IF-2 (22). The $K_a$ for the formation of an IF-2\textsubscript{mt}-GDP complex was too low (<10$^5$ M$^{-1}$) to be measured accurately by the filter binding assay. According to Pon et al. (22), the apparent $K_a$ (at 25 °C) for the formation of the E. coli IF-2-GDP and IF-2-GTP complexes are 8.0 $\times$ 10$^5$ and 7.0 $\times$ 10$^3$ M$^{-1}$, respectively.

The formation of an IF-2\textsubscript{mt}-fMet-tRNA complex was also weak. The $K_a$ for the formation of a binary complex (IF-2\textsubscript{mt}-fMet-tRNA) was estimated to be about 10$^5$–10$^6$ M$^{-1}$ (data not shown). The formation of this complex was inhibited by Mg$^{2+}$ suggesting that it does not play a physiological role in initiation. GTP did not affect the formation of the IF-2\textsubscript{mt}-fMet-tRNA complex. Hence, a ternary IF-2\textsubscript{mt}-fMet-tRNA-GTP complex does not appear to play a role in initiation in the animal mitochondrial system. These data and the results of the Airfuge centrifugation studies described above suggest that IF-2\textsubscript{mt} binds to mitochondrial ribosomes prior to its interaction with the initiator tRNA or GTP.

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