THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 9, Issue of March 1, pp. 7222–7230, 2002
Printed in U.S.A.

Unusual Polypeptide Synthesis in the Kinetoplast-Mitochondria from *Leishmania tarentolae*

IDENTIFICATION OF INDIVIDUAL *DE NOVO* TRANSLATION PRODUCTS*

Received for publication, October 9, 2001, and in revised form, December 12, 2001
Published, JBC Papers in Press, December 28, 2001, DOI 10.1074/jbc.M109715200

Anton Horváth‡§, Martina Neboháčová‡, Julius Lukeš¶, and Dmitri A. Maslov‖

From the Department of Biology, University of California, Riverside, California 92521

The *de novo* synthesis of cytochrome *c* oxidase subunits I, II (COI and COII), and apocytochrome *b* (Cyb) was investigated in kinetoplast-mitochondria of *Leishmania*. The organelles were isolated after breaking whole cells with nitrogen cavitation. Individual COI, COII, and Cyb polypeptides were identified by fractionation of the kinetoplast membranes, labeled with [^{35}S]methionine and cysteine, using two-dimensional (9 *versus* 14% and 20 *versus* 11%) denaturing gel electrophoresis. The reaction did not require exogenous energy sources or amino acids. On the contrary, the presence of amino acids other than methionine somewhat inhibited the labeling reaction probably by competing with the uptake of labeled amino acids. The synthesis reaction was insensitive to 100 μg/ml chloramphenicol, gentamycin, paromomycin, lincomycin, hygromycin, and tetracycline, as well as cycloheximide. The process showed a linear increase in the amount of synthesized polypeptides during the first 2 h of incubation, followed by a slower accumulation of products for up to 4 h. The *de novo* synthesized polypeptides were stable for several additional hours. Their assembly into respiratory complexes, investigated using two-dimensional Blue Native/*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine-SDS gels, began early during the incubation and continued throughout the course of the synthesis. This work represents the first unequivocal identification of the polypeptide synthesis in kinetoplasts.

The kinetoplast-mitochondrial genetic system of trypanosomes has revealed features that clearly distinguish it from animal, fungal, or plant mitochondria. The maxicircle component of kinetoplast DNA networks contains cryptic genes whose expression depends on post-transcriptional mRNA editing by uridylate insertions and deletions (1–3). This process is mediated by guide RNAs encoded in the kinetoplast DNA minicircles, which account for the bulk of DNA in these mitochondria. After the discoveries of RNA editing in 1986 (4) and guide RNAs in 1990 (5), there has been a steady progress toward elucidating the mechanism of this process, which has now been demonstrated to involve several enzymes and protein factors (6–10).

At the same time very little is known about other aspects of kinetoplast gene expression, especially at the level of translation. The very existence of a functional translation system remained unproven for almost 3 decades (summarized in Ref. 11). This question is important because kinetoplast translation must utilize templates modified or created by editing, and it is interesting to investigate interactions of these two systems. Early works demonstrated that cycloheximide at low concentrations effectively inhibited cytosolic translation in *Crithidia luciliae* and *Trypanosoma brucei in vivo*. A small fraction of the total cell protein synthesis was resistant to cycloheximide and at the same time insensitive to D-chloramphenicol and other inhibitors at concentrations that inhibit mitochondrial translation in other organisms (12, 13). However, high concentrations (~1 mg/ml) of chloramphenicol substantially inhibited total cell protein synthesis in *T. brucei* (13), whereas in *C. luciliae* the inhibition was close to 50% (14). Unexpectedly, no inhibition was observed in a closely related organism, *Crithidia fasciculata* (15). Consistent with the idea that some permeability barriers might be responsible for the apparent lack of chloramphenicol-sensitive translation in whole cells, the analysis of translation in the isolated mitochondrial fractions of *T. brucei* and *Crithidia oncopelti* showed that protein synthesis is indeed sensitive to lower concentrations of mitochondrial inhibitors (13, 16). A recent analysis of amino acid incorporation by digitonin-treated cells or the isolated kinetoplast-mitochondria of *T. brucei* (17, 18) apparently confirmed the existence of chloramphenicol-sensitive and cycloheximide-resistant kinetoplast translation system. In contrast, this idea was not supported by the results obtained in *C. fasciculata*, which showed that leucine incorporation by digitonin-treated kinetoplast-mitochondria was insensitive to 250 μg/ml chloramphenicol (19). The insensitivity to a broad range of chloramphenicol concentration in this organism was recently shown again, although no attempts to increase the permeabilization were made (20). Although incomplete inhibition in principle can be caused by impermeable barriers, the observed discrepancies can also be attributed to the presence of variable levels of bacterial contamination in kinetoplast preparations, as well as the presence of prokaryotic endosymbionts in some species.

Because of this controversy, a differential sensitivity of polypeptide synthesis to antibiotics, which in other systems served as a reliable criterion to distinguish cytosolic translation from mitochondrial, cannot be used as an evidence for translation in kinetoplasts. The problem can be solved, however, by demonstrating the *de novo* synthesis of individual polypeptides with known kinetoplast origin. We have recently

* This work was supported by Grant AI40634 from the National Institutes of Health (to D. A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.
§ Present address: Dept. of Biochemistry, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia.
¶ Present address: Inst. of Parasitology, Czech Academy of Sciences, and Faculty of Biology, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic.
‖ To whom correspondence should be addressed: Dept. of Biology, University of California Riverside, 3401 Watkins Dr., Riverside, CA 92521. Tel.: 909-787-6485; Fax: 909-787-4286; E-mail: dmitri.maslov@ucr.edu.

identified two such polypeptides, cytochrome *c* oxidase subunit I (COI)[1] and apocytochrome *b* (Cyb), which were found in enzymatically active respiratory complexes IV and III, respectively, from *Leishmania tarentolae* (21–23). The N-terminal sequences of these polypeptides matched the sequences of the corresponding mRNAs, which in the case of Cyb is modified by RNA editing, indicating that they are translated in the kinetoplasts. In this work we have identified the *de novo* synthesized COI, COII, and Cyb polypeptides in isolated kinetoplasts and demonstrated that they assemble into the corresponding respiratory complexes. This work for the first time clearly demonstrates the existence of protein synthesis in kinetoplasts and describes some unusual properties of this process.

EXPERIMENTAL PROCEDURES

Isolation of Kinetoplast-Mitochondria—Cells of *L. tarentolae*, strain UC, were cultivated in brain heart infusion medium supplemented with 10 μg/ml hemin and grown to a density 0.8–1.2 $\times$ 10^8 cells/ml as described earlier (24). The kinetoplast-mitochondrial fraction was isolated after cell rupture by nitrogen cavitation according to the procedure described previously with some modifications (25). The procedure was performed at 0–4 °C. Cells obtained from a 6-liter culture were pelleted by centrifugation at 6,000 $\times$ *g* for 10 min and washed with 20 mM sodium phosphate buffer, pH 7.9, containing 20 mM glucose and 150 mM NaCl. Washed cell were resuspended in 20 mM Tris-HCl, pH 7.5, 600 mM sorbitol, 2 mM EDTA (SoTE buffer) at 4 $\times$ 10^9 cells/ml in a beaker. The suspension was placed inside a Parr 4635 cell disruption chamber and compressed nitrogen was applied at the 65 bar pressure for 60 min. Following the quick release of gas pressure, the cell lysate was centrifuged at 16,000 $\times$ *g* for 10 min. The pellet was resuspended in the original volume of SoTE and $MgCl_2$ was adjusted to 6 mM. DNase I was added to 50 μg/ml, and the suspension was sheared by forcing through a 26-gauge needle and incubated for 30 min. EDTA was added to 6 mM. The unbroken cells and other large debris were removed by centrifugation at 500 $\times$ *g* for 10 min. The pellet was resuspended and centrifugation repeated. Kinetoplasts were recovered from the combined supernatants by centrifugation at 16,000 $\times$ *g* for 10 min. The pellet was resuspended in 30 ml of 60% Percoll made with 10 mM Tris-HCl, pH 7.9, 250 mM sucrose, 0.1 mM EDTA, and the suspension was layered underneath six 20–35% Percoll gradients. The gradients were centrifuged at 150,000 $\times$ *g* for 60 min in a Beckman SW 28 rotor. The kinetoplast band formed in the lower part of the gradient. The organelles were retrieved from the gradients and diluted with excess SoTE buffer. Percoll was removed by two rounds of centrifugation at 16,000 $\times$ *g* for 10 min. The final pellet was resuspended in SoTE at a protein concentration of 20 mg/ml, and kinetoplasts were frozen in liquid nitrogen and stored at −80 °C.

Measurements of Transmembrane Potential—The analysis was done using a Hitachi F-2000 fluorescence spectrophotometer at room temperature using the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) (Molecular Probes) at 622-nm excitation wavelength, 670-nm emission wavelength, and 0.5-s response time in 2-ml samples as described previously (25). The dye uptake was measured using 10–20 μl of the kinetoplast suspension (200–400 μg of protein). The dye release resulting from membrane depolarization was monitored after addition of valinomycin to a final concentration of 2.5 μM.

Polypeptide Synthesis in Whole Cells and Cell Lysates—Cells (10^6) were pelleted by centrifugation, washed with SoTE, resuspended in 100 μl of this buffer, and incubated with 100 μCi of EasyTag™ EXPRE^{35}S^{35}S protein labeling mix (PerkinElmer Life Sciences, >1000 Ci/mmol) at 28 °C for 60 min. Prior to the electrophoresis, labeled cells were extracted with 0.05% Triton X-100 diluted in 50 mM sodium phosphate, pH 7.4, on ice for 30 min and centrifuged at 16,000 $\times$ *g* for 20 min. For translation in cell lysates, we used the preparations obtained during the isotonic isolation of kinetoplasts (described above) directly after cell breakage by cavitation. Conditions of the labeling reaction for lysates were similar to the conditions for whole cells.

In Organello Translation—A translation mixture was prepared similar to the method described by Nabholz *et al.* (17) and contained 600 mM sorbitol, 40 mM Tris-HCl, pH 7.4, 5 mM KH_2PO_4, 20 mM $MgSO_4$, 50 mM KCl, 2.5 mg/ml bovine serum albumin, 4 mM ATP, 0.5 mM GTP, 5 mM NADH, 5 mM succinate, 12 mM creatine phosphate, and 0.1 mM amounts of each amino acid except methionine and cysteine.

The reaction was set according to Refs. 17 and 26 with the following modifications. Kinetoplasts (100–200 μg of protein) were briefly pelleted in a bench-top centrifuge, resuspended in 10 μl of SoTE, and combined with 100 μl of 1.2-fold concentrated translation buffer. After addition of creatine phosphokinase (Sigma) to 0.18 mg/ml and an appropriate antibiotic solution (see below), kinetoplasts were pre-incubated at 28 °C in 14-ml Falcon capped plastic tubes for 10 min with agitation at 100 rpm followed by the addition of 100 μCi (~8–9 μl) of EasyTag™ EXPRE^{35}S^{35}S protein labeling mix or L-[^{35}S]methionine (PerkinElmer Life Sciences, >1000 Ci/mmol). Most reactions included cycloheximide at 50 μg/ml added at the preincubation step. Incubation continued for the specified periods of time followed by chilling on ice, and kinetoplasts were recovered by a 5-min centrifugation at 2,000 $\times$ *g*. The pellet was resuspended in 200 μl of SoTE containing 0.1 mM phenylmethylsulfonyl fluoride and centrifuged again. This was followed by resuspension of the pellet in 50 μl of buffer containing 50 mM sodium phosphate, pH 7.4, and 0.05% Triton X-100, incubation on ice for 30 min, and centrifugation at 16,000 $\times$ *g* for 20 min. Before loading on the electrophoretic gel, the last pellet, resuspended in 50 μl of the Triton buffer, was mixed with an equal volume of denaturing buffer containing 125 mM Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, 30% glycerol, and 0.01% bromphenol blue, and incubated at 37 °C for 30 min.

Stock solutions of the antibiotics were prepared at 50 mg/ml in water (emetine, gentamycin, hygromycin, lincomycin, paromomycin, puromycin, tetracycline) or methanol (chloramphenicol, cycloheximide). Antibiotics were purchased from Fluka and Sigma.

Isolation of Yeast Mitochondria and Translation in Organello—This was done using the strain *S. cerevisiae* S288C according to published methods (26, 27) with minor modifications.

Electrophoretic, Autoradiographic, and Immunochemical Procedures—After translation, the kinetoplast pellets were resuspended in 50 μl of 50 mM sodium phosphate buffer, pH 7.4, containing 0.05% Triton X-100. After a 30-min incubation at 0 °C, the insoluble material was recovered by a 20-min centrifugation at 16,000 $\times$ *g* in an Eppendorf microcentrifuge. The samples were analyzed by single dimension Tris-glycine-SDS gels (28), two-dimensional Tris-glycine-SDS gels (29), and BN/Tricine-SDS-polyacrylamide gels (BN gels) (30) as described previously (23). After electrophoresis, the gels were fixed for 40 min in 10% acetic acid, 50% methanol and stained with Coomassie Blue R250 (Sigma). For autoradiography, stained gels were subjected to four 10-min washes in water followed by 40-min incubation in 1 M salicylate solution and dried with vacuum.

Western blot analysis was performed after semidry electroblotting. The source of anti-COI serum and its immunoaffinity purification were described previously (23). The blots were treated with SuperSignal enhanced chemiluminescence system (Pierce). The procedures utilized for N-terminal amino acid sequencing have also been described earlier (23).

RESULTS

Isolation of Intact Kinetoplast-Mitochondria—Because quality of the kinetoplasts was crucial for efficient *in organello* translation, we compared several isolation procedures. We used the level of electrochemical transmembrane potential in freshly isolated organelles and its maintenance after freezing-thawing as the criteria for intact kinetoplasts. Isolation by the traditional method of hypotonic cell lysis followed by purification in Renografin gradients (24) yielded organelles lacking a potential (data not shown). When kinetoplast were isolated isotonically by nitrogen cavitation (25) followed by Renografin gradient centrifugation, a measurable potential was present (data not shown); however, it was almost completely lost after one round of freezing-thawing. The use of Percoll gradients resulted in organelles with a higher potential (Fig. 1*A*), which was stably maintained after incubation of the organelles on ice for at least 3 h. A measurable fraction of the potential was observed after one cycle of freezing-thawing (Fig. 1*B*). Similar results were obtained after purification of kinetoplasts in Nycodenz gradients (25); however, contamination of the kinetoplasts with unbroken cells and cell debris, as seen in light microscope, was significantly higher, whereas the yield was

[1] The abbreviations used are: CO, cytochrome *c* oxidase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BN, Blue Native; ND, NADH dehydrogenase; Cyb, apocytochrome *b*; SoTE, Tris-HCl, sorbitol, EDTA.

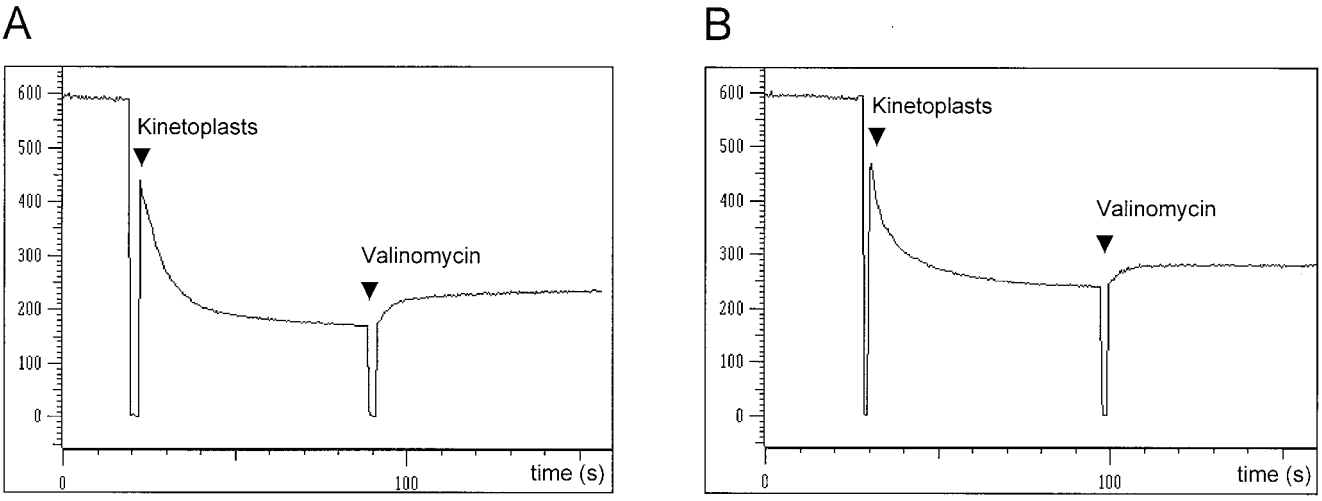

FIG. 1. **Fluorescence measurements of electrochemical transmembrane potential in kinetoplasts of *L. tarentolae* isolated by intracellular nitrogen cavitation and Percoll density gradient centrifugation.** *A*, freshly isolated organelles; *B*, organelles after freezing and thawing. Fluorescence (*vertical axis*) is shown in arbitrary units.

lower. The Percoll procedure was adopted for the subsequent work. It should also be mentioned that the highest potential was observed in kinetoplasts purified by several rounds of low speed differential centrifugation instead of gradients (data not shown). The potential was well maintained after freezing-thawing; however, contamination levels in such preparations were unacceptably high.

Translation in Whole Cells and Cell Lysates—After intact cells were incubated in SoTE buffer in the presence of radioactive methionine and cysteine, they were lysed and the labeled polypeptides were resolved by electrophoresis in denaturing gels. A specific pattern of bands representing the *de novo* synthesized polypeptides was observed (Fig. 2, *lane 1*). A nearly identical pattern was seen when the protein synthesis was performed using cell lysates (Fig. 2, *lane 2*). Cycloheximide and emetine at 50 μg/ml significantly inhibited isotope incorporation. After an exposure time equivalent to that shown in Fig. 2 (*lanes 1* and *2*), no bands were detected on the autoradiogram (data not shown). The observed labeling, therefore, was attributed to cytosolic translation, and the corresponding pattern of labeled bands is hereafter referred to as the whole cell or cytosolic pattern. Although the inhibition by cycloheximide was very efficient, it was not complete, and a longer exposure revealed a residual, cycloheximide-resistant incorporation pattern (Fig. 2, *lanes 3* and *4*). This pattern of bands could be traced to a specific subset of minor bands seen in the whole cell pattern. The "residual" pattern accounts for no more than 4% of the total incorporation as judged by the PhosphorImager analysis of a gel similar to that presented in Fig. 2. Cycloheximide at 1 mg/ml only slightly reduced the incorporation to a level of ~2.5%. The residual translation was also resistant to chloramphenicol at high concentrations (data not shown). Although it could be expected that the cycloheximide-resistant pattern represented kinetoplast translation, further analysis indicated that this was not the case.

Translation in Isolated Kinetoplasts: De Novo Synthesis of COI and Cyb—In the original experiments we used the complete translation mixture as described under "Experimental Procedures." In the course of this work, we found that, surprisingly, the presence of the remaining amino acids and the ATP regeneration system did not stimulate protein synthesis, and that this process can occur in SoTE buffer alone. This property is further addressed in the next section.

When isotonically isolated, gradient-purified organelles were incubated in SoTE buffer with ^{35}S-labeled amino acids without or with cycloheximide, the observed incorporation pattern largely resembled translation in unfractionated lysates or residual translation, respectively (data not shown). The presence of contaminating cytoplasmic membranes was not unexpected because a relatively low, ~3-fold, enrichment of kinetoplasts is achieved by this method (31); however, it had become clear that the residual incorporation could obscure kinetoplast translation.

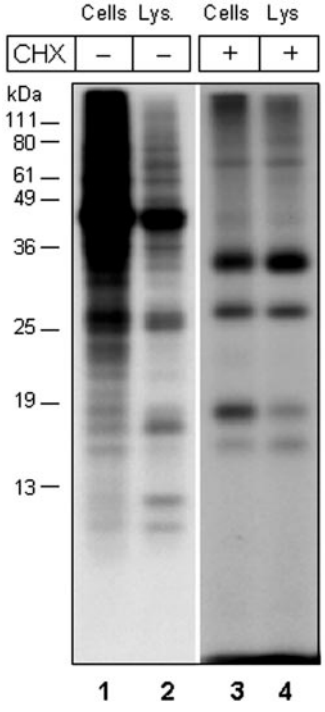

FIG. 2. **Translation in whole cells and cell lysates.** Addition of cycloheximide (*CHX*) is indicated *above* the *lanes*. *Lanes 1* and *3*, whole cells; *lanes 2* and *4*, cell lysate.

An additional complication was caused by the hydrophobic nature of kinetoplast-encoded polypeptides, such as COI and Cyb. We observed earlier that each of these molecules formed a series of aggregation products that were seen in single dimension SDS gels as broad, diffuse bands (22, 23). If these proper-

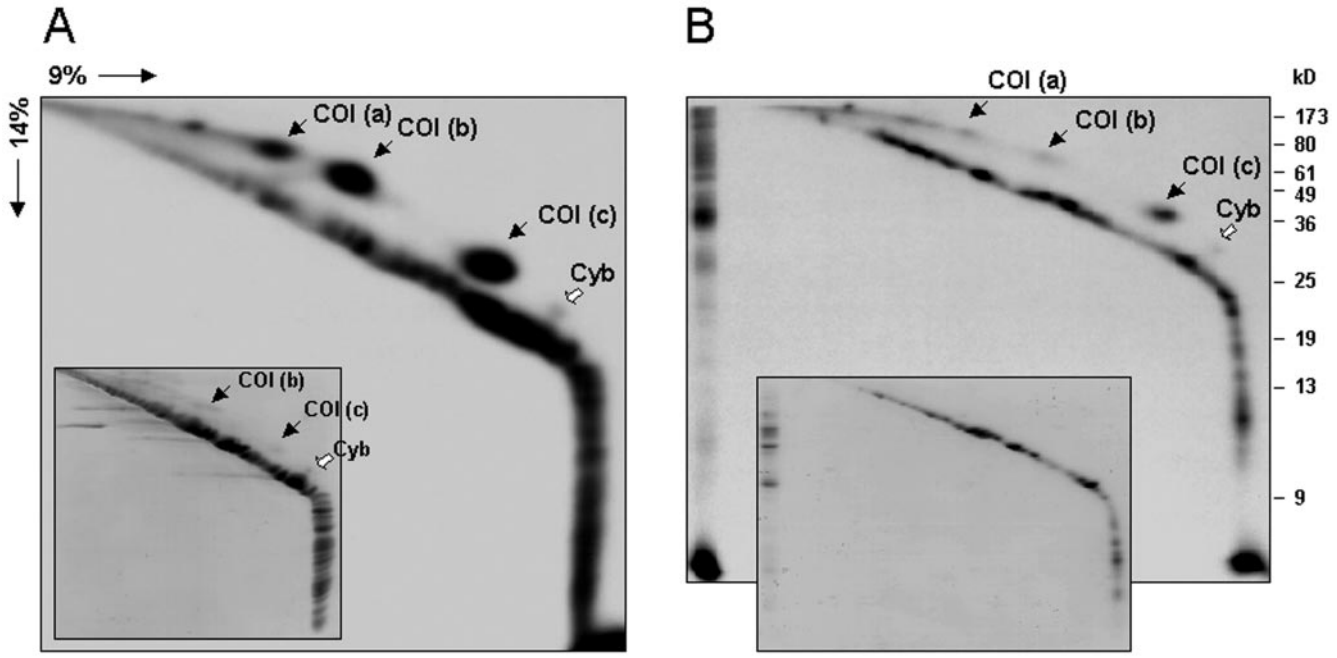

FIG. 3. **Analysis of translation products in isolated kinetoplasts using 9% *versus* 14% two dimensional SDS gels.** *A*, autoradiograph of the gel after kinetoplast labeling with EXPRE^{35}S^{35}S protein labeling mix; *B*, kinetoplasts labeled with L-[^{35}S]methionine; the *lane* at the *left side* of the *panel* represents the same material separated only by the second dimension gel. In both cases, the *inset* panel shows a corresponding gel stained with Coomassie. Positions of Cyb, monomeric COI (*c*), and aggregated COI (*a* and *b*) are indicated.

ties are shared by other kinetoplast-encoded polypeptides, which are also predicted to be hydrophobic, then an overlapping set of bands from the translation products will be represented by a smear.

These factors made single dimension gel electrophoresis unsuitable for the analysis of kinetoplast translation. We, therefore, had to look for an alternative procedure, which would allow us to separate the kinetoplast pattern and identify its individual components. We have previously shown that COI and Cyb polypeptides migrate in the form of well separated spots positioned off the main diagonal in 9 *versus* 14% two-dimensional polyacrylamide-SDS gels. Now, when we applied this gel system to the analysis of the products generated by *in organello* labeling in the presence of cycloheximide, we observed a labeled diagonal and a series of off-diagonal spots (Fig. 3*A*). By correlating the positions of these spots with the known positions of COI and Cyb spots, identified in the Coomassie-stained gel (Fig. 3*A*, *inset image*), we could easily identify the observed *de novo* synthesis products representing these polypeptides. We observed both the monomeric form of COI (spot *c*) and its aggregated forms (spots *b* and *a* and other high molecular weight spots) that we have identified previously (23). The monomeric form of Cyb was also present in its characteristic gel position (22), although its labeling was significantly lower level than that of COI. This difference contrasts sharply with the nearly equal ratio of the corresponding Coomassie-stained bands (Fig. 3*A*, *inset*). Although intensity varied in different experiments, the labeling of the COI spots was always higher than Cyb.

In the COI molecule there are twice as many predicted methionine residues (28 residues) as in Cyb (12 residues), whereas the cysteine content is nearly equal (19 and 20 residues, respectively). The labeling pattern in Fig. 3*A* was produced using *E. coli* ^{35}S-labeled hydrolysate in which [^{35}S]methionine and [^{35}S]cysteine accounted for 73 and 22% of the radioactivity, respectively. The intensity of COI was also greater than Cyb when labeling was done using methionine alone (Fig. 3*B*). Although the methionine content certainly contributes to the observed labeling difference, it is not a sufficient explanation. Therefore, other factors must be responsible.

It is noticeable that the COI aggregates (spots *b*, *a*, and others) were labeled less intensively with [^{35}S]Met than with the labeled mixture. This result is consistent with our previous observation that the spot *b* likely includes a heterodimer of COI and COIII. The COIII polypeptide contains 24 Cys residues and 5 Met residues.

Properties of the Polypeptide Synthesis in Organello—The influence of the components included in translation mixtures, such as amino acids and an ATP regeneration system, was investigated by the experiments presented in Fig. 4. Comparison of the labeling intensity in a complete system (Fig. 4*A*) to incubation in SoTE alone (Fig. 4*D*) showed that there was no stimulation of [^{35}S]Met and Cys incorporation by these additional components. Gel resolution, however, declined when a complete medium was used. Addition of the remaining 18 amino acids at 0.1 mM concentrations to the SoTE buffer medium slightly reduced the incorporation (Fig. 4*C*), whereas their omission from the complete medium slightly increased it (Fig. 4*B*). These results indicate that the reaction utilizes the endogenous pool of amino acids, whereas the exogenously added components may slightly compete with the uptake of the isotope.

The observed situation contrasted sharply with translation in the isolated yeast mitochondria, in which no incorporation was observed in SoTE with or without amino acids in a one h incubation (Fig. 4*E*). Translation medium without the 18 amino acids produced a smeared pattern that is probably the result of incomplete synthesis of polypeptides, which were hence misfolded and aggregated. On the other hand, very efficient polypeptide synthesis was observed after the reintroduction of the missing components. The overall efficiency of yeast mitochondrial synthesis, as judged by the comparison of band intensities, was at least an order of magnitude greater than in kinetoplasts.

The observed synthesis of kinetoplast Cyb and COI was not affected by chloramphenicol, hygromycin, paromomycin, linco-

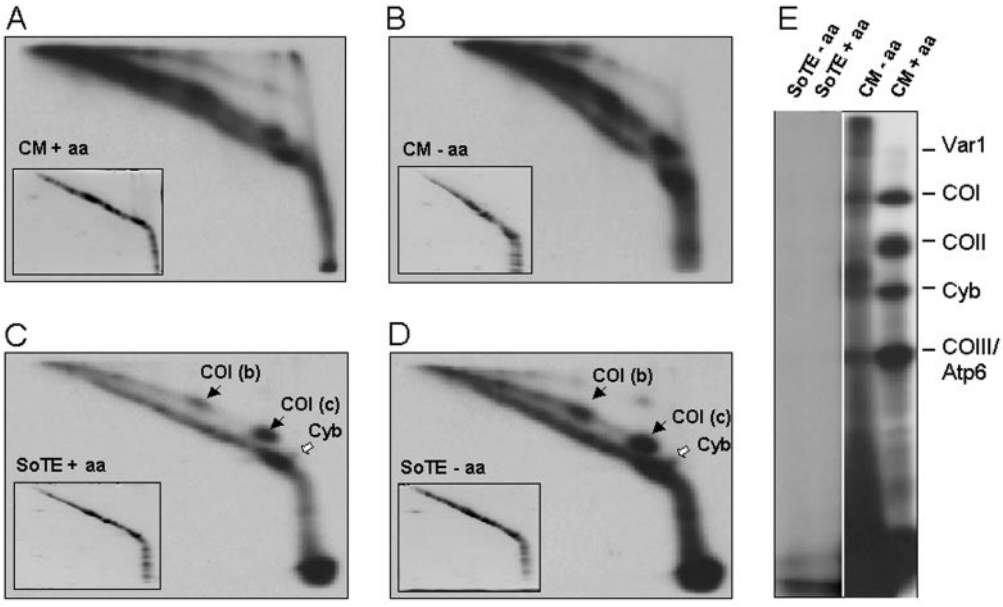

FIG. 4. **Effect of exogenous substrates on translation in isolated kinetoplasts.** *A*, autoradiograph of a 9 *versus* 14% gel of the translation products synthesized in the presence of a complete mixture, as described under "Experimental Procedures." *B*, translation in the identical mixture omitting the amino acids. *C*, translation in the sorbitol-Tris buffer with amino acids. *D*, translation in the sorbitol-Tris buffer without amino acids. *Inset panels* show the corresponding gels stained with Coomassie. *E*, polypeptide synthesis in isolated yeast mitochondria under conditions analogous to *panels A–D*. Labeled products are indicated; *CM*, complete mixture; *aa*, 18 additional amino acids.

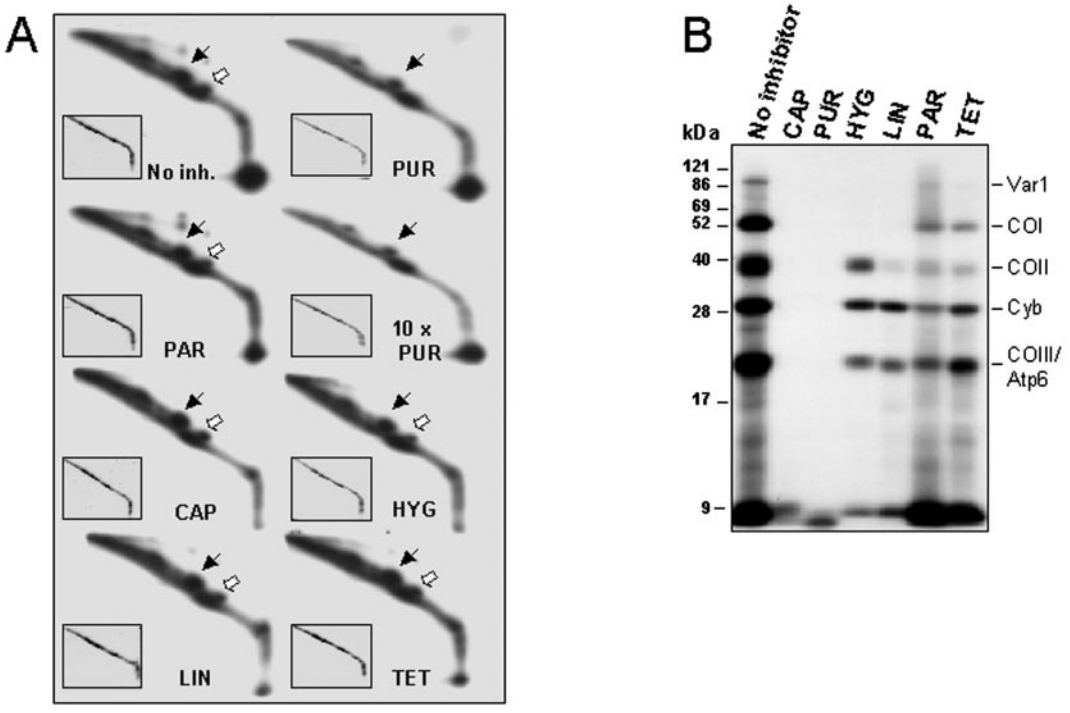

FIG. 5. **Effect of translation inhibitors on the polypeptide synthesis in isolated kinetoplasts.** *A*, autoradiographs of 9 *versus* 14% gels after labeling in the presence of 100 μg/ml paromomycin (*PAR*), chloramphenicol (*CAP*), lincomycin (*LIN*), puromycin (*PUR*), hygromycin (*HYG*), and tetracycline (*TET*). 10× puromycin corresponds to 1 mg/ml. All reactions also contained 100 μg/ml cycloheximide. *Black arrows* show monomeric COI, and *open arrows* show monomeric Cyb. In this set of gels, the COI and Cyb polypeptides migrated closer to the main diagonal than in most other presented gels. The additional off-diagonal spots seen above the COI and Cyb spots are caused by in-gel aggregation of these polypeptides after the first dimension electrophoresis. *B*, effect of inhibitors on translation in yeast mitochondria; autoradiograph of a 14% Tris-glycine-SDS gel.

mycin, tetracycline, and gentamycin at 100 μg/ml (Fig. 5*A*). Interestingly, puromycin strongly inhibited synthesis of Cyb, and synthesis of COI was also noticeably affected. Yeast translation, as expected, was completely inhibited by chloramphenicol and puromycin and was noticeably sensitive to other antibiotics (Fig. 5*B*).

A number of labeled polypeptides are always found in the main diagonal of the kinetoplast two-dimensional gels. We cannot exclude the possibility that some of these proteins correspond to unidentified kinetoplast-encoded translation products. However, taking into account the existence of cycloheximide-resistant residual translation, it is more likely that the diagonal represents translation products from contaminating cytoplasmic ribosomes. Contaminating cytosolic rRNA was de-

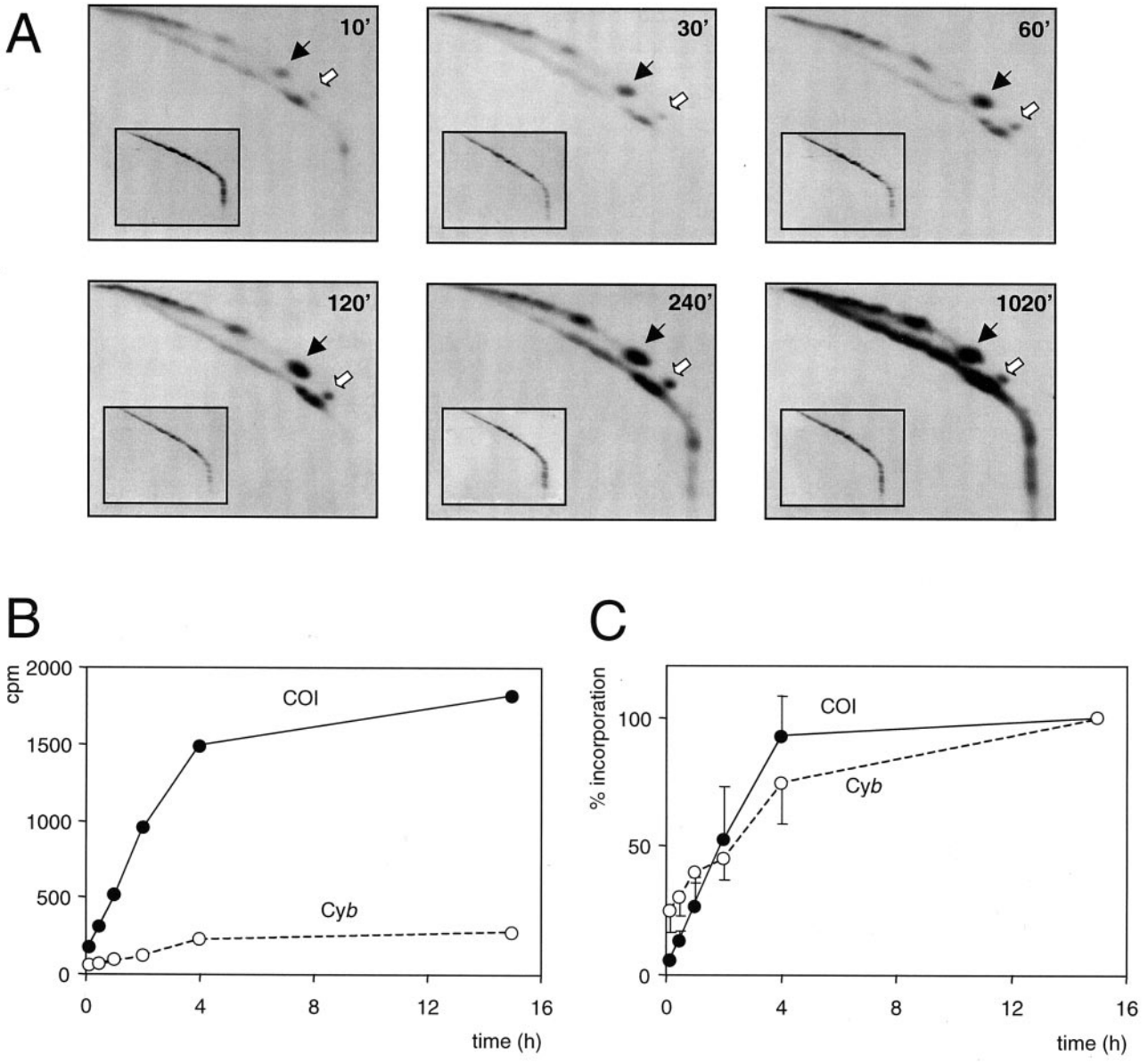

FIG. 6. **Time course of the polypeptide synthesis.** *A*, kinetoplasts were labeled for the various periods of time as indicated. The spots of monomeric COI (*black arrows*) and Cyb (*open arrows*) were excised and radioactivity determined by scintillation counting. *Insets* show the corresponding stained gels. *B*, the rates of incorporation of the isotope in COI and Cyb. *C*, relative rates of incorporation determined on the basis of several labeling experiments. *Vertical bars* show the standard deviation.

tectable in the RNA extracted from the kinetoplast preparations used in these experiments, although it was much less abundant than the kinetoplast 12 and 9 S rRNAs (data not shown). The intensity of the diagonal generally correlated with the level of contamination by unbroken cells and cell debris, as judged by light microscopy. The reasons why a fraction of cytoplasmic ribosomes was insensitive to cycloheximide remain unclear.

Time Course of the Synthesis—Freshly isolated kinetoplasts were incubated in SoTE with isotope for the specified periods of time. Chloramphenicol at 100 μg/ml was included to prevent bacterial growth during long incubations. Control experiments conducted with and without the antibiotic did not show any significant difference in the initial rate of the reaction (data not shown). The aliquots were withdrawn and chilled on ice. The organelles were pelleted by brief centrifugation, washed with SoTE, and lysed with the electrophoretic sample dye. The overall handling time from sample withdrawal to lysis did not exceed 5 min. The samples were analyzed by the two-dimensional gel electrophoresis. After fluorography of the gels, the radioactive spots were excised, and the amount of radioactivity was determined by scintillation counting.

These experiments were performed several times; each time a new preparation of kinetoplasts was used. Some variations in kinetics of labeling were observed, which can be attributed to the differences in physiological conditions of the kinetoplasts, in particular to their intactness after isolation. Results of one experiment are shown in Fig. 6. The amount of newly synthesized COI and Cyb polypeptides accumulated linearly for more than 2 h, followed by a gradually decreasing rate during the overnight incubation (Fig. 6, *A* and *B*). In other experiments the plateau was reached after 4 h, and the products were stably maintained for at least 12 h (data not shown). As no evidence for the product degradation could be seen, the rate of accumulation represented the rate of synthesis. The amount of radioactivity associated with the monomeric COI spot was always 15–20 times higher than with Cyb (Fig. 6*B*). Relative rates of synthesis (Fig. 6*C*) were calculated on the basis of four inde-

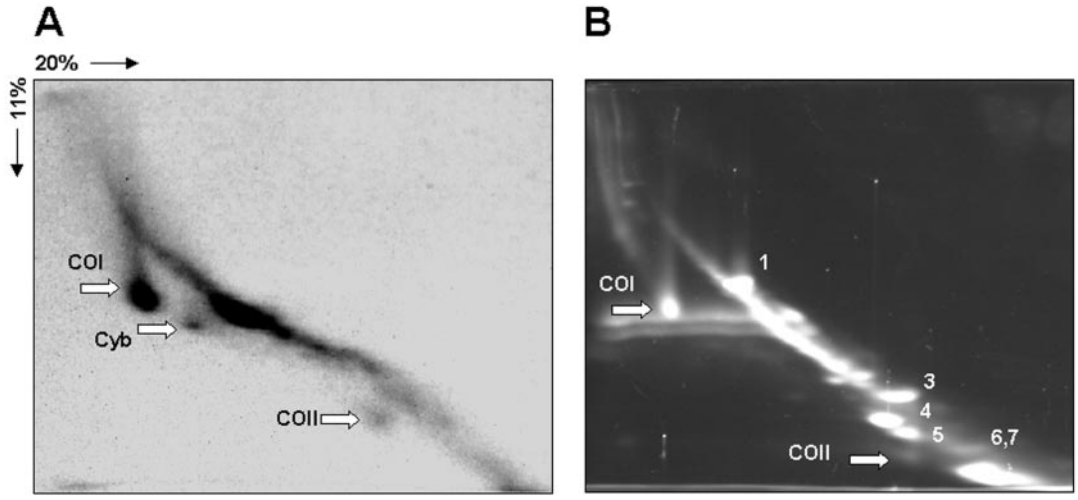

FIG. 7. **Identification of COII.** *A*, analysis of the kinetoplast labeling products in a 20 *versus* 11% two dimensional gel. *B*, separation of cytochrome *c* oxidase subunits in a similar gel. The gel was stained with SYPRO-RED. *Arabic letters* designate several largest nuclear encoded subunits. Mitochondrial encoded subunits COI and COII are as indicated.

pendent data sets with COI and two data sets with Cyb. An apparent decrease of Cyb synthesis at 2 h is most likely the result of experimental error. Nevertheless, the relative rate for Cyb appears to be somewhat slower than for COI. Whether these differences reflect the fact that RNA editing is required for maturation of the Cyb mRNA is unknown. Addition of excess cold methionine completely stopped the incorporation, and the labeled pattern and intensities remained unchanged during an additional incubation for several hours (data not shown).

Identification and de Novo Synthesis of COII—To identify smaller size translation products, we utilized a 20 *versus* 11% two-dimensional gel (Fig. 7*A*). High molecular weight components did not resolve well in these gels, and therefore COI was seen as a single spot. Cyb was represented by a faster migrating spot. An additional weaker radioactive spot was seen off the main diagonal. With the purpose of its identification, we analyzed purified cytochrome *c* oxidase, which was isolated by a procedure described recently (21). The gel was stained with SYPRO-RED, a fluorescent dye with a higher efficiency of staining for hydrophobic polypeptides than Coomassie (Fig. 7*B*). The COI spot stained very well with this dye. In addition, there were also several brightly stained spots near the main diagonal that are the nuclear-encoded subunits of the enzyme (subunit 2 is usually lost from the preparations obtained by DEAE chromatography) (21), as well as contaminating polypeptides. Several low intensity spots were observed off the diagonal. The N-terminal amino acid sequence of the spot positioned near the nuclear-encoded subunit 5 was MAFIL(S/A)F*XX*I. This is nearly identical to the expected sequence of COII (MAFILAFWMI). The staining intensity of the COII spot was substantially lower than COI. Because these subunits are stoichiometric in the holoenzyme, the observed difference in their abundance must be the result of a preferential loss of COII by aggregation.

The position of COII in this gel approximately corresponds to the position of the additional spot in Fig. 7*A*, thus making it very likely to be the same polypeptide. The loss of COII by aggregation can also explain, at least in part, the lower labeling intensity of COII compared with COI. Although the weakly labeled spot could also represent other polypeptides, the BN gel analysis of translation products described below supports the conclusion that it is subunit II of cytochrome *c* oxidase.

The monomeric COIII, which in *Leishmania* is larger than COII, has not yet been identified. It is possible that this highly hydrophobic polypeptide aggregates completely or that the gel used does not separate this polypeptide from the diagonal.

Assembly of the de Novo Synthesis Products into the Respiratory Complexes—The stability of newly synthesized polypeptides may be associated with their incorporation into respiratory complexes. When a dodecyl maltoside extract of kinetoplasts that had been incubated with the isotope for 12 h was analyzed in a two-dimensional BN/Tricine-SDS gel, we observed respiratory complexes with a characteristic subunit composition, similar to what we observed earlier (Fig. 8*A*) (23, 32). The use of 3.5–13% gradient polyacrylamide gels noticeably improved the resolution of ATPase complexes compared with the 6% gels we have used in our previous works. A fluorography of the same gel revealed a series of intensive radioactive bands, some of which were comigrating with respiratory complexes IV and III (Fig. 8*B*). The intensive bands seen in complex IV (*black arrows*) did not match with any nuclear encoded subunits, suggesting that these bands represent COI. Probing the blot of a similar gel with the anti-COI serum (20) confirmed that these bands represent monomeric and multimeric forms of COI (Fig. 8*C*). A number of the faster migrating bands with less labeling intensity and also associated with this complex possibly represent COII, COIII, or aggregates thereof. Specific sera to verify this identification are not yet available, but according to the results described above, a low abundance COII band must be localized to this region.

A single, relatively intensive band comigrated with complex III (Fig. 8*B*, *open arrow*). This polypeptide represents monomeric Cyb, whereas the few products labeled with lower intensity above this band likely represented the Cyb aggregates that we observed earlier (22). Because hydrophobic polypeptides in general, and the kinetoplast polypeptides in particular, migrate anomalously in SDS gels, it was impossible to confirm these identifications by estimating the polypeptides' molecular masses. However, this interpretation of the BN gel data is consistent with the polypeptides' relative labeling intensities described above.

A number of labeled polypeptides migrate at the front of the native dimension including an intensively labeled band with a size close to monomeric COI (Fig. 8*B*). It is possible that some of these polypeptides represent a free pool of kinetoplast translation products; however, the anti-COI serum failed to recognize a cognate polypeptide in this region (Fig. 8*C*), indicating that its free pool must be much lower in abundance than the amount which is found within the assembled complexes. This

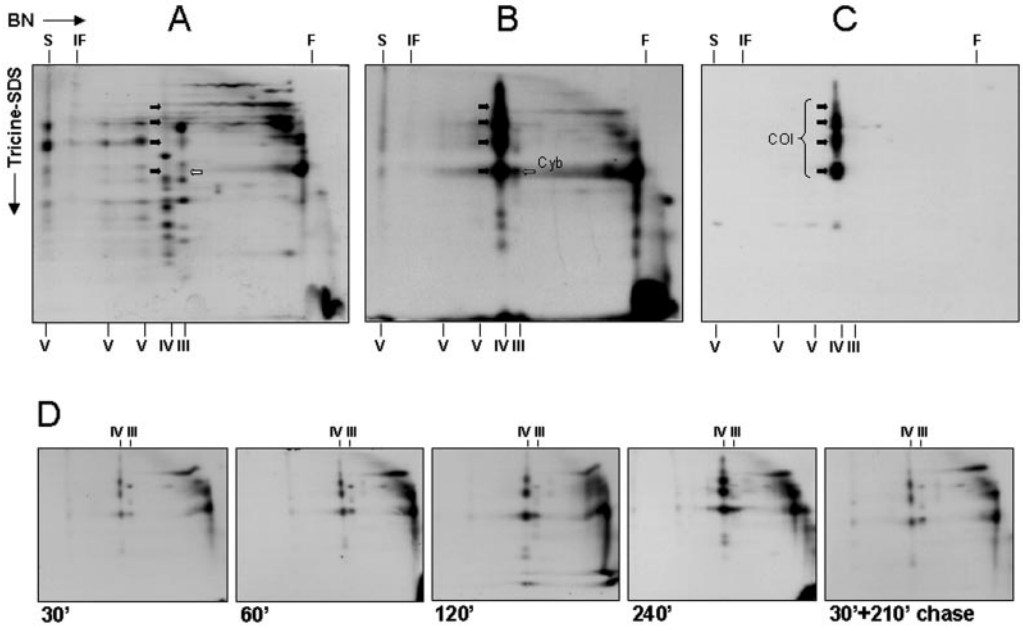

FIG. 8. **Assembly of the synthesized polypeptides into respiratory complexes.** *A*, analysis of the complexes extracted with dodecyl maltoside in a two-dimensional BN/Tricine-SDS gel (staining with Coomassie). Kinetoplasts were labeled for 12 h prior to isolation of the complexes. *S*, start of the native dimension; *IF*, interface of the stacking 3% gel and resolving 3.5–13% gradient gel; *F*, mobility front of the native dimension. The complexes are: *V*, complex V (ATPase); *IV*, complex IV (cytochrome *c* oxidase); *III*, complex III (cytochrome bc_1). *B*, autoradiograph of the same gel. *Black arrows* show COI, and *open arrow* shows Cyb. *C*, results of the probing of the blot of a similar gel with an anti-COI serum. *D*, time course of the assembly. Kinetoplasts were labeled for the specified periods and time and analyzed in BN two-dimensional gels similar to *A*. In the last panel, kinetoplasts were labeled for 30 min followed by chase for 210 min.

also suggests that the newly synthesized subunits are rapidly incorporated into complexes. The labeled material in the front region may contain a low amount of nascent kinetoplast polypeptides, but these labeled polypeptides are probably largely the result of the contaminating cytosolic translation. Therefore, the polypeptides in the front of BN gels are the same as the polypeptides in the diagonal of denaturing two-dimensional gels.

The kinetics of complex assembly was investigated by labeling kinetoplasts for various periods of time up to 4 h, followed by a two-dimensional gel analysis (Fig. 8*D*). Incorporation of the labeled polypeptides into complexes was already seen after labeling for 30 min. The amount of the incorporated material increased steadily during the incubation, and the pattern of labeled bands remained the same as in Fig. 8*B*. The 30-min labeling pattern and its intensity did not change after a 3.5-h chase with the excess of cold methionine and other amino acids. Thus, it appears that the observed kinetics of complex assembly (Fig. 8*D*) is similar to the kinetics of product accumulation (Fig. 8*A*), confirming the conclusion that the entire amount of the newly synthesized polypeptides is rapidly incorporated into respiratory complexes.

DISCUSSION

In this work we have investigated *de novo* polypeptide synthesis in isolated kinetoplasts of *Leishmania tarentolae*. Although it has been known for decades that isolated mitochondria from a number of organisms are capable of faithful translation of the endogenous messengers (33, 34), the progress toward investigating the process of translation in kinetoplasts has been very slow. In fungi, mammals, and plants, a differential sensitivity of the mitochondrial and cytosolic translation systems to cycloheximide and chloramphenicol provided a reliable criterion to distinguish one system from another (35, 36). We found that, in *Leishmania*, although the bulk of the total cell synthesis was highly sensitive to cycloheximide, a so-called residual incorporation had taken place even at high concentrations of the antibiotic. This process, however, could not be attributed to kinetoplast translation mainly because the identified authentic kinetoplast polypeptides, COI and Cyb, were clearly derived from a different translation pattern. Residual synthesis was also insensitive to emetine at 100 μg/ml, as well as chloramphenicol (data not shown).

By the identification of newly synthesized COI and Cyb polypeptides, we were able for the first time to provide clear evidence for the existence of authentic kinetoplast translation *in organello* and investigate some of its properties. The hydrophobicity of these polypeptides, a property usually detrimental for analytical procedures, was utilized for their visualization in denaturing two-dimensional gels off the main diagonal. We could demonstrate that the kinetoplast protein synthesis is resistant to the usual inhibitors of mitochondrial translation. This is at odds with a number of earlier works, in which kinetoplast translation was found to be sensitive to chloramphenicol (16–18), but consistent with the others, which indicated that it is resistant to this antibiotic (12, 15, 19, 20). It should be mentioned, however, that in none of the previous cases were individual polypeptides investigated, so it is very difficult to ascertain that the authentic kinetoplast process was considered in each case.

A possible explanation for the insensitivity of kinetoplast translation to various inhibitors lies in the unusual structure of the kinetoplast ribosomes. The ribosomal large (12 S) and small (9 S) RNAs are the smallest among their counterparts. Several structural domains are entirely missing, and other domains are reduced in these molecules (37–39). Their remaining sequences, including the region of 12 S rRNA that represents the peptidyl transferase center and that is highly conserved in most other organisms, are significantly diverged. The part of the peptidyl transferase region, implicated in chloramphenicol binding that inhibits translation, is partially missing whereas the remainder is highly diverged. Kinetoplast translation had been predicted earlier to be resistant to this antibiotic (39), and our results confirm this.

An unexpected and distinguishing property of the *in or-*

ganello kinetoplast translation is the absence of requirements for exogenous amino acids and additional sources of energy. On the contrary, addition of other amino acids to the translation medium resulted in a slightly decreased incorporation of the labeled methionine. We can explain these results by proposing that most amino acids enter kinetoplasts through a common port and thus compete with the methionine. On the other hand, there is possibly an endogenous pool sufficient for the reaction to proceed over the time. Kinetoplasts also show significantly lower intensity of labeling than yeast mitochondria. This may be caused by a slower rate of importation resulting from a smaller surface to volume ratio in kinetoplasts, as well as a dilution of the imported labeled methionine with the endogenous pool of amino acids in these relatively large organelles. In addition, the kinetoplast translation machinery may be intrinsically slow, as suggested by a linear accumulation of the labeled products over the period of 2–3 h, whereas in yeast and HeLa mitochondria this process usually continues for 30–60 min (40, 41).

The identifiable synthesized products were found to be extremely stable, with little if any degradation after a 15-h incubation. Although little is known about the mechanisms of protein turnover in the kinetoplast, our data show that the stability is associated with rapid incorporation of the synthesized translation products into respiratory complexes. This indicates that there is either an efficient mechanism to exchange individual subunits within the complex or, more likely, that a pool of the imported nuclear-encoded subunits is sufficiently large to entirely absorb the newly synthesized kinetoplast subunits during the assembly process.

The amount of radioactivity associated with COI is 15–20 times higher than with Cyb. As mentioned above, this difference cannot be attributed solely to different methionine content. A preferential loss of Cyb caused by aggregation is also unlikely, because both polypeptides can be visualized by Coomassie staining with nearly equal, although low, intensity (Fig. 3*A*, *inset panel*) (22, 23). Relative rates of labeling are also similar (Fig. 6). Assuming that both polypeptides are equally stable, these results indicate that a higher amount of COI is synthesized in isolated organelles. A primer extension analysis of relative amounts of these mRNAs in the steady state population indicated that the level of COI mRNA is similar to edited Cyb mRNA, and that the level of unedited Cyb mRNA is lower.[2] So, the reason for a higher amount of synthesized COI in isolated organelles remains unclear. It also needs to be investigated whether production of these polypeptides is more balanced *in vivo*.

The nature of additional labeled products observed in BN gels remains unknown. One kinetoplast-encoded polypeptide (MURF 4) was previously identified as ATPase subunit 6 (A6) (42). Although some radioactivity was observed in the regions of complex V in Fig. 8*B* and similar experiments, a trailing of the intensive COI bands in the, native dimension may obscure weakly labeled A6 bands. Because another trypanosomatid, *Phytomonas serpens*, lacks cytochrome *c* oxidase, this species can be used in future to identify A6 (32, 43).

The labeled spots in BN gels migrating with the mobility front may represent products translated on contaminating cytosolic membranes. It is not clear yet whether any of these bands represent subunits of NADH dehydrogenase. Seven such subunits are encoded in kinetoplast DNA (ND1, ND3, ND4, ND5, ND7, ND8, and ND9) (44). However, there is no high molecular weight complex that could be a candidate for being complex I. It is possible that this complex is very fragile in *Leishmania* and falls apart during solubilization of membranes generating multiple subcomplexes or that it is down-regulated under culture conditions in this organism. This problem requires further investigation.

[2] D. A. Maslov, unpublished observations.

Acknowledgments—We thank L. Simpson and M. Peris for critical reading of the manuscript, A. Schneider for details of the kinetoplast isolation protocol, T. Kingan for protein sequencing, and M. Grunstein for the yeast strain.

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Unusual Polypeptide Synthesis in the Kinetoplast-Mitochondria from *Leishmania tarentolae* : IDENTIFICATION OF INDIVIDUAL DE NOVO TRANSLATION PRODUCTS

Anton Horváth, Martina Nebohácová, Julius Lukes and Dmitri A. Maslov

J. Biol. Chem. 2002, 277:7222-7230.
doi: 10.1074/jbc.M109715200 originally published online December 28, 2001