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Short technical report

Identification of the mitochondrially encoded subunit 6 of F_1F_O ATPase in *Trypanosoma brucei*

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ABSTRACT

Kinetoplast maxicircle DNA of trypanosomatids encodes eighteen proteins. RNA editing is required to confer translatability to mRNA for twelve of these. Sequence conservation of the predicted hydrophobic polypeptides indicates that they represent functional components of the respiratory chain. Yet, so far only two of those, cytochrome *c* oxidase subunit I and apocytochrome *b* of cytochrome *c* reductase, have been identified with biochemical methods. Here we report on identification of A6 subunit of F_1F_O ATPase encoded by a pan-edited mRNA in *Trypanosoma brucei*. The polypeptide was present among the ^{35}S-labeled mitochondrial translation products characterized by anomalous migration in denaturing 2D gels. It was identified as an ATPase subunit by co-migration with this complex in Blue Native 2D gels. A partial N-terminal sequence of the corresponding polypeptide present in the gel-purified ATPase complex from *Leishmania tarentolae* was consistent with the predicted A6 sequence.

The trypanosomatid-type RNA editing is a complex and costly process which serves to produce translatable mRNA out of pre-edited genome-encoded sequences, with the end result of making a set of subunits for mitochondrial respiratory complexes and one ribosomal protein [1]. The extent of the required editing varies greatly among the genes for different subunits of the same complex, or for the same gene in different species, and it does so without an apparent rationale [2]. However, the protein sequences are remarkably conserved indicating that the final products of this cumbersome process are functional. Numerous studies have indicated that mitochondrial gene expression is indispensable and subject to regulation during the life cycle. In *Trypanosoma brucei*, the agent of Human African Trypanosomiasis, also known as 'sleeping sickness', the mitochondrion is fully up-regulated in procyclic cells, the form found in the tsetse fly vector, and down-regulated in bloodstream (BS) 'long slender' trypanosomes which proliferate in a mammal [3]. The upregulation involves the mitochondrial synthesis of subunits I - III (COI-COIII) for cytochrome *c* oxidase and apocytochrome *b* (Cyb) of cytochrome *c* reductase (cytochrome bc_1 complex), while the down-regulation leads to cessation of their production. According to the current knowledge of the mitochondrial metabolism in BS trypanosomes, some other proteins have to be expressed constitutively: these include several subunits of NADH dehydrogenase, ribosomal protein S12, and, most notably, subunit 6 (A6, formerly MURF4) of F_1F_O ATPase [4,5]. This enzymatic complex is active throughout the life cycle but plays the opposite roles in procyclic and BS cells [6,7]. Subunit 6 (also known as subunit **a** in other organisms) is an integral membrane protein forming part of the transmembrane proton channel and interfacing the multimeric subunit 9 (subunit **c**) [8]. In *T. brucei* the predicted A6 protein (231 amino acids, 28.7 kDa) is translated from the pan-edited mRNA [9] and contains six transmembrane α-helices (as determined by TMpred, Supplementary Fig. 1). Although this protein is crucial for the function of the F_1F_O ATPase, the respective polypeptide was not detected during the previous analyses of this enzyme [10].

A similar problem had been encountered at the analysis of cytochrome *c* oxidase and cytochrome *c* reductase complexes which contain three and one mitochondrially encoded subunits, respectively [11,12]. It was resolved by employing an extreme hydrophobicity of these proteins, the very property which makes them refractory to the standard approach, to separate the mitochondrial subunits from the nuclear-encoded, less hydrophobic subunits [13,14]. The anomalous electrophoretic migration of hydrophobic polypeptides in SDS gels results in their positioning off the main diagonal in two-dimensional (2D) gels. Such separation is crucial because hydrophobic polypeptides show tendency to precipitate, aggregate and appear in gels as diffuse poorly

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stained bands or spots, which would, therefore, be effectively masked by 'normal' polypeptides. The identity of the major off-diagonal spots observed in the 2D gel analysis of the purified cytochrome *c* oxidase and cytochrome *c* reductase complexes in *Leishmania tarentolae* (COI and Cyb, respectively) was determined by N-terminal sequencing (Edman degradation following removal of the N-terminal formyl group) [13,14]. The COII polypeptide was later found among the minor components of cytochrome *c* oxidase, apparently this polypeptide, as well as COIII, is more prone to aggregation compared to COI. By combining the 2D gel approach with metabolic labeling of cells with ^{35}S-amino acids in the presence of cycloheximide it was possible to detect some of the *de novo* synthesized mitochondrial proteins [15]. The two most abundant labeled spots represented COI and Cyb polypeptides.

A similar approach has resulted in detection of several hydrophobic polypeptides in *T. brucei* [16]. The two most abundant labeled components were identified as COI and Cyb by comigration with the off-diagonal components observed in the purified cytochrome c oxidase and reductase complexes, respectively (I.Š. and D.A.M., unpublished observations). This identification proved to be invaluable to investigate the interface of RNA editing/maturation and translation in *T. brucei* [16–18]. However, the dearth of identifiable mitochondrial translation products represents an impediment to furthering such studies. As a partial remedy, in this work we have identified an additional mitochondrially encoded product — subunit A6 of F_1F_O ATPase, a constitutively expressed protein defined by a pan-edited mRNA.

In order to detect the products of mitochondrial translation in procyclic *T. brucei*, strain 29-13, 10^7 cells were labeled with [^{35}S]-amino acid mix in the presence of 100 μg/ml cycloheximide to inhibit the cytosolic translation for 1 h and then chased with the excess of cold methionine and cysteine for 2 h [15,16]. Proteins in the total cell lysate were separated using a 2D (9% vs 14%) polyacrylamide Tris–glycine–SDS gel. The labeled products were detected by fluorography (Fig. 1A). In addition to COI and Cyb, several additional labeled polypeptides were also present. With the purpose of identification of these products, we then studied the incorporation of the labeled products into mitochondrial respiratory complexes. Crude mitochondrial fraction was obtained from 0.5×10^9 cells labeled with ^{35}S as described above. Mitochondrial membranes were lysed with 1% dodecyl maltoside and the complexes were separated in a 3–13% Blue Native /10% Tris–tricine–SDS 2D gel [19] (Fig. 1B). Renografin-purified cold mitochondria were analyzed in parallel (Fig. 1C). The respiratory complexes in gels were identified by their relative migration in the native dimension, a characteristic banding pattern in the denaturing dimension and by probing with antibodies against the F_1F_O ATPase subunit p18, cytochrome *c* reductase Rieske protein and cytochrome *c* oxidase trCOIV subunit as described previously [20]. It should be mentioned that the gradient 3–13% BN gel shown in Fig. 1B and 1C is a trade-off chosen for its optimal resolution of the oligomeric ATPase, as opposed to F_1 moiety of the enzyme and the cytochrome *c* oxidase and reductase complexes which are better resolved in a 6% uniform BN gel (see below). Under these conditions, the bulk of the radioactivity associated with the COI spot is found in the BN gel region encompassing cytochrome *c* oxidase indicating that most of the newly synthesized COI protein gets assembled into this respiratory complex (Fig. 1B). A fraction of the radioactive COI material is also seen at the origin of the BN dimension testifying to the presence of the incompletely solubilized or aggregated material. Additional labeled material present in the cytochrome c oxidase may represent oligomerized COI (migrating slower than monomeric COI in Tris–tricine dimension), as well as COII and/or COIII subunits (migrating faster than monomeric COI). The radioactivity found in the Cyb spot, which represents the most intensively labeled spot in Fig. 1A, can be only partially chased into the assembled cytochrome *c* reductase. A broad streak formed by the Cyb labeled material in the BN dimension suggests that this polypeptide, probably the one synthesized in excess, is found in partially assembled and/or chaperon complexes; however, this has not been investigated further.

The F_1F_O ATPase complex is easily detectable by the characteristic presence of the two abundant subunits from its F_1 moiety: α and β (Fig. 1C). The fastest migrating form of the complex, found in the vicinity of the cytochrome *c* oxidase and reductase complexes represents F_1, and two additional forms seen in gel represent oligomeric forms of the holoenzyme [21]. It is remarkable that the radioactivity represented by the putative A6 labeled spot in Fig. 1A is nearly completely chased into the F_1F_O ATPase complex (Fig. 1B). Due to its absence in F_1 it is likely part of F_O with A6 being the most likely candidate.

In order to verify this tentative conclusion, the polypeptide with properties of the predicted A6 proteins was searched using the isolated F_1F_O ATPase complex. N-terminal sequencing by Edman degradation was chosen for sequence analysis instead of mass-spectrometry because mitochondrially encoded polypeptides were conspicuously absent in the recent analyses on mitochondrial proteome in trypanosomatids [22]. We used *L. tarentolae* instead of *T. brucei* because it was easier to obtain a large amount of the material necessary for analysis of this polypeptide which was expected to be inefficiently blotted and poorly retained on the sequencing membrane, as was the case with other studied mitochondrial polypeptides. Additional advantages of using *L. tarentolae* is that mitochondria are usually isolated in high purity and solubilization of membranes with dodecyl maltoside is highly effective. Examples of Blue Native gel separation of *L. tarentolae* respiratory complexes are shown in Fig 1D and F, and that of a BN/Tricine–SDS 2D gel in Fig. 1G. The high level of similarity in the composition of respiratory complexes in these two species is obvious (see also Fig. 5 of Ref. [20]). Preparative BN gels, each loaded with 3.2 mg of mitochondrial protein were employed to purify the ATPase complex from renografin-purified mitochondria of *L. tarentolae*. The electro-eluted material from several such gels was pooled and analyzed in denaturing 2D gels as shown in Fig. 1F. Stainable off-diagonal spots were present in the area approximately matching position of the labeled A6 spot of *T. brucei* (Fig. 1A). After blotting on a PVDF membrane and the N-terminal deblocking, a partial N-terminal sequence of two of those polypeptides was determined. The sequence of one of them (*x*TVAISxQGL), where *x* denoted unidentified residues, matched the predicted sequence of ATPase subunit 9 (subunit **c**) from the related organism *L. major*, strain Friedlin, as determined by Blastp (http://www.genedb.org/blast/submitblast/GeneDB_Lmajor). This subunit represents a hydrophobic component of the F_O moiety [23] and is, therefore, also prone to anomalous gel migration. If the cleavage site of the A9 (**c**) polypeptide is conserved among species, this result indicates that the N-terminal signal sequence of 28 amino acids is cleaved off in *L. major*, and that of 39–40 amino acids is removed in *T. brucei* (Supplementary Fig. 3).

The partial N-terminal sequence of another spot was *x*FV(a/f)Iv*x*DLV(h/i)M, where *x* denoted unidentifiable residues and low case letters denote residues identified with lower confidence. Remarkably, this sequence was consistent with the predicted sequence of the mitochondrially encoded A6 polypeptide: MFVF-FVCDLVIM (considering that cysteine residues are not identifiable by the utilized sequencing protocol). We concluded from this analysis that A6 polypeptide was indeed present in the specific area of the electrophoretic gel supporting the identification based on the gel migration a labeled translation product described above.

Identification of mitochondrially encoded proteins represents an important pre-requisite to investigation of mechanisms coordinating mRNA processing and translation in trypanosomatids, as well as, at least in *T. brucei*, the developmental aspects of this inter-