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A putative novel nuclear-encoded subunit of the cytochrome *c* oxidase complex in trypanosomatids[☆]

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Abstract

A relatively large nuclear-encoded polypeptide, designated trCOIV, is found in the cytochrome *c* oxidase (CO) complex of trypanosomatids. In order to determine if this polypeptide represents a bona fide subunit of the complex, we have characterized the cDNA and the gene for this polypeptide in *Leishmania tarentolae*. Its nuclear gene has no sequence similarity to mammalian COIV. The trCOIV preprotein has a long mitochondrial targeting sequence of 31 residues. The mature polypeptide cofractionates with kinetoplast-mitochondria and its preferential mitochondrial localization was confirmed by immunofluorescence and immunoelectron microscopy. Based on the hydropathy plot analysis, the protein lacks pronounced transmembrane domains and likely occupies a peripheral position within the CO complex. The corresponding genes are also present in the sequenced portions of the *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major* genomes, and the same polypeptide is found in cytochrome oxidase isolated from procyclic *T. brucei* and promastigote *Leishmania mexicana amazonensis*. However, the trCOIV gene, the mRNA and the polypeptide could not be detected in a respiration-deficient trypanosomatid *Phytomonas serpens*.

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1. Introduction

The adaptation of digenetic (two host) trypanosomatids to specific conditions in their vertebrate or invertebrate hosts is accompanied by drastic changes in energy metabolism, as illustrated by the reversible inactivation of Krebs cycle, mitochondrial cytochrome-mediated electron transport and oxidative phosphorylation in bloodstream *Trypanosoma brucei* [1–3]. Since subunits of the mitochondrial respiratory complexes are encoded in the nuclear and kinetoplast-mitochondrial genomes, both types of genes are expected to undergo a life cycle

stage-specific regulation. Unraveling the mechanisms involved requires identification and a detailed characterization of the subunits. While all kinetoplast-encoded sequences from several species are now available (see [4] and references therein), only a few nuclear-encoded subunits of cytochrome *bc*₁, cytochrome *c* oxidase (CO) and ATPase have been described so far [5–11]. We have purified the CO complex from *Leishmania tarentolae* and analyzed its nuclear-encoded subunits by N-terminal sequencing [12,13]. The extremely hydrophobic subunits translated from kinetoplast mRNAs were difficult to identify, being first tentatively detected by mass spectroscopy [14] and, only recently, directly by N-terminal sequencing [12,15].

We have found previously that the largest component observed in the preparations of CO isolated by ion-exchange chromatography [13] or Blue Native gel electrophoresis [12] did not correspond to any of the three mitochondrially-encoded subunits COI, COII and COIII. These are usually the largest subunits directly

[☆] Note: The nucleotide sequence reported in this work is deposited in the GenBank™ database under the accession number AF205379.

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observed in electrophoretic gels [16], but in trypanosomatids they can only be detected using special procedures [12,15]. So, the largest observed subunit must be nuclear-encoded and we originally called it COIV to reflect its sequential number. Previously, it was also found that the homolog of mammalian COIV is one of the smallest subunits of the enzyme, termed subunit 8 by the authors [7] and COXI by us [12]. In order to avoid a possible confusion due to identical names assigned to different polypeptides, we hereby propose a designation 'trCO' for the nuclear-encoded subunits in the trypanosomatid enzyme. The existence of a relatively large nuclear-encoded subunit as trCOIV is without a precedence in other organisms, so the question arises whether this component is a bona fide subunit of the trypanosomatid enzyme and not a polypeptide spuriously copurifying with it in *L. tarentolae*.

To this end, we have cloned the gene and cDNA for this polypeptide, confirmed its preferential mitochondrial localization and investigated the presence of this subunit in other species of trypanosomatids with and without active CO complexes.

2. Materials and methods

2.1. Cell growth and isolation of kinetoplasts

The cultures of *L. tarentolae* UC, *Crithidia fasciculata* Cf-C1 and *P. serpens* 9T were grown in the BHI medium with 10 $\mu\text{g ml}^{-1}$ hemin [17]. Promastigotes of *Leishmania mexicana amazonensis* LV78 were grown in the 199 medium supplemented with 10% fetal bovine serum [18]. Procyclics of *T. brucei* 427 MiTat 1.2 were cultivated in SDM-79 [19]. The kinetoplast-mitochondrial fractions were isolated by the hypotonic lysis procedure and were further purified by flotation in Renografin density gradients as described earlier [17].

2.2. Nucleic acid procedures

Isolation of total cell DNA, restriction digestions, agarose gel electrophoresis, and blotting onto nylon membranes were performed by standard protocols [20]. The DNA probes labeled by random priming (Prime-It II kit, Stratagene) were hybridized to genomic blots at 65 °C in 6 \times SSC, 5 \times Denhardt's solution, 0.1 mg ml⁻¹ denatured sonicated salmon sperm DNA and 0.5% SDS for 12–16 h in rotating hybridization oven followed by several extensive washes of the blots at 50 °C in 2 \times SSC, 0.1% SDS.

Polyadenylated total cell RNA was isolated from 10⁸ exponentially growing cells of *L. tarentolae* by extraction with TRIzol Reagent followed by purification with MESSAGEMAKER mRNA Isolation System (Gibco BRL) or High-Pure RNA isolation kit (Roche). For Northern

hybridizations, 20 μg of total RNA per lane was separated in 1% agarose–formaldehyde gels and transferred onto the Hybond N⁺ membranes (Amersham Pharmacia Biotech). The hybridization was performed in 0.5 M Na₂HPO₄, pH 7.2, 1 mM EDTA, 7% SDS, 1% BSA at 55 °C overnight, and the membranes were washed in 2 \times SSC, 0.1% SDS at room temperature for 20 min and in 0.2 \times SSC, 0.1% SDS at 55 °C for 15 min.

Reverse transcription, PCR, cloning and sequencing were performed as described earlier [21].

2.3. Oligonucleotides

Following oligonucleotides were used in the study:

M087:	RCAYTCNSWRTRAAAYTTR-TARTTRTG (antisense oligonucleotide for trCOIV cDNA synthesis);	124
M088:	GTRTCNARYTCNARNGCRTGNCRTA (trCOIV antisense downstream PCR oligonucleotide);	125
M089:	AACTAACGCTATATAAGTATCAGTTT (spliced leader-specific sense upstream PCR oligonucleotide);	126
M097:	TGTTTACGCGTCGTGCCGTGTCTTC (trCOIV sense upstream genomic PCR oligonucleotide);	127
M122:	AATTTTGTTTGCCAGTTTCCGCCATTC (trCOIV antisense downstream genomic PCR oligonucleotide);	128
XY2:	<i>GGATCCGACCACGACCGCTGG-TACGGC</i> (expression-cloning sense adapter oligonucleotide, <i>Bam</i> HI site is italicized);	129
XY3:	<i>GAATTCCTAAATTTTGTTTGCCAGTTTC</i> (expression-cloning antisense adapter oligonucleotide, <i>Eco</i> RI site is italicized).	130

2.4. Expression and purification of recombinant protein

The entire trCOIV coding region (936 bp) was PCR-amplified from the genomic clone with the oligonucleotides XY2 and XY3. The amplicon was digested with *Bam*HI and *Eco*RI, gel-purified, and cloned into the pRSET A expression vector (Invitrogen). The resulting expression plasmid with the N-terminal hexahistidine tag in frame with the ORF was propagated in *Escherichia coli* strain BL21(DE3)pLysS (Novagen). Upon induction, the His-tagged overexpressed protein was purified from the supernatant using ProBondTM Ni²⁺-chelating resin under denaturing conditions as specified by the manufacturer (Invitrogen).

2.5. Preparation of antibodies and Western blotting

Polyclonal antibodies were prepared by immunizing a rabbit at 2-week intervals with four subcutaneous

injections of 0.5 mg purified recombinant protein emulsified with complete (first injection) and incomplete (following injections) Freund's adjuvant. Serum was collected 7–10 days after the third and fourth injections and tested by Western blotting as described below. The overexpressed protein was immobilized on the AminoLink® Plus Coupling Gel (Pierce) and the polyclonal antibodies were affinity purified on a column as suggested by the manufacturer.

Cell lysates of *L. tarentolae*, *P. serpens* and *T. brucei* were prepared as described elsewhere [22] and separated in 12% Tris–glycine–SDS gels [23]. Analysis of respiratory complexes in dodecyl maltoside lysates of purified kinetoplast-mitochondria was performed by two dimensional Blue Native/Tricine–SDS PAGE [24]. Separation in the first and second dimensions was performed in 6% Blue Native gels and in 12% Tricine–SDS gels [12]. Proteins were electroblotted onto Hybond–ECL nitrocellulose membranes (Amersham Pharmacia Biotech) using a MilliBlot semidry electroblotting system (Millipore) as described earlier [12]. After incubating the blot with primary antibodies against recombinant trCOIV (used at 1:2000 dilution), the immunoreactive polypeptides were detected using the SuperSignal® West Pico chemiluminescence system (Pierce). The polyclonal mouse antiserum against *L. tarentolae* Rieske iron-sulfur protein and the rabbit antiserum against ATPase subunit b (p 18) (provided by L. Simpson) were used at 1:4000 and 1:10 000 dilutions, respectively. The rabbit serum against *T. brucei* pyruvate kinase (provided by P.A.M. Michels) was used at 1:2000 dilution.

2.6. Immunocytochemistry

Cells were resuspended in phosphate buffered saline (PBS) at a concentration 10^8 cells per ml. The following procedures were performed at room temperature. The suspension (20 μ l) was spotted onto poly-L-lysine-coated slides and cells were allowed to adhere for 30 min in a humidity chamber. The slides were submerged into 4% paraformaldehyde in PBS for 3 min, and fixation was stopped by washing the slides with 0.1 M glycine, pH 8.6, in PBS for 5 min, followed by two washes in PBS for 5 min. Cells were permeabilized in 1% Tween-20 in PBS supplemented with a blocking solution (20% goat serum) in a humid chamber for 1 h. After a single wash in PBS containing 0.05% Tween-20 (PBST) for 5 min, slides were incubated with primary antibody diluted in PBST and the incubation was performed under the same conditions for 2 h. The affinity-purified anti-trCOIV serum and the anti-p18 serum were used at 1:100 and 1:500 dilutions, respectively. The polyclonal rabbit antiserum against purified *C. fasciculata* ATPase complex (provided by R. Benne) was used at 1:500 dilution. Slides were washed four times in PBST for 5 min each and then incubated with FITC-conjugated

goat anti-rabbit IgG antibodies (used at 1:160 dilution; Sigma) for 1 h in a humid chamber. The incubation was followed by three washes in PBST and one wash in PBS for 5 min each. Finally, the slides were incubated in PBS containing $0.1 \mu\text{g ml}^{-1}$ DAPI for 3 min, rinsed with distilled water and mounted into the antifade. The slides were examined with a Zeiss Axioplan 2 microscope, black and white images were recorded with a cooled Mega F-View II CCD camera (Soft Imaging Systems, Münster), pseudo-colored and then superimposed using ADOBE PHOTOSHOP, version 5.0.

2.7. Immunoelectron microscopy

Cells were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in PBS at 4 °C overnight followed by a low-speed centrifugation ($1250 \times g$) and three washes in PBS for 2 min each. The washed cells were embedded in 2% agarose (Fluka), the agar block was sliced into small pieces and these were cryoprotected by 2.1 M sucrose for 2 h, placed onto a pin, and frozen by plunging into liquid nitrogen. Cryosections (100 nm thick) were prepared at $-100 \text{ }^\circ\text{C}$ with a Leica ultramicrotome UCT equipped with the FCS cryochamber, and transferred onto 200 mesh copper grids with a carbon-coated formvar membrane. The grids were picked upon a drop of 2.3 M sucrose and thawed in 0.3% agarose containing 1% gelatine (Serva), $0.01 \times$ PBS, and placed into a moist chamber. The following incubations were performed at room temperature: blocking in fetal bovine serum containing 2% glycine, 10% BSA and 1% fish-skin gelatine (Sigma) in $0.1 \times$ PBS for 30 min, incubation with primary antibodies for 1 h, five 2 min washes in PBS containing 1% fish-skin gelatine; incubation with protein A coupled with 10 nm colloidal gold (Sigma) for 1 h and five 2 min washes in deionized water. The grids were contrasted in 2% methylcellulose (Sigma) and 3% uranyl acetate and examined in JEOL JEM 1010 transmission electron microscope. The anti-trCOIV serum (affinity-purified) and the anti-p18 serum were used at 1:50 and 1:200 dilutions, respectively.

2.8. Protein sequencing

Respiratory complexes were solubilized with 2% dodecyl maltoside and resolved in a single dimension 6% Blue Native gel [24]. The bands of individual complexes were excised, proteins were denatured by incubating gel slices in 1% SDS, 1% β -mercaptoethanol for 30 min at 37 °C, and the entire slices were placed atop a 12% Tris–glycine–SDS gel. Electrophoretically separated polypeptides were electroblotted onto Trans-Blot PVDF membrane (Bio-Rad). The bands were detected by staining with Coomassie R-250 (Sigma). N-terminal microsequencing was performed by Edman

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259 degradation at the UCR Genomics Institute Core
260 Instrumentation Facility.

261 3. Results

262 3.1. Cloning and characterization of the trCOIV mRNA 263 and genomic sequence

264 The determined N-terminal sequence of trCOIV,
265 DHDRWYGHAELELDTHNYKFNGEP, excluding
266 the first five residues was back-translated to generate
267 two nested antisense primers M087 and M088 (Fig. 1A).
268 Complementary DNA enriched for the trCOIV mRNA
269 was synthesized by reverse transcription of poly(A)⁺
270 RNA with M087 and used as a template for amplifica-
271 tion of the 5' end region of this mRNA with M088 and
272 the upstream spliced leader-specific primer M089. After
273 cloning and sequencing of the amplified DNA, several
274 clones were obtained in which the N-terminal sequence
275 DHDRW was present immediately upstream of M088,
276 thereby, indicating that the amplification was specific.

277 The database search with the amplified DNA se-
278 quence and the partial amino acid sequence yielded
279 homologous genes within the sequenced regions of the
280 *T. cruzi*, *T. brucei* and *L. major* genomes. No similarity
281 to CO subunits from organisms outside Trypanosoma-

282 tidae was detected. The downstream primer M122 was
283 then derived from the *T. cruzi* sequence and used for
284 amplification of the missing portion of the *L. tarentolae*
285 gene in combination with the upstream primer M097.

286 The 5' sequence of the trCOIV cDNA in *L. tarentolae*
287 including the 5'UTR and the beginning of the reading
288 frame is shown in Fig. 1A. The first and only in-frame
289 ATG initiation codon is found 31 amino acid residues
290 upstream of the N-terminus of the mature trCOIV,
291 indicating that translation of this ORF generates the
292 precursor with the cleavable N-terminal peptide. This
293 peptide demonstrates characteristic features of the
294 kinetoplast targeting sequences: it is composed of basic,
295 hydroxyl-containing and hydrophobic residues while
296 negatively charged residues are absent. According to
297 the analysis by the GCG program HELICALWHEEL, this
298 region can be folded into an amphipathic helix. One side
299 of the helix contains twelve polar residues including all
300 four positively charged arginines and only four non-
301 polar residues, while the other side is hydrophobic with
302 five polar residues and ten nonpolar residues.

303 The polypeptides in other three trypanosomatids are
304 remarkably similar within the region corresponding to
305 the mature *L. tarentolae* protein. Less similarity is seen
306 among their N-termini (Fig. 1B), however, they too
307 contain features expected for the kinetoplast signal
308 peptides including a characteristic tandem of arginines

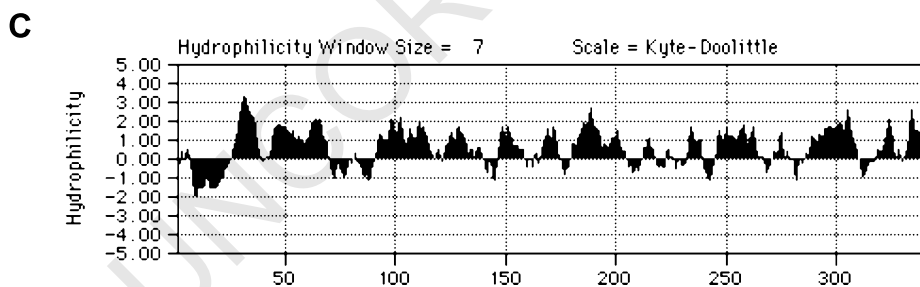
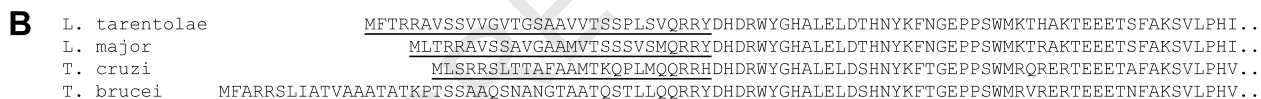
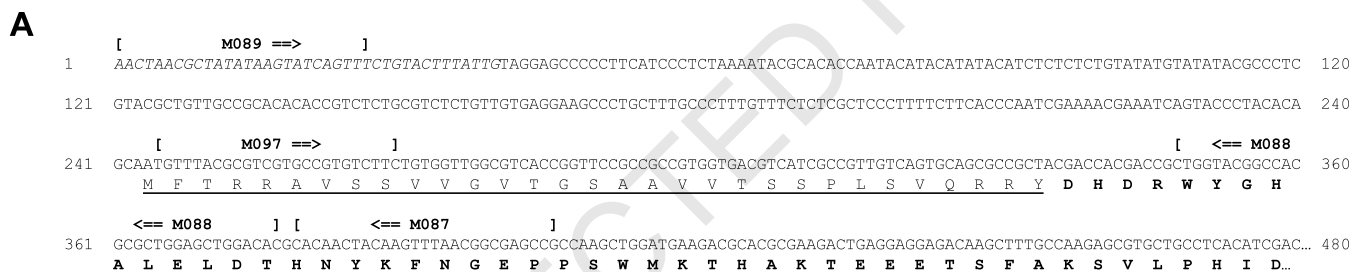


Fig. 1. (A) The partial sequence of cDNA for trCOIV mRNA from *L. tarentolae*. Positions and orientations of the amplification primers are given above the sequence, its translated amino acid sequence is given below. The minioxon sequence is given in italics, and the putative mitochondrial targeting peptide is underlined. (B) Comparison of the N-terminal regions of *L. tarentolae* trCOIV with *L. major* (GenBankTM accession number AL390114), *T. cruzi* (AF052427), and *T. brucei* (AL359782). The putative signal peptides are underlined. (C) Hydropathy plot of the *L. tarentolae* trCOIV polypeptide. The plot was generated with the MACVECTORTM program, version 5.0, using the Kyte–Doolittle scale (positive and negative values correspond to hydrophilic and hydrophobic regions, respectively) and a seven amino acid window.

309 which is often found close to the first methionine residue
310 [25,26].

311 The predicted mature protein of *L. tarentolae* is 36
312 kDa in size and that corresponds to the experimentally
313 determined value. Hydropathy plot analysis (Fig. 1C)
314 indicated that the protein is devoid of large transmem-
315 brane domains with the only notable hydrophobic
316 region corresponding to the targeting peptide. A rela-
317 tively low level of hydrophobicity indicates that this
318 subunit most likely occupies a peripheral position with
319 respect to the inner membrane. No meaningful sequence
320 motifs could be recognized in the protein by the
SCANPROSITE program using PROSITE database, re-
322 lease 17.6 (<http://us.expasy.org/tools/scanprosite/>). A
323 weak match with the N-terminal domain of the ami-
324 noacyl class II tRNA synthetases was recognized by the
325 program MOTIFSCAN in the Pfam collection of hidden
326 Markov models (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>).
327 This domain's function is ATP-binding and the poten-
328 tial significance of this match is discussed below.

329 A single band of 2.5 kb was detected on the Northern
330 blot of *L. tarentolae* total cell RNA and similar size
331 transcripts were found in *C. fasciculata* (Fig. 2) and *T.*
332 *brucei* (data not shown).

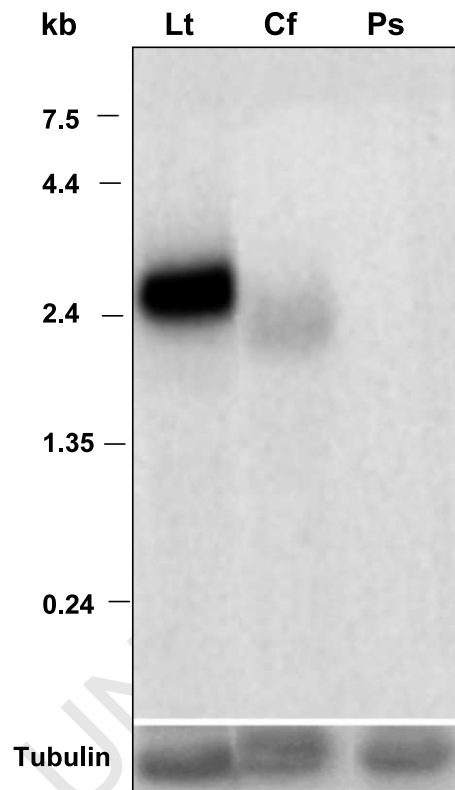


Fig. 2. Northern blot hybridization of trCOIV transcripts in trypanosomatids. Lt, *L. tarentolae*; Cf, *C. fasciculata*; Ps, *P. serpens*. Marker sizes (0.24–9.5 Kb RNA ladder, Gibco BRL) are shown to the left. Hybridization of a similar blot with the uniformly labeled *T. brucei* β -tubulin cDNA probe (bottom panel) was used as a control.

3.2. Preferential association of the trCOIV polypeptide with CO complexes

335 The entire trCOIV coding sequence was expressed in
336 *E. coli* and the recombinant polypeptide was purified to
337 homogeneity (Fig. 3). The polyclonal serum raised
338 against the purified trCOIV polypeptide was used to
339 investigate its intracellular localization. In parallel with
340 *L. tarentolae*, we also investigated the procyclic forms of
341 *T. brucei*, known to possess active CO (reviewed by [3]),
342 and the culture forms of *P. serpens*, in which no
343 detectable CO could be found [27].

344 On a blot of the total cell and kinetoplast lysates of *L.*
345 *tarentolae*, the polyclonal antibodies against trCOIV
346 recognized a polypeptide of the expected size, which,
347 thus, represented trCOIV (Fig. 4). A stronger signal was
348 obtained with the kinetoplast lysate than with the
349 equivalent amount of the total cell lysate, indicating
350 that the immunoreactive protein cofractionated with
351 kinetoplast-mitochondria. The same result was obtained
352 with *T. brucei*, in which the serum recognized a
353 polypeptide of the similar size. No polypeptide of the
354 appropriate size was recognized in *P. serpens*. This
355 pattern paralleled that of the Rieske iron-sulfur protein,
356 a subunit of cytochrome bc_1 complex which represents a
357 component of the mitochondrial inner membrane [5].
358 The same fractionation pattern was also observed with
359 p18, a subunit of mitochondrial ATPase, except that this
360 polypeptide was present in *P. serpens* which is known to
361 have the active enzyme [27]. On the contrary, pyruvate

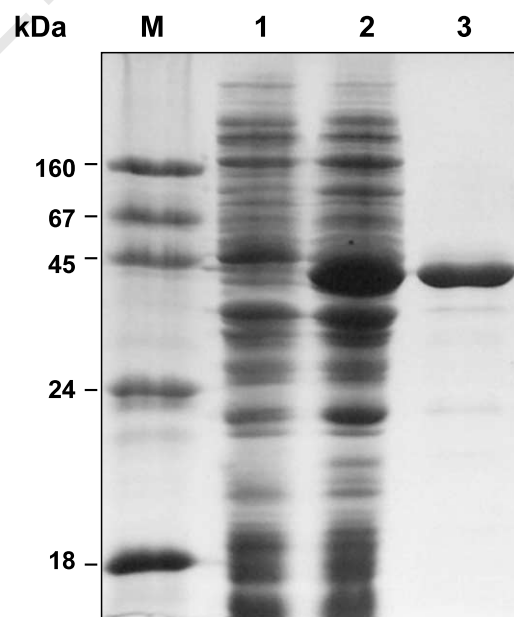


Fig. 3. Expression of trCOIV ORF in *E. coli*. The polypeptides in the uninduced bacterial lysate (lane 1), the induced lysate (lane 2) and the purified recombinant protein (lane 3) are shown. Lane M represents the markers from Molecular Weight Marker Kit (ICN), the band sizes are given to the left.

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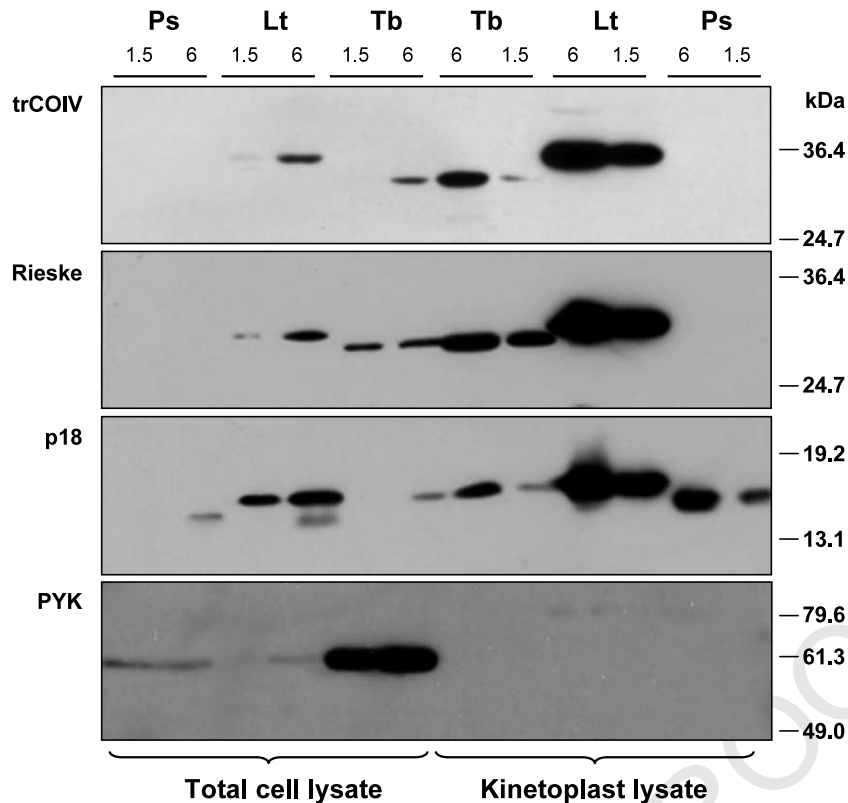


Fig. 4. Cofractionation of the trCOIV polypeptide with kinetoplast-mitochondria. Total cell lysate and kinetoplast lysate (6 or 1.5 μ g protein, as indicated above the lanes) were fractionated in a 12% Tricine–SDS gel, blotted and probed with the sera indicated to the left. Lt, *L. tarentolae*, Tb, *T. brucei*, Ps, *P. serpens*. The sizes of the marker bands (BenchMark Protein Ladder, Gibco BRL) are shown to the right.

kinase, a cytosolic marker [2,28], did not cofractionate with trCOIV, at least in *T. brucei*, as seen on the blot probed with the serum against the enzyme from this species (the serum seems to only weakly cross-react with the other two species).

Respiratory complexes of *L. tarentolae* and *T. brucei* were solubilized with dodecyl maltoside and separated by two-dimensional gel electrophoresis (Fig. 5A and B). Identification of the complexes in *L. tarentolae* was described earlier [12,27]. The respiratory complexes in *T. brucei* were found to be similar in electrophoretic migration and composition to the complexes from *L. tarentolae* except for an additional ATPase-related complex in *T. brucei* which migrated ahead of cytochrome bc_1 . This complex might represent an F_1 moiety, but we did not investigate this further. The original identification of ATPase complexes was performed using the sera against the entire *C. fasciculata* enzyme [8] and *L. tarentolae* subunit b (p18) [29] (data not shown). N-terminal sequencing of the anticipated subunit α produced the sequence DSTLGKVDTGAPNIV, which matched the known sequence [11]. Cytochrome bc_1 complex was originally identified by probing the blot of a Blue Native two-dimensional gel with the serum

against *L. tarentolae* Rieske protein (data not shown) followed by N-terminal sequencing of this subunit. The obtained sequence, VSLVFKQLEGSNPLT, also matched the corresponding known sequence [30].

The remaining complex which in both species represents the monomeric CO complex migrates in a well-defined position in the Blue Native dimension. A minor (possibly aggregated) fraction of it is found in the interface between the 3% stacking and 6% resolving gels, together with other aggregated complexes. When blots of such gels were probed with the anti-trCOIV serum, the main spot of immunoreactivity corresponded to the cognate polypeptide, which was found within the monomeric form of the CO complex. On the *L. tarentolae* blot (Fig. 5A) it was possible to detect a smaller amount of trCOIV migrating with the front of the native dimension, which contains free polypeptides. In addition, a relatively small amount of the peptide was also observed in the undefined low abundance complex migrating faster than the monomeric CO complex, and also in the aggregated CO complexes found in the stacking gel and in the interface of the stacking and resolving gels. In *T. brucei*, trCOIV was detected mainly in the monomeric CO complex (Fig. 5B). The sequence

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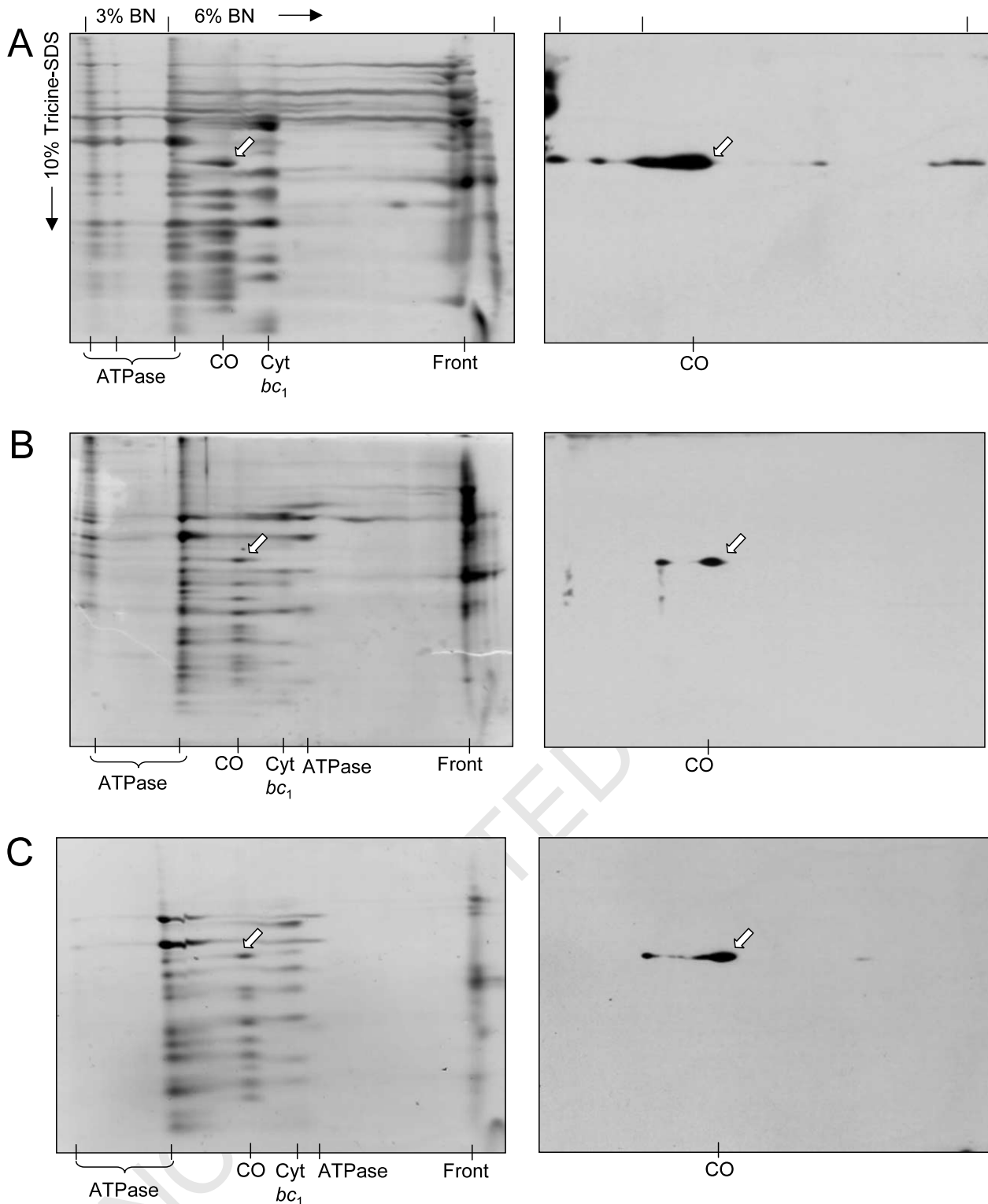


Fig. 5. Preferential association of trCOIV with the CO complexes. Respiratory complexes of *L. tarentolae* (A), *T. brucei* (B) and *L. m. amazonensis* (C) were resolved in the two-dimensional 6% Blue Native (3% stacking)/12% Tricine–SDS gels (left panels). Parallel gels were blotted and probed with the anti-trCOIV serum (right panels). The trCOIV polypeptides contained within the monomeric CO complexes are shown by the open arrow. Positions of the respiratory complexes are indicated under the panels.

410 obtained by N-terminal sequencing of this subunit,
 411 DHDRxYGHLELDH, matched the sequence de-
 412 rived from the genomic clone (Fig. 1B).

In addition, the N-terminal sequence of a faster
 413 migrating band from the *T. brucei* CO complex,
 414 SGGSTGPTPY(Y/Q)D(D/K)xY, was similar to the
 415

416 sequence of *L. tarentolae* trCOV [12], the second largest
417 nuclear-encoded subunit of the enzyme.

418 The subunit pattern in *Leishmania mexicana amaz-*
419 *nensis* complexes was very similar to *L. tarentolae* and
420 the largest CO subunit was specifically recognized by the
421 antiserum against the *L. tarentolae* trCOIV subunit
422 (Fig. 5C). Therefore, trCOIV is localized in the kine-
423 toplast-mitochondria and inside the organelle this poly-
424 peptide exists mainly in association with the CO
425 complexes in the analyzed species. These data also
426 indicate that other large nuclear-encoded subunits may
427 be conserved as well.

428 3.3. Immunolocalization of trCOIV

429 The intracellular localization of trCOIV was also
430 investigated using immunofluorescence microscopy
431 (Fig. 6). The antibodies against trCOIV were immu-
432 noaffinity purified in order to reduce nonspecific
433 reactivity. Control immunostaining (Fig. 6A and B)
434 performed with the sera against membrane specific
435 markers, ATPase subunit b (p18) and the entire ATPase,
436 showed typical rounded mitochondria usually observed
437 in cells prior to the division [31]. With the anti-trCOIV
438 serum, we have also observed these forms (Fig. 6C and
439 D). The staining showed an even distribution of trCOIV
440 in the mitochondrion.

441 A more detailed view was obtained using immunoe-
442 lectron microscopy which confirmed that the trCOIV
443 polypeptides are preferentially localized in the kine-
444 toplast-mitochondrion (Fig. 7). A statistically significant
445 departure from random distribution was confirmed by
446 χ^2 analysis ($P < 0.005$ for the null hypothesis of random
447 distribution). The immunogold particles were present in
448 the mitochondrial tube (Fig. 7B and C) as well as in the
449 vicinity of the kinetoplast DNA disk (Fig. 7D). The dots
450 of immunogold were preferentially seen in association
451 with the tubular cristae, and to a lesser extent with the
452 organelle's envelope (Fig. 7C and E) indicating that the
453 polypeptide is associated with the inner mitochondrial
454 membrane. A similar distribution was observed for p18
455 (Fig. 7A).

456 3.4. Apparent absence of the gene for trCOIV in *P.* 457 *serpens*

458 Southern blot with restriction digests of the total cell
459 DNA from *P. serpens* was probed with the DNA
460 fragment containing the *L. tarentolae* coding region
461 amplified with primers M097 and M122. Two trypano-
462 somatid species, *L. tarentolae* and *Trypanosoma cruzi*,
463 with the known trCOIV genes were used as a control.
464 The observed restriction band pattern in these species
465 was consistent with the occurrence of restriction sites in
466 the gene sequences confirming that the hybridization
467 was specific (Fig. 8A). A simple band pattern observed

468 in both cases also indicated that the gene is most likely
469 single copy. Remarkably, no hybridization signal was
470 observed in *P. serpens*, although the amount of loaded
471 DNA was the same as with the other two species (Fig.
472 8B). When the blot of a total cell mRNA of *P. serpens*
473 was probed with the trCOIV probe under low stringency
474 conditions, no signal was detected (Fig. 2).

475 4. Discussion

476 The trypanosomatid CO is distinguished from this
477 enzyme in other organisms by the presence of five to six
478 relatively large nuclear-encoded polypeptides in the 20–
479 40 kDa size range [8,12–14,32]. The largest of them,
480 trCOIV is 36 kDa, while the largest bovine subunit
481 COIV is only 17 kDa [16]. These subunits are not
482 homologous, and the homolog to the bovine COIV
483 subunit is one of the smaller subunits, trCOXI (earlier
484 designated 'cox subunit 8') [7]. With the progress of the
485 genomic sequencing projects, complete sequences of
486 other trypanosomatid subunits will soon be available
487 and their homologs, if there are any, will become
488 known. The presence of the large subunits in trypano-
489 somatids raises questions about whether they represent
490 genuine subunits of the enzyme. Indeed, one of these
491 subunits, trCOV, which is the second largest, was
492 present in the enzymes separated by native gel electro-
493 phoresis ([12] and this work) and hydrophobic interac-
494 tion chromatography [32], but was absent in the enzyme
495 isolated by ion-exchange chromatography [13], indicat-
496 ing that this may be a relatively weakly associated
497 component.

498 The uncertainty regarding what is to be considered a
499 genuine component of a multisubunit complex is not
500 unique to the trypanosomatid enzyme. Although the
501 structure of mammalian CO is now known in detail
502 [33,34], its subunit composition was also debated in the
503 past ([35,36] and references therein). First of all, a
504 composition of the enzyme is not well conserved in a
505 broad phylogenetic sense. Only three mitochondrially-
506 encoded subunits with a catalytic function (COI–III)
507 are universal, and their homologs are present in the
508 prokaryotic enzymes as well. A variable number of
509 additional subunits are present in different eukaryotes,
510 and these subunits are nuclear-encoded. Sequences of
511 the nuclear encoded subunits are usually poorly con-
512 served across the taxa and even within the same
513 organism some of these subunits may have a few
514 isoforms with substantially different sequences (re-
515 viewed in [16]). Also considering that no specific
516 function could be assigned to any of these subunits, it
517 should not be surprising that concerns were raised that
518 some of these polypeptides might be spuriously asso-
519 ciated components rather than genuine subunits. A
520 partial solution to this question was found in demon-

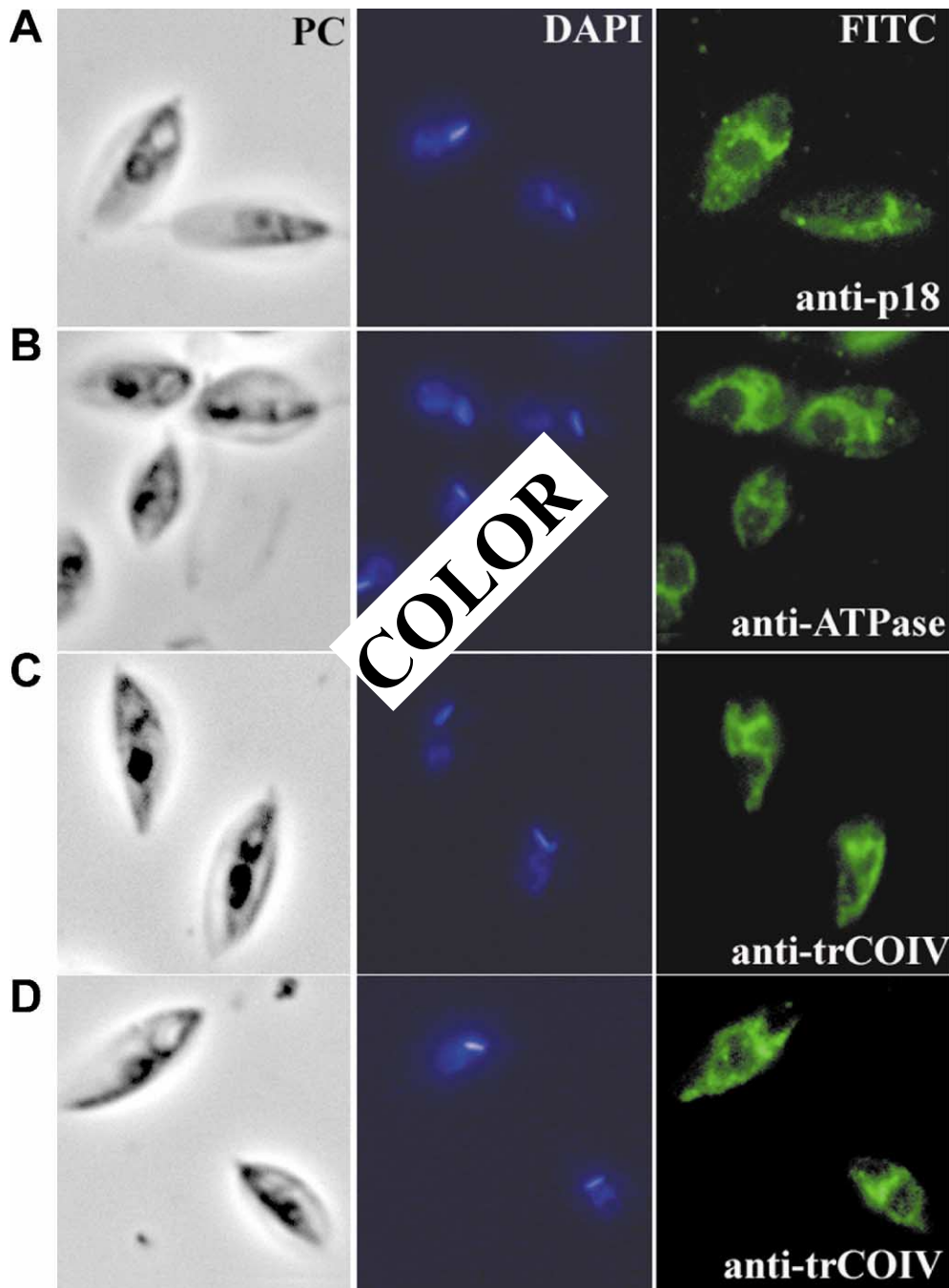


Fig. 6. Immunolocalization of trCOIV (panels C and D) in the *L. tarentolae* cells. Immunostaining with the anti-p18 (panel A) and anti-ATPase (panel B) sera were used as controls. Fluorescence from DAPI (central column of panels) and FITC (right columns) is shown for cells also shown in phase contrast (left columns).

521 strating that these polypeptides were present in enzyme
 522 preparations isolated by different procedures in the
 523 equimolar ratio with other subunits and learning their
 524 other properties [35]. It was hypothesized that these
 525 subunits control the enzyme's activity and abundance or
 526 participate in its assembly and positioning in the
 527 membrane, or provide an insulating function [16]. The
 528 recent crystallographic studies [33,34] indicated that
 529 none of the ten nuclear-encoded subunits in the bovine

enzyme is implicated in formation of the redox sites of
 the enzyme, consistent with their proposed regulatory or
 other noncatalytic functions, and three of these subunits
 are even localized entirely outside of the membrane.

In light of these properties of the nuclear-encoded
 subunits, the evidence presented in this work is consis-
 tent with a role of trCOIV as a bona fide subunit of
 the CO complex. Using cell fractionation and micro-
 scopy, we have found that this polypeptide is preferen-

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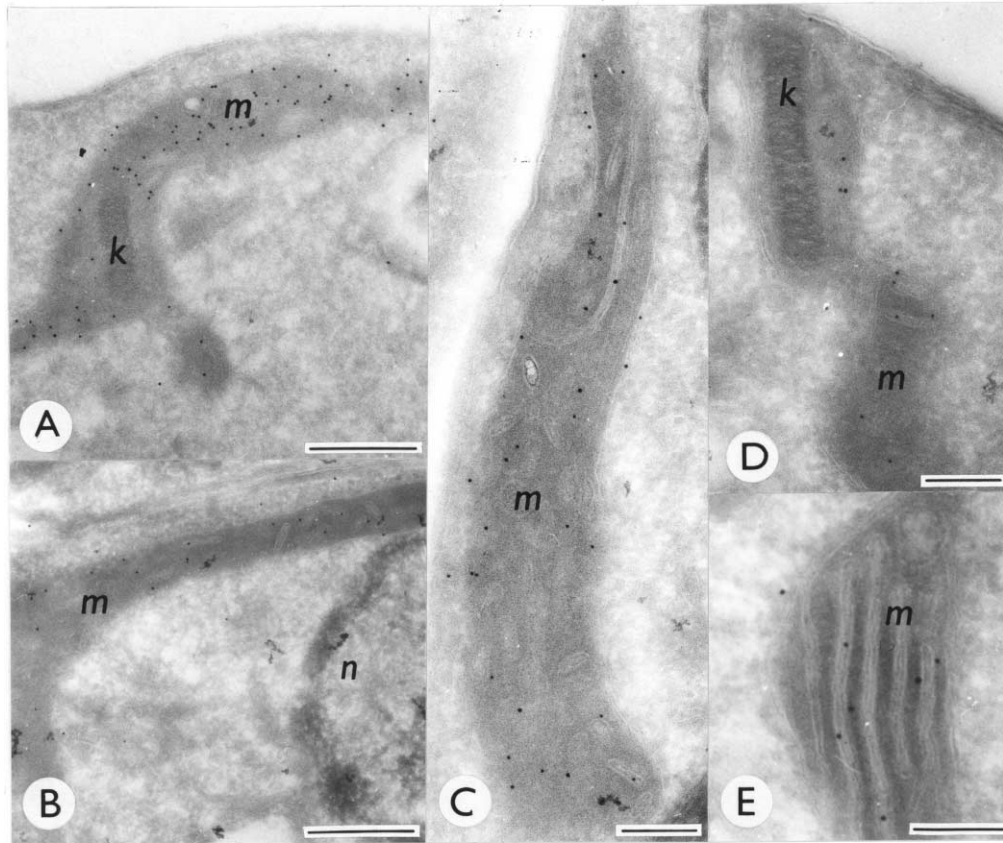


Fig. 7. Immunoelectron microscopy of the *L. tarentolae* cells with the immunogold-conjugated antibodies against trCOIV (B–D) and p18 (A). (A, B) Longitudinal section of the mitochondrion (*m*) including the kinetoplast disk (*k*) and nucleus (*n*). (C–E) Higher magnification of the labeled mitochondrion, showing a well-preserved kinetoplast disk and cristae. Bars: 750 nm (A, B), 300 nm (C, D, E).

539 tially localized within the kinetoplast-mitochondrion in
 540 association with the membranes. Moreover, after solu-
 541 bilization of the kinetoplast membranes with the deter-
 542 gent and fractionation of respiratory complexes by Blue
 543 Native gel electrophoresis, this component is largely
 544 found in association with monomeric CO. The trCOIV
 545 polypeptide is reproducibly found in this association in
 546 all tested trypanosomatids with active respiration. This
 547 subunit seems to be tightly bound to the enzyme since it
 548 is found in an apparently equimolar ratio to other
 549 subunits after ion-exchange chromatography [12] and
 550 hydrophobic interaction chromatography [32]. Finally,
 551 the attempts to find this polypeptide, the corresponding
 552 gene or the mRNA in the respiration-deficient trypano-
 553 somatid *P. serpens* invariably failed. Our preliminary
 554 data (not shown) indicate that this subunit is down-
 555 regulated in the bloodstream stages of *T. brucei*.

556 A potentially peripheral localization of this subunit
 557 with regard to the membrane is analogous to that of
 558 subunits Va, Vb of the bovine enzyme which are located
 559 on the matrix side or subunit VIb located on the
 560 opposite side [33]. Several ATP/ADP binding sites
 561 were identified in the bovine enzyme, and one of them,
 562 located in the matrix domain of subunit IV, was

563 implicated in allosteric regulation of cytochrome *c*
 564 oxidase by the ATP/ADP ratio (reviewed in [37]). This
 565 regulation is abolished by binding of 3,5-diodothyronine
 566 to the adjacent subunit Va. The allosteric inhibition is
 567 enhanced by phosphorylation of some subunits includ-
 568 ing Vb. The known functions of subunit VIb include a
 569 stabilizing contact between the monomers in the dimeric
 570 bovine enzyme, and a possible involvement in the
 571 cooperativity between the cytochrome *c* binding sites.
 572 It is not known whether or not any of these mechanisms
 573 operate in the trypanosomatid enzyme, and the proper-
 574 ties of trCOIV subunit itself have not yet been fully
 575 investigated. However, the available data allow to
 576 suggest a regulatory role for this polypeptide with an
 577 extramembraneous localization and a possible presence
 578 of an ATP-binding domain. Nevertheless, in *L. tarento-*
 579 *lae* and possibly in other species the polypeptide may
 580 have a dual function and its presence in a low
 581 abundance complex smaller in size than cytochrome
 582 oxidase (Fig. 5A) is consistent with this possibility.

583 The case of *Phytomonas* requires a particular atten-
 584 tion. Previous phylogenetic analyses showed that the
 585 genera *Leishmania* and *Phytomonas* are more closely
 586 related to each other than either is to the genus

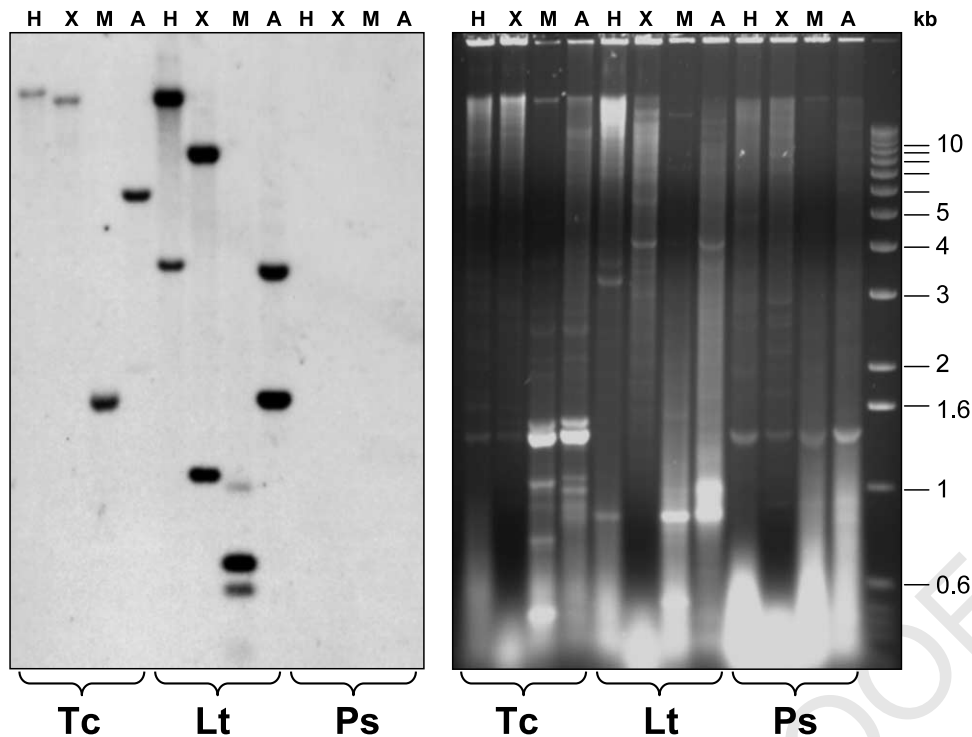


Fig. 8. Southern hybridization analysis of the trCOIV gene in trypanosomatids. Total cell DNA (5–10 µg per lane) from *T. cruzi* (Tc), *L. tarentolae* (Lt) and *P. serpens* (Ps) was digested with *Hind*III (H), *Xho*I (X), *Msp*I (M) and *Acc*I (A), resolved in a 0.7% agarose gel (right panel), blotted and hybridized with the *L. tarentolae* trCOIV coding region amplified with the primers M097 and M122 (left panel). Positions of the 1 kb DNA ladder bands (Gibco BRL) are given to the right.

Trypanosoma [38,39]. Therefore, the lack of a hybridization signal with *P. serpens* using the *L. tarentolae* probe (Fig. 8) indicated that the gene was lost or had an unusually high level of sequence divergence. Both events could occur when the gene structure is no longer under the pressure of maintaining selection due to a loss of function. Earlier works established that in *Phytomonas* spp. there is no cytochrome-mediated electron transport chain and oxidative phosphorylation [40,41]. We found previously that CO and cytochrome *bc*₁ complexes are absent, and genes for the corresponding mitochondrially-encoded subunits are deleted from the maxicircles of kinetoplast DNA [27,42]. The results presented here suggest that the loss of active CO was also accompanied by the inactivation or loss of the gene for one of its nuclear-encoded subunits.

Cleavable N-terminal sequences of the imported kinetoplast proteins in general are rather diverse in length and sequence. The smallest of them are found in cytochrome *c*₁ and trCOIX (earlier designated cox VI or subunit 6) which contain only a single cleavable methionine residue [6,43], and in the latter case it was shown that the targeting information is contained within the N-terminus of the mature protein [43]. Larger sequences of eight to nine residues are exemplified by mitochondrial hsp60 and several kinetoplast DNA-associated proteins [25,44]. In spite of their small size, they represent functional mitochondrial targeting sig-

nals [26]. Another group of presequences contains 16–27 residues and is found in a variety of membrane-associated proteins [7,10,29,30], as well as in some matrix proteins [45–47]. Even larger cleavable sequences were reported recently [11,46], although the actual targeting signal may represent only a portion of these peptides. Homologous proteins in different species usually possess the same presequence type. The trCOIV sequences apparently fall into the category of large presequences, however, they show an unusual variability among the species (Fig. 1B). The determined sequence in *L. tarentolae* is 31 residues long, while the predicted sequences in the other three species varied from 25–44 residues. The presequences started with a consensus M(F/L)(T/S/A)RR(A/S) and ended with QRR(Y/H), with most variability occurring in the region between these motifs. This variability contrasts sharply with the conservation observed in the hsp60 [44], Rieske iron-sulfur protein [30] and trCOXI (cox VIII) cleavable presequences [7,43], and resembles the situation so far observed only in the trCOXIII (cox X) presequences of *T. brucei* and *L. major* [43]. The reasons why the presequences are conserved in some proteins and variable in others and why a particular presequence type is associated with a particular protein remain unknown. This may be related to minor differences in functions of such proteins in different species, however, nothing is known about the functions of trCOIV at this time. It

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643 should also be noted that the trCOIV presequences in *L.*
 644 *major* and *T. cruzi* contain internal methionines fol-
 645 lowed by conserved arginines. As the actual initiation
 646 codons are unknown, a possibility exists that alternative
 647 presequences are utilized under different physiological
 648 conditions. Additional studies, including silencing of the
 649 expression of this protein by RNA interference, are
 650 under way and can shed light on these problems.

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