

Short communication

The I-complex in *Leishmania tarentolae* is an uniquely-structured F_1 -ATPase[☆]

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The enzymatic machinery for the gRNA-mediated U-insertion/deletion RNA editing of pre-mRNAs encoded by the maxicircle mitochondrial genome of kinetoplastid protozoa involves several high molecular weight complexes that interact via RNA. In *Leishmania tarentolae*, the core L-complex contains approximately 16 polypeptides including the two RNA ligases, REL1 and REL2 [1,2]. Initially, the L-complex was identified by labeling the component RNA ligases with $\alpha^{32}\text{P}$ -ATP. This labeled complex sedimented in a glycerol gradient from 20 to 25S and migrated in a native gel as a single band with an apparent molecular weight of approximately 1200–2000 kDa. A second more intensely labeled complex which sedimented at around 12S–15S and migrated in a native gel as a doublet band at an apparent molecular weight of approximately 700 kDa was termed the intermediate or I-complex [3]. We have shown that the I-complex could be labeled with $\alpha^{32}\text{P}$ -ATP in the absence of Mg^{2+} ; since Mg^{2+} was required for adenylation of the REL1 and REL2 RNA ligases, this indicated that I-complex labeling was not due to adenylation of REL1 and REL2 [2]. We have also shown that the labeling of the I-complex is not covalent since the label was released by 55 °C heat treatment of the gel and by exposure to low pH (data not shown); furthermore, extraction of the $\alpha^{32}\text{P}$ -ATP-labeled I-complex yielded predominately $\alpha^{32}\text{P}$ -ADP (not shown).

In order to further characterize the I-complex, it was purified from *L. tarentolae* mitochondrial extract by glycerol gra-

dient fractionation followed by cationic and anion exchange chromatography, and preparative native gel electrophoresis. The resulting six major stained bands (Fig. 1A) were eluted, digested with trypsin, and peptides sequenced by mass spectrometry. The peptide sequences allowed the identification of all six bands as components of the F_1F_0 -ATPase. Fig. 1B shows the localization of the *L. tarentolae* peptides within the amino acid sequences of the *Leishmania major* proteins. Five bands (α_N , α_c , β , γ , and δ subunits) are components of F_1 -ATPase and one band, subunit b (=p18) [4], is a component of F_0 -ATPase. The latter is a common contaminant of F_1 -ATPase purified in the absence of chloroform extraction [5]. All of these full length *L. major* conceptual sequences have highly significant predicted mitochondrial targeting sequences at their amino-termini (Fig. 1B).

The canonical H^+ F_1F_0 -ATP synthase is structured as a rotary motor, with a water-soluble F_1 portion extrinsic to the mitochondrial membrane and composed of five different subunits in a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$, and a membrane-embedded F_0 portion composed of at least three different subunits in a $a_b_2c_{9-12}$ ratio [6]. The crystal structure [7,8] of isolated F_1 -ATPase indicates that the α and β subunits are arranged alternately to form a hexagon with nucleotide-binding sites at the α/β interfaces; three of these are catalytic and principally associated with the β subunits and three are noncatalytic and associated with the α subunits. In addition, the α and β subunits, although only weakly homologous, are folded into almost identical structures consisting of three domains: the amino-terminal domain contains a 90-amino acid β -barrel structure that forms a crown for the top of the F_1 ; the central domain with nine α helices and nine-associated β strands contains the catalytic and noncatalytic

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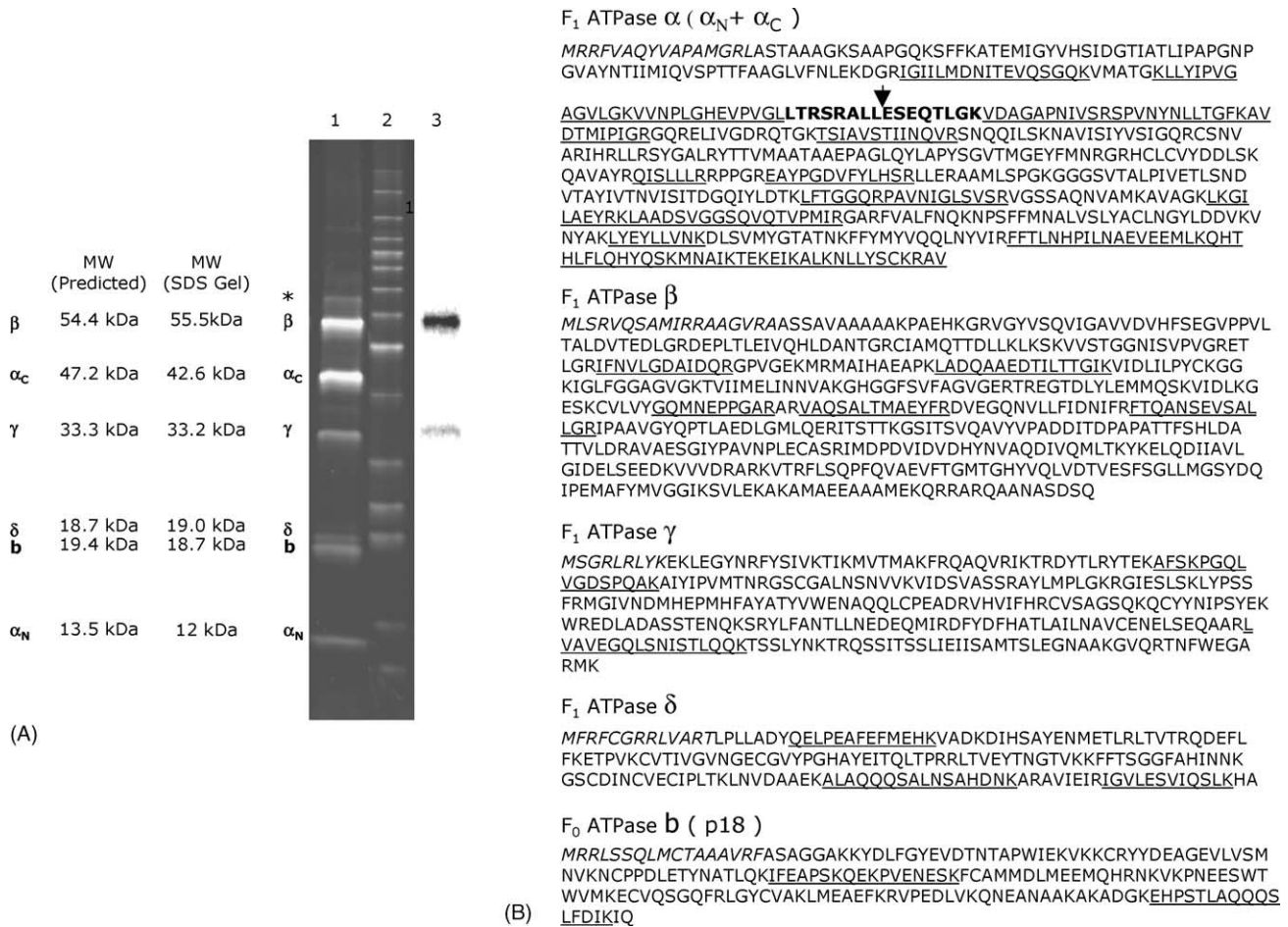


Fig. 1. Identification of the components of the I-complex. Extracts were prepared from 25 g (wet weight) of a Renografin density gradient-purified mitochondrial fraction [21] and fractionated by sedimentation in glycerol gradients. The I-complex containing fractions were identified by adenylation, pooled and adjusted to 50 mM HEPES, pH 7.5, 30 mM KCl, 0.5 mM Na₂EDTA, 1 mM DTT, 10% glycerol with protease inhibitors in a total volume of 50 ml. This was chromatographed through a 5 ml HiTrapTM SP HP column. After addition of CHAPS to 2 mM, the flow-through was concentrated by centrifugal filtration (UltraFree-15 5K, Millipore) to 2.5 ml and adjusted to 25 mM Tris-HCl, pH 7.5, 30 mM KCl, 0.5 mM Na₂EDTA, 1 mM DTT, and 10% glycerol, in a total volume of 10 ml. This was loaded on a 1 ml HiTrapTM Q HP column and fractionated by anionic exchange with a 22 ml, 60–600 mM KCl linear gradient. The I-complex (1 ml, 350 mM salt) was further purified by preparative native gel electrophoresis; after location by negative staining (Zinc Stain Kit, Bio-Rad), the portion of the gel containing the I-complex was crushed, extracted with 0.2% SDS and 40 mM DTT, and the extract fractionated by SDS gel electrophoresis. Polypeptide bands were located by colloidal blue staining (Novex). Mass spectrometric analysis of proteins was performed as described in detail previously [2]. Briefly, the protein spots of interest were excised and digested with trypsin *in situ*. The recovered peptides were purified using ν C18 ZipTips (Millipore). Mass spectrometric measurements were performed using an Applied Biosystems 4700 Proteomics Analyzer, which is a tandem time-of-flight instrument (TOF/TOF) with a MALDI ion source [22]. Peptide sequences were determined by manual interpretation of the MS/MS spectra. The inferred sequences were searched using Protein Prospector (UCSF) against the NCBI database, as well as the parasite genome databases (<http://www.genedb.org>). (A) Lane 1. Sypro Ruby-stained I-complex polypeptides. Lane 2. BenchmarkTM Protein Ladder (Invitrogen). Lane 3. Peak I-complex fraction after anionic exchange chromatography was incubated with α^{32} P-ATP, purified by native gel electrophoresis, and the I-complex located in the gel by brief autoradiography and irradiated with 254 nm UV for 4 min. Protein was eluted and fractionated in an SDS gel, which was subjected to autoradiography. (B) Alignment of peptide sequences determined by mass spectrometry of the *L. tarentolae* I-complex (underlined) with corresponding sequences in conceptual genes from the *L. major* genome project. The predicted mitochondrial signal peptides are in italics and the peptide sequences are underlined. The minor band labeled ‘*’ contains tryptic peptides for keratin, a common contaminate. The sequences labeled F₁-ATPase a ($\alpha_N + \alpha_C$), F₁-ATPase b, F₁-ATPase g, and F₀-ATPase b (p18) are from GeneDB, entries Q9BL30, LmjF25.1170c, Q9NE84, and Q25423, respectively. The F₁-ATPase d sequence was assembled from shotgun DNA reads in GeneDB.

nucleotide-binding sites; and the carboxy-terminal domain of 100 amino acids contains seven (α subunits) or six (β subunits) α helices. Finally, the first and the second, and the second and third domains are separated by interdomain sequences that lie on the surface of the folded structure.

It is possible that the ϵ subunit [9] and possibly the IF1 regulatory subunit of F₁-ATPase are also present in the pu-

rified I-complex as minor bands below 10 kDa but this was not analyzed experimentally. What makes it likely that IF1 (9 kDa in bovine ATPase) is also part of the I-complex is that the size of the complex, after treatment with CHAPS, remained stable at \sim 700 kDa throughout purification. This would indicate a dimeric rather than a monomeric organization of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ (see also [2]) and several studies have

shown that stable dimers are held together by a bridge of dimerized IF1 [10–12].

To determine which subunit was responsible for the non-covalently bound $\alpha^{32}\text{P}$ -ADP derived from the $\alpha^{32}\text{P}$ -ATP which was used to initially identify the I-complex, the purified I-complex was incubated with $\alpha^{32}\text{P}$ -ATP and then cross-linked with UV at 254 nm (Fig 1A, lane 3). The majority of the cross-link was in the β subunit, a small amount in the γ subunit, and an almost undetectable amount in the α subunit. Therefore, the ADP is bound to the catalytic β subunit rather than the noncatalytic α nucleotide-binding site in the F_1 -ATPase. We presume that the essential Mg^{2+} is derived from that which is bound during the initial extraction of the mitochondria and which remains bound during purification, since the purification and the $\alpha^{32}\text{P}$ -ATP labeling were accomplished without added Mg^{2+} [13].

What is of some interest about the *Leishmania* F_1 -ATPase is the apparent specific cleavage of the α subunit within the first interdomain region, yielding α_N , the N-terminal β -barrel domain, and α_C , the C-terminal region (Fig 1A, lane 1; Fig. 1B, $\alpha(\alpha_N + \alpha_C)$). Since the conceptual *L. major* α subunit is a continuous open reading frame with a single mitochondrial targeting sequence, this polypeptide must be synthesized in the cytoplasm, transported into the mitochondrion, and then cleaved into α_N and α_C . The cleavage site was localized between the carboxy terminus of the C-terminal-most tryptic peptide from α_N and the amino terminus of the N-terminal-most tryptic peptide from α_C , within the sequence, LTRSRALLESEQTLGK (Fig. 1B). The cleavage is most likely not due to proteolysis during the isolation since no peptides belonging to an uncleaved 55 kDa α subunit were detected.

Our observations extend those previously reported on the mitochondrial F_1 -ATPases from other trypanosomatid species [9,14–16]. A chloroform-purified F_1 -ATPase from *Trypanosoma brucei* contained five major polypeptides and several minor species. From the relative sizes, the major species were assigned to the five canonical ATPase subunits [16]. By direct amino-terminal sequencing, the largest two bands were determined to be β and what we refer to as α_C [14]; the other bands were not experimentally identified. The coding structure of the predicted *T. brucei* α subunit gene is very similar to that of the *L. major* gene, with a single mitochondrial targeting sequence followed by the complete α subunit sequence. The experimentally determined N-terminus of the *T. brucei* α_C polypeptide was within the region that we have determined for the cleavage site in the *Leishmania* protein. The α_N cleavage fragment was not identified in this study by Western analysis due to the use of an antibody specific for the C-terminal portion of the α subunit [14]. In fact, it was suggested that the α_N portion of the α subunit was degraded as part of an unusually large mitochondrial presequence [14]. Additional evidence for this cleavage occurring in vivo and not as an artifact of isolation was provided by the observation that the chloroform-purified F_1 -ATPase from *T. brucei* (in which

α is 100% cleaved) when reconstituted with F_1 -depleted inner membrane vesicles exhibited ATPase activity and oligomycin sensitivity and to a limited extent, ATP synthesis, [16]. Similarly, an F_1F_0 complex eluted from a native acrylamide gel of *Crithidia fasciculata* mitochondrial proteins had both oligomycin-sensitive ATPase activity and, apparently, a cleaved α subunit [9]. The retention of α_N (the β -barrel domain) in the assembled ATP synthase is not surprising given the abundant evidence that it is indispensable for assembly and function in other organisms [17–20].

Alignment of the conserved sequences from the α proteins of *L. major*, *T. brucei* and *C. fasciculata* suggest a localization of the cleavage site in the *L. major* protein as indicated below (/), but this must be experimentally confirmed.

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... PVGLLTRSRALL/ESEQTLGKVDAGAPNIVSRSP
   VNY... (L. major/L. tarentolae)
... PVGLVTRSRRL/DS-TLGKVDTGAPNIVSRSPV
   NY... (T. brucei)/DSEQVVGKVDAGAPNIVSRSPV
   GY... (C. fasciculata)
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The experimentally determined sequences are indicated by underlining.

It is likely that this site-specific cleavage of the α subunit is characteristic of all trypanosomatid species: in the plant pathogen, *Phytomonas serpens*, the largest F_1 -ATPase polypeptide was identified by amino-terminal sequencing as the β subunit, while an immunoreactive species the size of the *Leishmania* α_C could be identified with anti- F_1 -ATPase antibody as the second largest polypeptide [15] (D. Maslov, personal communication). The cleavage may be unique to trypanosomatid or kinetoplastid protists since a cleaved α subunit has never been reported for purified ATP synthases in other organisms. In fact a comparison of the α subunit sequences from 450 different organisms showed a unique short insertion sequence at the predicted cleavage site only in trypanosomatid sequences (see supplementary figure). We speculate that the biological role of this site-specific cleavage of the α - F_1 ATPase subunit may be to confer greater flexibility in the properties of the non-catalytic nucleotide binding site of the α subunit and thereby to regulate the activity of the ATP synthase.

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