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Characterization of the respiratory chain from cultured *Crithidia fasciculata*

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Abstract

Mitochondrial mRNAs encoding subunits of respiratory-chain complexes in kinetoplastids are post-transcriptionally edited by uridine insertion and deletion. In order to identify the proteins encoded by these mRNAs, we have analyzed respiratory-chain complexes from cultured cells of *Crithidia fasciculata* with the aid of 2D polyacrylamide gel electrophoresis (PAGE). The subunit composition of F₀F₁-ATPase (complex V), identified on the basis of its activity as an oligomycin-sensitive ATPase, is similar to that of bovine mitochondrial F₀F₁-ATPase. Amino acid sequence analysis, combined with binding studies using dicyclohexyldiimide and azido ATP allowed the identification of two F₀ subunits (b and c) and all of the F₁ subunits. The F₀ b subunit has a low degree of similarity to subunit b from other organisms. The F₁ α subunit is extremely small making the β subunit the largest F₁ subunit. Other respiratory-chain complexes were also analyzed. Interestingly, an NADH: ubiquinone oxidoreductase (complex I) appeared to be absent, as judged by electron paramagnetic resonance (EPR), enzyme activity and 2D PAGE analysis. Cytochrome c oxidase (complex IV) displayed a subunit pattern identical to that reported for the purified enzyme, whereas cytochrome c reductase (complex III) appeared to contain two extra subunits. A putative complex II was also identified. The amino acid sequences of the subunits of these complexes also show a very low degree of similarity (if any) to the corresponding sequences in other organisms. Remarkably, peptide sequences derived from mitochondrially encoded subunits were not found in spite of the fact that sequences were obtained of virtually all subunits of complex III, IV and V. © 1997 Elsevier Science B.V.

Keywords: RNA editing; Trypanosomes; Mitochondrion; Respiratory chain; 2D gel analysis; Kinetoplast

Abbreviations: BNPS-skatol, 2-(2-Nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; cox, cytochrome c oxidase; DCCD, N,N'-dicyclohexyldiimide; DCPIP, 2,6-dichlorophenolindophenol; HIC, hydrophobic interaction chromatography; mt, mitochondrial(ly); nt, nucleotide; NBT, 4-nitro-blue-tetrazolium; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride.

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1. Introduction

Trypanosomatid protozoa are unicellular parasitic organisms belonging to the order kinetoplastida. One of the most striking peculiarities of these organisms is the intricate way in which mitochondrial (mt) pre-mRNAs, encoded by the mt maxicircle, are post-transcriptionally edited by insertion and deletion of uridylyate residues under the direction of guideRNAs (for reviews, see [1–5]). The extent of editing varies between transcripts in a species-dependent fashion, but in virtually all cases editing is essential for the production of translatable RNAs.

It is not known why the expression of mitochondrial genes in trypanosomatids requires the extra step. In principle, RNA editing could provide an extra level of regulation (see [1–5]), but Riley et al. show that the abundance of unedited apocytochrome *b* RNA and not that of the necessary gRNAs determines the frequency of editing [6]. This suggests that, at least in this case, regulation does not occur at the level of RNA editing, but rather at the level of transcription or RNA stability. It has further been observed that RNA editing speeds up the rate at which mt protein sequences evolve [7], but it is difficult to see this as a specific role for RNA editing, since a high rate of evolution seems at odds with the highly conserved structure and function of many of the mitochondrial proteins. As a third possibility, it could be envisaged that RNA editing functions in allowing the production of multiple proteins from one gene via alternative editing of mRNAs, in analogy to alternative splicing. Partially and, to a lesser extent, differentially edited RNAs have indeed been observed (reviewed in [2,4,5,8]) and at least in theory, the potential to create protein heterogeneity via their translation is enormous. However, the lack of mt protein sequence data has hampered the verification of this hypothesis and it has not even been formally proven yet that the edited mRNAs are indeed used by the mitochondrial protein synthesizing machinery.

For these reasons, we have initiated the analysis of mt proteins, supposedly encoded by edited mRNAs, in cultured cells of the insect trypanosoma-

matid *Crithidia fasciculata*. The trypanosomatid mt genes can be classified as follows [1–5]: seven genes encode products with a low identity but distinct homology to complex I (NADH-dehydrogenase, ND) subunits found in other eukaryotes (called ND1,3–5,7–9, respectively), one gene encodes apocytochrome *b*, a subunit of complex III (cytochrome *c* reductase), whereas three genes encode subunits 1, 2 and 3 of complex IV (cytochrome *c* oxidase, *cox*). The assignment of the MURF4 gene to encode subunit 6 of complex V (F_0F_1 -ATPase, ATP) has been controversial due to a marginal level of similarity to ATP6 from other organisms [3,9]. Of the RNAs encoded by trypanosomatid mt genes, only the *cox1* and the ND1,4 and 5 mRNAs are completely unedited.

So far, complex III and IV have been purified from the insect trypanosomatid *Crithidia fasciculata* [10,11]. Unfortunately, candidate mt encoded proteins appeared to have a blocked N-terminus [10,11] and attempts to obtain internal sequences have failed so far, presumably due to the extreme hydrophobicity of the proteins in question ([10], D. Speijer et al., unpublished observations). Little information is available on the subunit composition of the other complexes of the trypanosomatid respiratory chain. The F1 part of mt F_0F_1 -ATPase has been characterized from a number of species and appeared to consist of either four or five subunits (reviewed in [12]). The F_0 part has not yet been studied in any detail and it is unknown whether it contains the ATP6/MURF4 gene product. It is also unknown which (if any) of the genes encoding (potential) subunits of complex I are expressed in cultured trypanosomatids. Insect form *Trypanosoma brucei* and other African trypanosomes metabolize succinate via the respiratory chain, starting with succinate dehydrogenase [13,14]. At this developmental stage, they do not appear to synthesize a conventional complex I, although there is one recent report of the existence of a rotenone-sensitive NADH dehydrogenase in these organisms [15]. In cultured *T. brucei*, the concentration of fully edited (i.e. translatable) ND subunit mRNAs is generally (much) lower than that of their unedited counterparts, whereas in laboratory strains of *C. fasciculata* and *Leishmania tarentolae* translatable mRNAs for some ND subunits are absent [1–5,16].

In this paper, we specifically focus on the detailed characterization of *C. fasciculata* F_0F_1 -ATPase, but complex III and IV were also analyzed. Our analysis failed to provide evidence for the presence of a typical complex I, however.

2. Materials and methods

2.1. Cell growth and preparation of mitochondrial vesicles or extracts

C. fasciculata was grown with shaking and aeration in batches of 10 l, as described in [17], to a density of approximately 1.1×10^8 cells ml^{-1} . Mt vesicles from *C. fasciculata* were isolated according to the method described in [18]. Routinely, the mt vesicle preparation was enriched 50–100-fold, as judged by Northern-blot analysis with a mt DNA segment containing the rRNA genes as a probe. *T. brucei* procyclic form was grown as described [19] in batches of 125 ml. Mt vesicle isolation was performed with the aid of a 20–35% renografin gradient, essentially as described by Feagin et al. [20]. Mt vesicles from 10 l of cells were lysed in 15 ml 0.1–0.5% (v/v) Triton X-100 (depending on the experiment) in 50 mM potassium phosphate, pH 7.5 (or 20 mM Hepes–KOH, pH 7.6) and 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by centrifugation at $10\,000 \times g$ for 15 min at 4°C. The pellets were extracted with 3% (w/v) lauryl maltoside in the presence or absence of 300 mM KCl. After centrifugation at $10\,000 \times g$ for 15 min at 4°C to remove insoluble debris, the resulting supernatant contained all of the respiratory-chain complexes, except complex I (see below). Protein concentrations were determined by the method of Lowry [21].

2.2. Hydrophobic interaction chromatography of mitochondrial extract

Mt extract (50 mg) was loaded onto a 20 ml methyl-hydrophobic interaction chromatography (HIC) column (Biorad), equilibrated in 1.5 M $(\text{NH}_4)_2\text{SO}_4$ in 50 mM potassium phosphate, pH 7.5 and 0.05% (w/v) lauryl maltoside, as described in [11]. A fraction enriched for respiratory-chain

complexes was prepared by eluting the column at 850 mM $(\text{NH}_4)_2\text{SO}_4$ in the same buffer (see Section 3 and [11]).

2.3. Purification of bovine mt and spinach chloroplast F_0F_1 -ATPase

After sonication of bovine mitochondria and the isolation of submitochondrial particles the ATPase was separated from the other respiratory-chain complexes by differential precipitation in the presence of 1 mM ATP and 0.5 M Na_2SO_4 as described in [22]. Spinach chloroplast F_0F_1 -ATPase was isolated as described in [23].

2.4. Purification of the F_1 fraction from F_0F_1 -ATPase

Mt vesicles from 1.1×10^{11} cells of cultured *C. fasciculata* were lysed by sonication (3×10 s) on ice in the presence of 1 mM ATP, followed by extraction with chloroform, as described by Beechey et al. [24], to obtain the F_1 part from the F_0F_1 -ATPase. Although not completely pure, proteins of 50, 40, 33, 23 and 10 kDa, found to comprise purified F_1 -ATPase from *C. fasciculata* [25] were among the major protein components in this preparation (see Section 3). Bovine F_1 -ATPase was isolated according to the procedure of Knowles and Penefsky [26].

2.5. Electron paramagnetic resonance (EPR) and visible spectrophotometry

EPR and visible spectrometry were performed with complete mt vesicles and a fraction enriched for respiratory-chain complexes (see above). EPR measurements at X-band (9 GHz) were performed with a Bruker ECS 106 EPR spectrometer with a field-modulation frequency of 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 cryostat equipped with ITC4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter. The X-band frequency was measured with an HP 5350B microwave frequency counter.

Succinate dehydrogenase (complex II) was identified by the presence of the ubiquinone/-

ubiquinone' biradical signal (approximately 2 μM) and the specific signal for Fe-S cluster 1 of succinate dehydrogenase ($g_{\text{Syz}} = 1.919, 1.935, 2.0255$, approximately 1.3 μM) upon reduction of the sample with sodium dithionite [27]. Cytochrome *c* reductase (complex III) was identified by the presence of the Rieske Fe-S cluster (approximately 3.2 μM) with $g_z = 2.026$ and $g_y = 1.89$ in the (reduced minus oxidized) difference spectrum [27]. Cytochrome *c* oxidase (complex IV) was identified by the presence of the EPR signal of the Cu_A centre [11] and the signal of cytochrome *a* ($g_{\text{Syz}} = 1.49, 2.23, 2.99$; approximately 4 μM) of the haem *a*. No EPR signal indicating the presence of a normal complex I (e.g. the typical signal coming from the Fe-S clusters 2 [27]) could be identified in any mt preparation.

Visible spectra were obtained with the aid of a Beckman DU-70 spectrophotometer, as described in [11]. The presence of complex III and IV was indicated by a peak in reduced minus oxidized spectra at 560 and 605 nm, respectively.

2.6. Enzymatic activity measurements

F_0F_1 -ATPase activity was measured as described in [28] in the presence or absence of oligomycin (4 $\mu\text{g ml}^{-1}$), with a 2 min preincubation step with 1% lecithin. Activity of complex III was measured by following the reduction of cytochrome *c* at 550 nm by ubiquinone₂H₂, in the presence or absence of antimycin A (2 $\mu\text{g ml}^{-1}$), according to [29]. Complex IV activity was measured as KCN (2 mM) sensitive oxidation of 25 μM reduced horse-heart cytochrome *c* (Sigma), in a buffer containing 30 mM sodium/potassium phosphate, pH 7.4, 0.1% lauryl maltoside and 2 mM antimycin A, by following the disappearance of 550 nm absorption, as described in [11]. The possible presence of complex I was investigated by following the oxidation of NADH (monitoring the decrease in absorption at 340 nm) in the presence or absence of 2 $\mu\text{g ml}^{-1}$ of rotenone in a buffer containing 25 mM potassium phosphate, pH 7.4, 0.1% lauryl maltoside, 2 mM KCN, 2 $\mu\text{g ml}^{-1}$ antimycin A, 40 mM ubiquinone₀ and 2.5 mg ml^{-1} bovine serum albumin (BSA) [30]. Blue

native first dimensions (see next section) were also tested directly for the presence of NADH dehydrogenase activity by staining with 4-nitro-blue-tetrazolium (NBT) in the presence of menadione according to [31]. The possible presence of complex II was investigated by following the oxidation of succinate coupled to the reduction of 2,6-dichlorophenolindophenol (DCPIP), monitoring the decrease in absorption at 600 nm, in a buffer containing 25 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 0.1% lauryl maltoside, 2 mM KCN, 2 $\mu\text{g ml}^{-1}$ rotenone, 2 $\mu\text{g ml}^{-1}$ antimycin A, 20 mM succinate and 50 μM 2,6-dichlorophenolindophenol [32].

2.7. Polyacrylamide gel electrophoresis analysis

The 'blue native' 2D polyacrylamide gel electrophoresis (PAGE) system, developed for the analysis of respiratory-chain complexes from bovine mitochondria [33] was used with *C. fasciculata* mt lysate and methyl-HIC fractions, essentially as described in [11]. A varying amount of protein/lane, as indicated in the figure legends, was layered onto the non-denaturing first dimension minigel in 1.1 M aminocaproic acid, 60 mM Bistris, 0.5–3.0% laurylmaltoside and 0.4% Serva blue G. The first dimension was run for 4 h at 60 V. Individual lanes were excised, incubated for 1 h in 1% SDS, 1% 2-mercaptoethanol and put on top of a second dimension Tris–tricine/SDS gel, followed by electrophoresis for 4 h at 100 V.

To elute the complexes, gel slices (100 μl) were incubated for 3 h at 4°C in 500 μl 50 mM Tricine, 15 mM Bistris pH 7 and 0.1% laurylmaltoside. The subunit composition of eluted complexes was analyzed by conventional Tris–tricine/SDS gels, for activity measurements, see previous section.

2.8. *N,N'*-dicyclohexyldiimide and 2-Azido-ATP modification

About 1 mg of protein was incubated in 200 μl with 2 μCi (40 nmol) of ¹⁴C-labeled *N,N'*-dicyclohexyldiimide (DCCD, 50 mCi mmol⁻¹), as described in [34] or with α -³²P-labeled 2-Azido-ATP, as described in [35]. The labeling of proteins was analyzed by 2D gel analysis followed by autora-

Table 1
Composition of the respiratory chain in *C. fasciculata*

Source	Complex I ^a	II ^b	III ^c	IV ^d	V ^e
Mt membranes	–	+	+	+	+
850 mM salt fraction	–	+	+	+	+
Complex A ^f	–	–	–	–	+
Complex A	–	–	–	–	+
Complex B ^f	–	–	–	+	–
Complex B	–	–	–	–	–
complex C	–	–	+	–	–
Complex D	–	–	–	–	–
Complex E	–	–	–	–	–

^a–^e Elution and spectroscopic and activity measurements of respiratory-chain complexes were carried out as described in Section 2. The 850 mM salt fraction refers to material eluting from a methyl HIC column at 850 mM (NH₄)₂SO₄.

^f Rotenone-sensitive oxidation of NADH and complex I specific EPR signals could not be detected in any of the fractions.

^b Complex II activity could not be detected in any of the gel eluates, but could be detected in mt membranes and the 850 mM salt fraction. Activity: 200 nmol succinate oxidized min⁻¹ mg⁻¹.

^c–^e Complex III to V activity was detectable in mt membranes and the 850 mM salt fraction.

^c Complex III activity could only be detected in the gel eluate containing complex C. Activity: 100 nmol cytochrome *c* reduced min⁻¹ mg⁻¹, which could be fully inhibited by 2 μg ml⁻¹ antimycin A (compare [33]).

^d Complex IV activity could only be detected in the gel eluate containing complex B^f but not in B, due to the large amount of Coomassie blue that co-elutes with the complex and interferes with the activity measurement. Activity: 60 nmol cytochrome *c* oxidized min⁻¹ mg⁻¹, which could be fully inhibited by 2 mM cyanide (compare [33]).

^e Complex V activity could only be detected in the gel eluates containing complexes A to A^f. Activity: 2 μmol ADP generated min⁻¹ mg⁻¹, which could be inhibited to 1.4 μmol ADP generated min⁻¹ mg⁻¹ by 4 μg ml⁻¹ oligomycin (compare [33]).

diography with X-omat AR film. For ¹⁴C-DCCD-labeled proteins the gels were soaked in 250 mM salicylic acid for 10 min prior to drying.

2.9. Peptide micro-sequencing

PAGE was followed by semi-dry blotting onto polyvinylidene difluoride (PVDF) membranes (Biorad or Immobilon-P), according to the protocols provided by the manufacturers. Blotted proteins were either sequenced directly (from Biorad membranes) or digested with 2-(2'-Nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine (BNPS-skatol) according to [36] (on Immobilon-P membranes). The resulting peptides were run on a 15% SDS-PAGE minigel and blotted again onto PVDF membranes (Biorad). N-terminal and internal sequencing was done with a Perkin Elmer/Applied Biosystems Procise 494 protein sequencer, or a Beckman-Porton LF 3200 protein sequencer.

3. Results

3.1. Composition of the respiratory chain in cultured *C. fasciculata*

We first determined composition and properties of the respiratory chain in cultured *C. fasciculata* by assaying the enzyme activity of the different complexes in the crude mt membrane fraction in standard assays for complexes I, II, III, IV and V, in combination with visible and EPR spectrometry (see Section 2). As summarized in Table 1, we find clear evidence for the presence of active complexes II, III, IV and V, with properties similar to those of eukaryotic respiratory-chain complexes in other organisms (compare e.g. [27–30] and [32]). Remarkably, complex I scored negative in all assays: all EPR signals were missing (e.g. the typical Fe-S clusters 2) and no rotenone-sensitive NADH dehydrogenase activity could be detected (see Table 1 and Section 2). Table 1 also shows that the same complement of respiratory-chain

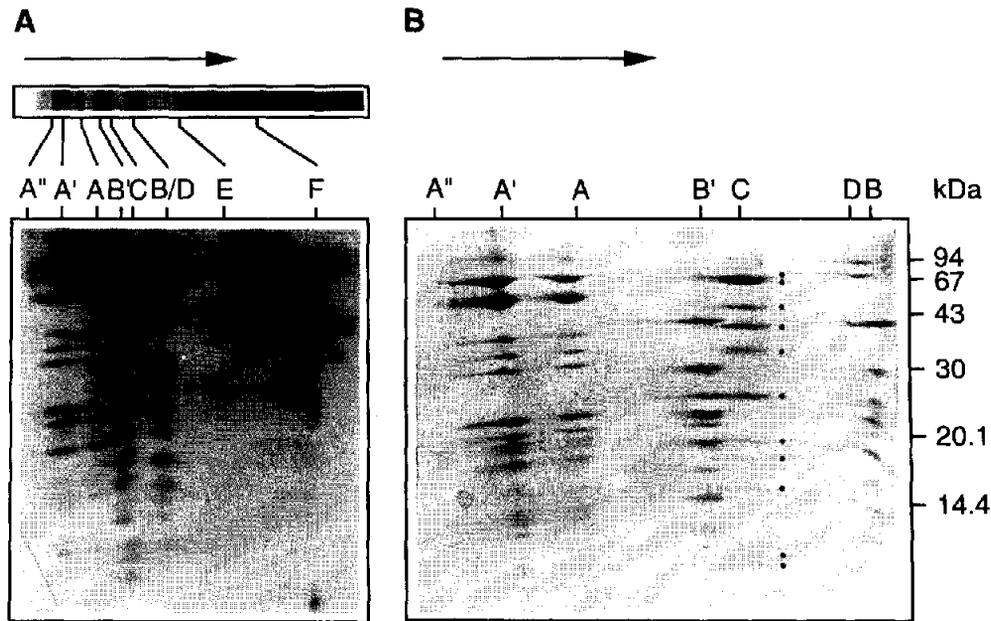


Fig. 1. 2D PAGE of mt membrane proteins of *C. fasciculata* (A) 5 μ g of protein of a mt membrane preparation obtained by lysis of mt vesicles with 0.5% (v/v) Triton X-100 was analyzed by 2D 'blue native' PAGE with a gradient (5–13% w/v) first dimension gel, as described in Section 2. The beginning of the arrow above the figure marks the top of the first dimension gel (shown above the 2D gel). The 2D gel was calibrated with marker proteins the sizes of which are indicated. Staining was with silver. A, A', A'': mono and multimeric forms of complex V; B, B': mono and dimeric forms of complex IV; C: complex III; D: unknown complex; E: putative succinate dehydrogenase; F: front. (B) 20 μ g of protein of a 850 mM $(\text{NH}_4)_2\text{SO}_4$ fraction from a methyl-HIC column (see Section 2) was analyzed by 2D 'blue native' PAGE with a linear (5% w/v) first dimension gel. This gel was stained with Coomassie brilliant blue. For other details, see A.

activities is present after purification by chromatography of the mt membrane fraction on a methyl-HIC column (see Section 2).

As a tool in the identification of the mt encoded subunits of respiratory-chain complexes of *C. fasciculata*, we employed the 2D PAGE procedure used in the analysis of the bovine respiratory chain: the 'blue native' gel system [11]. One of the major advantages of this gel system is the combination of a first dimension non-denaturing gel, which facilitates the identification of (at least some of) the respiratory-chain complexes via activity assays of gel-eluted material, with a second dimension SDS-PAGE to analyze their respective subunit compositions. Fig. 1A shows the results of a typical experiment in which the subunit composition was analyzed of the different complexes (labeled A–E, F representing the front), that are present in the

crude mt membrane preparation of *C. fasciculata*. In Fig. 1B, the results are shown of a similar experiment with the methyl-HIC column fraction. Although the gel percentages used were different, it is clear that at least complexes A–D are still present. Since the use of the methyl HIC column fraction consistently results in better separation of respiratory-chain complexes in 2D PAGE (presumably because it is freed from lipids and other components present in the crude membrane fraction), it is used in most of the experiments reported below. It should be stressed however that all experiments in which we searched for complex I were also performed with solubilized mt vesicles (see Section 2) to avoid its possible loss.

Next, the identity of most of the complexes was determined in enzyme activity assays with gel-eluted material (Table 1). Oligomycin-sensitive

Table 2
Peptide sequences of subunits of complex B, C, D and E.

Complex C (complex III)			Complex III [10]		
Band	MM	Sequence	Band		
1	68	IYQYKFGQTP	1		
2	62	IYEYKFGQPSLkAFGTNI	3		
3	48	Blocked N-terminus (int.) WDEEFVkkHLTP (int.) WvcLgPAQLiyT	2(?)		
4	40	VSLLVKQLEGTTP	4		
5	35	AGKKAHPIKRDWY	5		
6	27	MAQGIWAGFRYYIGHFFYPNMYREFII	6		
7	19	APPKASLPARLFAGDFMGI	7		
Complexes D and E			Complex B (complex IV [11])		
Band	MM	Sequence	Band	MM	Sequence
D1	94	ALSRAYPV	1	41	Blocked N-terminus (int.) WDIIDAL
D3	41	VHSVAVVIINSVcAGGAE			(int.) WYKRNT
D5	29	IYTEwGSVPcE			
D8	18	SDSQKVRAADAWQ	2/3	30	Blocked N-terminus (int.) WSYELTYL
F1	48	Blocked N-terminus			(int.) WVQFRAFNFKK
E2	30	Blocked N-terminus			(int.) WVaRVPEFM
E3	28	REVE <u>ELN</u> V <u>PQELV</u> L <u>e</u>	4	26	Blocked N-terminus (int.) WeMNFNMT

N-terminal and internal sequences are given of complex subunits numbered according to decreasing size, as indicated with dots in Fig. 1B for complex C (III) and in Fig. 2A for complex D. Numbering of complex B (IV) and N-terminal sequences of subunit 5 to 10 can be found in [11]. Lower case letters indicate that the identity of the amino acid was uncertain. Internal (int.) sequences are from peptides generated with 2-(2'-Nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine. 2-(2'-Nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine cleaves the peptide bond C-terminal to tryptophan [36], therefore the W at the beginning of the internal sequences is inferred. Bold type amino acids differ from the sequence of complex III subunits published in [10], the sequence in italics is an extension. The identification of our band 3 as band 2 in [10] is tentative, based on the fact that it is the only blocked major band in both preparations migrating at approximately the same position. Only the indicated subunits of complex D were sequenced; no obvious homologies were found. Residues of subunit 3 of complex E that are conserved in subunit 3 of yeast complex II [38] are underlined. The molecular mass (MM) is given in kDa.

ATPase activity was found to be associated with complexes A'', A' and A, indicating that these complexes represent different forms of F₀F₁-ATPase (complex V). In other organisms multimerization of F₀F₁-ATPase has been observed [37] so A'' and A' most likely represent multimeric forms. Antimycin A-sensitive cytochrome *c* reductase (*bc*₁ complex, complex III) activity and cyanide-sensitive cytochrome *c* oxidase activity were found to co-localize in the region containing complexes B' and C. We had already identified complex B' and B as different forms of cytochrome *c* oxidase [11] and we inferred, therefore, that complex C is complex III. This was confirmed by comparison

of subunit pattern and protein sequences to those obtained by Priest et al. for purified complex III [10] (Table 2). Since succinate dehydrogenase (complex II) in other species usually consists of three to four subunits, complex E seems the best candidate for the *C. fasciculata* complex II. Indeed, sequence analysis of the smallest (28 kDa) subunit shows similarity to a part of the yeast complex II cytochrome *b* subunit (see Table 2 and [38]). Succinate dehydrogenase activity could not be measured in a gel eluate containing complex E, but the loss of activity of complex II upon elution after separation with blue native electrophoresis has been reported for other organisms [33]. Fi-

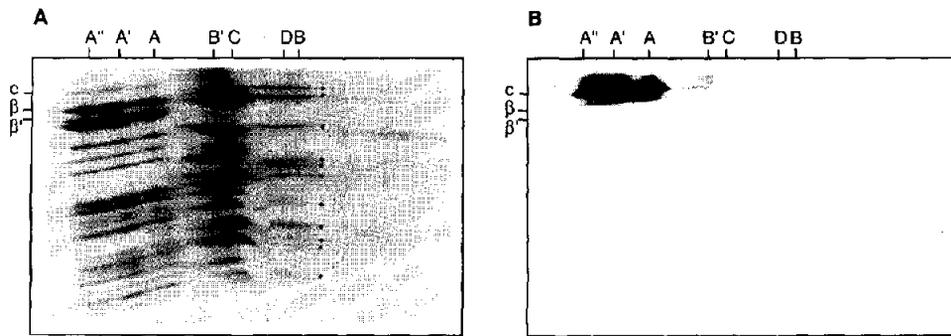


Fig. 2. Respiratory-chain complexes modified with [^{14}C]DCCD. Protein (1 mg) of the methyl-HIC column fraction was modified with [^{14}C]DCCD followed by 2D PAGE. In the first dimension a blue native gel of 5% (w/v) was used. The 2D gel was stained with Coomassie brilliant blue (A) and autoradiographed (B). For other details, see legend to Fig. 1. The position of subunits c , β and β' has been indicated.

nally, activity and sequence analyses provided no clues as to the identity of complex D (Table 2).

The use of the 'blue native' gel system allowed the identification and characterization of complex I from bovine mitochondria [33]. A large multi-subunit complex that could represent *C. fasciculata* complex I (which should be larger than complex V, see e.g. [33,39]), is conspicuously absent from our gels. In addition, we confirmed the results obtained with crude mt membranes and the methyl HIC column fractions by showing the absence of any NADH dehydrogenase activity in the first dimension gel (by use of the NBT color assay; see Section 2) or any of the gel slice eluates.

3.2. Modification with *N,N'*-dicyclohexyldiimide and 2-azido ATP

We further analyzed the different components of the respiratory chain of *C. fasciculata* by labeling experiments with [^{14}C]DCCD and with [α - ^{32}P]2-azido ATP. DCCD can be linked to an acid residue embedded in a hydrophobic region of proteins. In most organisms this results in strong labeling of subunit c of complex V and to a lesser extent in labeling of subunit β of complex V, *cox 3* (complex IV), cytochrome *b* (complex III) and ND 1 (complex I) [40,39,41,42]. 2-azido ATP can photolabel the β subunit of the F_0F_1 -

ATPase and the ADP-ATP carrier protein [43]. The DCCD-labeling experiment is shown in Fig. 2. A comparison of the stained gel and the autoradiogram (Fig. 2A and B, respectively) revealed that a weakly staining band in the high molecular mass range of the F_0F_1 -ATPase complex had the highest affinity for DCCD and that the largest major band was also labeled. In analogy to complex V subunit labeling patterns in other organisms, we tentatively identify these bands as (a multimeric form of) subunit c and β , respectively (see below). Two other complexes were also labeled by DCCD: complex IV subunits are labeled at the top of the gel in a rather broad smear and a weak but specific labeling is found in the smallest of the eleven subunits of complex D. No further labeling was observed (see Section 4).

The results of the 2-azido ATP labeling experiments, given in Fig. 3A and B, show that the putative complex V β subunit indeed became labeled by 2-azido ATP. In addition, a second subunit of complex V was labeled which presumably represents a β subunit degradation product (β' , see below). Other specifically labeled proteins (i.e. with affinity for ATP) were found: complex III subunit 8, the function of which is unknown (see Table 2) and complex D, subunit 2. Labeling was also found in a strongly staining, non-complexed protein, which probably represents the ADP-ATP carrier protein.

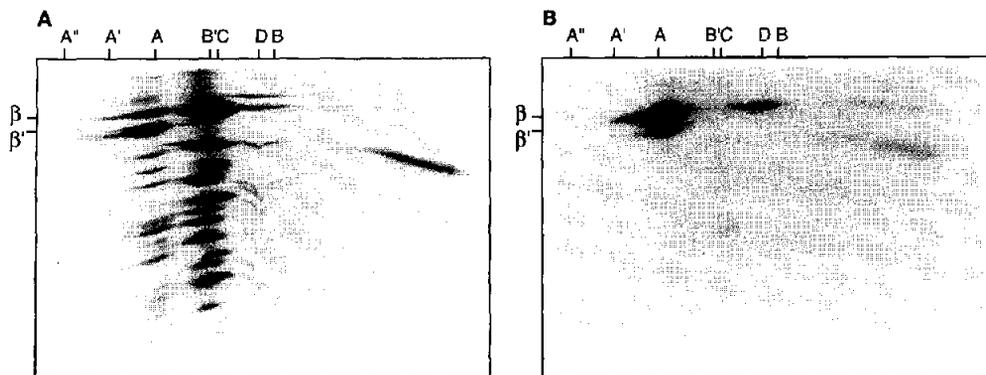


Fig. 3. Photolabelling of respiratory-chain complexes with [32 P] 2-azido ATP. Protein (1 mg) of the methyl-HIC column fraction was labeled with 2-azido ATP followed by 2D PAGE. The 2D gel was stained with Coomassie brilliant blue (A) and autoradiographed (B). For other details, see legend to Fig. 1. The position of subunits β and β' has been indicated.

3.3. The subunit composition of F_0F_1 -ATPase

C. fasciculata F_0F_1 -ATPase was further analyzed by high resolution SDS PAGE (Fig. 4) and peptide microsequence analysis (Table 3). Fig. 4, lane 5 shows the subunit composition of the combined mono and multimeric forms of the complex (A, A' and A'') following their elution from a first dimension gel. Fig. 4 also shows the composition of *C. fasciculata* F_1 , isolated from mitochondrial vesicles by chloroform extraction (lane 4), see [24], bovine mitochondrial F_1 and F_0F_1 (lanes 1,2) and spinach chloroplast F_0F_1 (lane 3). We first addressed the identity of the proteins in the F_1 preparation. In most other organisms, the F_1 part of F_0F_1 -ATPase is composed of five protein subunits ($\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$) and the subunit that binds DCCD and ATP is the second largest (the β subunit), see e.g. [43,44]. The modification experiments of Figs. 2 and 3 suggested that in *C. fasciculata* F_1 the β homologue is in fact the largest subunit. This was confirmed by N-terminal sequence analysis of subunits of the complex (Table 3, Fig. 5A), which indeed revealed significant similarity of this subunit to the β subunits of bovine mitochondrial and *E. coli* ATPases. The same sequence was found in the protein migrating at 44 kDa, which also could be cross-linked to 2-azido ATP. We termed this protein β' to indicate that it is closely related to β and most likely

represents a proteolytic breakdown product. Of the other proteins present in the F_1 fraction, also the ϵ subunit could be identified on the basis of a clear (N-terminal) protein sequence similarity to its counterpart in other organisms (Fig. 5B). An alanine rich N-terminal sequence, similar to that of the bovine F_1 δ subunit (Fig. 5C) is found in two proteins of about 23 and 12 kDa. Given the fact that the smaller protein is about 50% of the size of the larger one, we assume that we are dealing with mono and dimeric forms of the δ subunit (δ and δ' , respectively), but alternative explanations (proteolysis?) cannot be excluded. The N-terminus of the second and third largest proteins in the F_1 fraction proved to be blocked, and an internal peptide sequence of 14 residues displayed no significant similarity to any of the known sequences (Table 3). Nevertheless, it seems likely that these proteins represent the *C. fasciculata* homologues of F_1 subunit α and γ , respectively, given their abundance and size: although the putative α subunit is smaller than its homologue in other species, the putative γ subunit migrates at exactly the same position as its bovine mt counterpart.

An additional abundant 19 kDa protein in the F_1 fraction (see Fig. 4, lane 4) proved to be the *C. fasciculata* homologue of *Leishmania tarentolae* p18, a mt membrane protein picked up in a search for gRNA binding proteins [45], as judged from

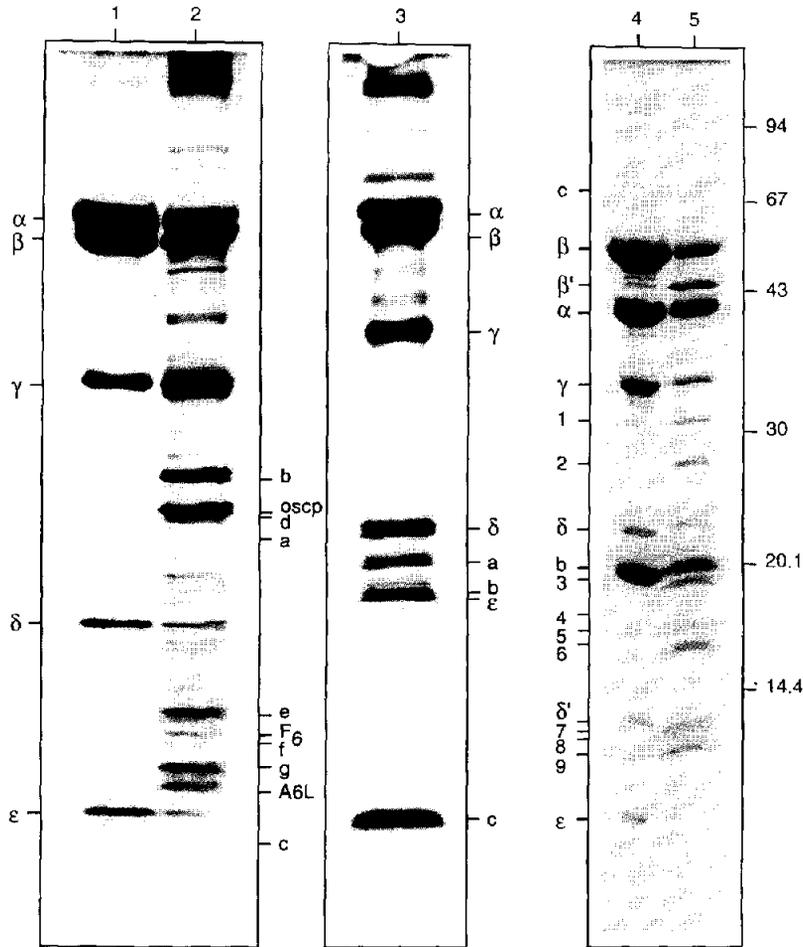


Fig. 4. The subunit composition of *C. fascicularis* F₀F₁-ATPase. F₁ (150 μg, lane 4) and F₀F₁ (100 μg, lane 5)-ATPase from *C. fascicularis* were analyzed by SDS-PAGE on a 16% (w/v) Tris/tricine gel [33], in comparison with F₁ (250 μg, lane 1) and F₀F₁ (200 μg, lane 2) from bovine mitochondria and 250 μg F₀F₁ from spinach chloroplasts (lane 3). The gel was calibrated with molecular-mass markers: their size is indicated on the left. Subunits are indicated left and right of the different lanes (see text). Staining was done with Coomassie brilliant blue.

an almost complete sequence identity: 13 out of the 14 N-terminal and 10 out of the 11 internal amino acids given in Table 3 were identical. A systematic comparison of the p18 sequence with published F₀F₁-ATPase subunit sequences revealed a significant C-terminal sequence similarity with ATPase b subunits from *E. coli* and bovine mitochondria (see Fig. 5D). This similarity together with characteristics displayed by ATPase b in other organisms, such as a high abundance and a tendency to partially co-purify with the F₁

fraction upon extraction with organic solvents [46], make it likely that this protein is the trypanosomatid F₀F₁-ATPase subunit b, despite the lack of any obvious homology in the remaining 75% of the protein. Although the N-terminal part is less hydrophobic than its known counterparts (see Section 4), Bringaud et al. [45] demonstrated localization on/in the mt inner membrane (with the aid of immunofluorescence).

Apart from subunits b and c (see previous section), this analysis provided little information

with respect to the identity of other F_0 subunits. None of the sequences obtained, either N-terminal or internal, showed any similarity to known proteins. These proteins are numbered 1–9 in Fig. 4A and Table 3. In conclusion, we find the trypanosomal F_0F_1 -ATPase to be composed of at least 16 different subunits assuming that none of the bands is derived from multimerized subunits. This would result in a complexity similar to that seen in higher eukaryotes (compare bovine mt F_0F_1 -ATPase, Fig. 4, lane 2), although the degree of subunit sequence similarity is low.

Table 3
Peptide sequences of subunits of F_0F_1 -ATPase

Band	MM	Sequence
c	69 (multimer)	Blocked N-terminus
β	50	DSEQVVGKVDAGAPNIVSR- SPVGYDii
β'	44	Identical to β
α	40	Blocked N-terminus
γ	33	Blocked N-terminus (int) WKEDLADASsTEnQ
1	30	Blocked N-terminus (int) W-DNIETe LR Y
2	26	Blocked N-terminus (int) WERTVVVGDKVEF
δ'	23 (dimer?)	ASSAAAATAT (see δ)
b (p18)	19	ASAGAKKYDLFFEYF (int) WIEKVKKCQ(F/Y)Y
3	18.5	MKVELTLQYLDDWMLR- KFQTE
4	17.5	aVPHDIPEAFEGF
5	16.5	AAAPAAAAAASSDPKM- SALHKKLLTgEAQFR
6	16	VLFSTYRSKRIVAKGFLNG- PVM
δ	12	ASSAAAATATAT
7 ^a	11	Blocked N-terminus
8 ^a	11	Blocked N-terminus
9 ^a	11	VVGNSKVDpTLVFQ
ϵ	10	NWRDQGVSYVKYLN- VCTETL

The nomenclature of subunits is that of Fig. 4. — Indicates that no residue could be identified. For further details, see Legend to Table 2. ^asubunits 7, 8 and 9 migrate closely together in SDS-PAGE, therefore the assignment of the sequence VVGNSKVDpTLVFQ to subunit 9 is tentative. The molecular mass (MM) is given in kDa.

4. Discussion

4.1. Composition of the trypanosomatid respiratory chain

In this paper we report on a series of experiments designed to get a comprehensive picture of properties and composition of the respiratory chain in cultured cells of the trypanosomatid *C. fasciculata*, our long term goals being to identify the mt encoded subunits and to shed some light on the hypothesis that RNA editing is used to create protein sequence heterogeneity. Trypanosomes diverged very early from the main eukaryotic lineage and this is reflected in the very low degree of identity of protein sequences that is generally observed when trypanosome sequences are compared to those of other organisms (see e.g. [47,48]). This also applies to the nuclearly encoded subunits of respiratory-chain complexes such as complex III (Table 2 and [10,49]), complex IV [11,50] and complex V (Fig. 5, Table 3). However, apart from the differences in size of some subunits (such as the F_1 -ATPase α homologue, Fig. 4), the overall complexity (and function) of the trypanosomatid respiratory-chain complexes III, IV and V appears not dissimilar from that found in mitochondria of other eukaryotes (Figs. 1 and 4), see also [10,11,51,52]. This most likely also applies to complex II, although the identification of complex E as its trypanosomal representative is uncertain (see Table 1). Clearly the main features of respiratory-chain composition must have arisen early in eukaryotic evolution and seem to be the result of a single endosymbiotic event.

Nevertheless, a complex I-type NADH dehydrogenase seems to be missing, which is in agreement with the absence of translatable mt mRNAs for NADH dehydrogenase subunits in cultured strains of *C. fasciculata* and *Leishmania tarentolae* [16,53,54]. Other lower eukaryotes, like the yeast *Saccharomyces cerevisiae*, do not have a complex I-type NADH dehydrogenase either, but in contrast to trypanosomes the yeast lacks mt genes encoding ND subunits altogether [55]. This raises the question in which other life cycle stage of trypanosomatids, the products of the mt encoded ND genes are required. Since *C. fasciculata* is

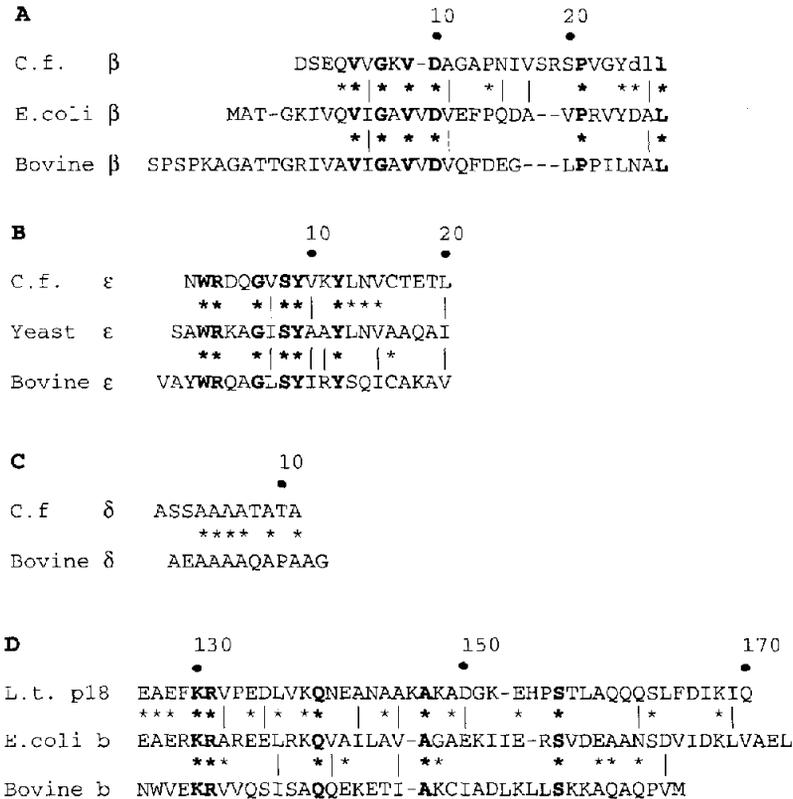


Fig. 5. C.f. Amino acid sequence alignments of ATPase subunits. (A) Alignment of the N-terminal sequence of the *C. fasciculata* (C.f.) ATPase β subunit with its homologues from *E. coli* [64] and bovine mitochondria [64]. An asterisk indicates conservation of an amino acid with respect to *C. fasciculata*, a bold asterisk indicates the conservation in all three sequences. | indicates conservative substitutions (V,I,A,L; D,E; N,Q). Lower case residues in the *C. fasciculata* sequence indicate uncertain identity. - indicates an insertion to maximize similarity. (B) Alignment of the N-terminal sequence of the *C. fasciculata* (C.f.) ATPase ϵ subunit with its homologues from yeast [65] and bovine mitochondria [51]. (C) Alignment of the N-terminal sequence of the *C. fasciculata* (C.f.) ATPase δ subunit with its homologue from bovine mitochondria [51]. (D) Alignment of the C-terminal sequence of the *L. tarentolae* (L.t.) ATPase b subunit [45] with its homologues from *E. coli* [63] and bovine mitochondria [52].

monogenetic, having an insect host only, it could be assumed that it goes through different stages, e.g. during the transmission from insect to insect, a functional complex I not always being required. Alternatively, it could be that *C. fasciculata* has no different life cycle stages, but that complex I activity is dispensable when the organisms are cultivated in rich media for a prolonged period of time and that the loss of complex I is an artifact of culturing. In addition, it should be pointed out that the degree of sequence similarity between the mt 'ND' genes in trypanosomes and those of other organisms is only marginal in most cases

(see e.g. [54,56]) and that the ND genes in *T. brucei* are expressed predominantly in the bloodstream phase, when cytochromes and a functional respiratory chain are absent [57]. The possibility remains, therefore, that trypanosome ND genes do not encode subunits of a complex I-type but rather of a different type of NADH dehydrogenase (e.g. coupled to an alternative oxidase involved in direct oxidation of ubiquinone by molecular oxygen (TAO, [58]).

As a consequence of the absence of complex I it is unclear how the NADH produced in mitochondria of cultured trypanosomatids is oxidized. This

could be accomplished by a fumarate reductase converting fumarate to succinate (coupled to the oxidation of NADH), as found, e.g., in *T. brucei* [59]. Also rotenone-insensitive oxidation of NADH without site 1 linked phosphorylation could exist, as found in yeast [60,61]. Alternatively, a peripheral NADH dehydrogenase (similar to that found in *Neurospora crassa* mitochondria [62]) composed of nuclearly encoded subunits only, could shuttle the electrons from NADH via ubiquinone to complex III. The nature of the mt NADH oxidation in *C. fasciculata* culture form remains obscure. No NADH oxidation could be measured in any of the slices and eluates from the first dimension blue native gel, not even in the area of complex D whose abundance, size and subunit pattern are similar to that of *N. crassa* peripheral NADH dehydrogenase. More work, e.g. checking the presence and identity of NADH-dehydrogenases in recently isolated strains of *C. fasciculata* and *L. tarentolae* will be required to shed further light on these matters.

4.2. Subunit composition of the F_0F_1 -ATPase

The blue native gel system is ideally suited for the rapid analysis of the subunit composition of respiratory-chain complexes, also in trypanosomatids. The subunit composition of complex IV as determined by this method is identical to that of purified complex IV [11], whereas our complex III (Table 2) is very similar to that of an extensively purified preparation, except for two extra small subunits that may have been lost during the purification procedure used in [10]. Other minor differences such as slightly different apparent molecular masses for some subunits and a reversal of bands 2 and 3 must stem from the use of different SDS-PAGE conditions. With three exceptions that could be derived from strain polymorphisms or, more likely, sequencing inaccuracies, the amino acid sequences found were identical in the two preparations. With complexes III and IV as internal control, we are confident that the analysis by this method of the subunit composition of the F_0F_1 -ATPase is also highly reliable. A number of its subunits could be

identified by cross-linking and/or peptide microsequence analyses: β , (δ), ϵ , b and c (Figs. 4 and 5; Table 3). Subunit b of *C. fasciculata* has a very low overall identity to its known counterparts and does not have a hydrophobic N terminal domain (compare the sequence in [45] with, e.g. [52,63]). This could imply a novel way of interacting with the other F_0F_1 subunits and/or the mt inner membrane.

4.3. Mitochondrially encoded subunits of respiratory-chain complexes

So far, our analysis has not resulted in the identification of the (edited) MURF4/ATP6 gene product as a subunit of the F_0F_1 -ATPase of *C. fasciculata*. The internal sequences that we obtained for subunits 1 and 2, which have a molecular mass in the range expected for the MURF4/ATP6 gene product and those obtained from smaller subunits did not match any of the sequences inferred from MURF4/ATP6 cDNAs (see Table 3). Similar results have been obtained in a search for cox I, II and III (see Table 2 and [11]) and cyt b (see Table 2 and [10]). In all instances internal sequences from N-terminally blocked proteins did not match the expected amino acid sequence. Therefore these are not the mt encoded subunits. Priest et al. explained the lack of results with respect to the identification of cytochrome b as a subunit of complex III by assuming that it may form aggregates in SDS [10]. The predicted extreme hydrophobicity and cysteine richness not only of cytochrome b but of all the mt encoded subunits may indeed promote aggregation. The multimerization of mt encoded subunits (and consequently of the respiratory-chain complexes themselves) has been frequently reported ([10,11], and references therein). Our results with DCCD are in agreement with this, suggesting that cytochrome b is in fact completely absent from complex III on 2D PAGE and that cox III localizes to an unexpected position in the high molecular mass range of complex IV subunits. The fact that certain subunits are absent or end up in unexpected places in 2D PAGE could indeed explain why, so far, no mt encoded proteins have been identified (or sequenced). As a

tool in the identification of these proteins, we are currently generating antisera against fusion proteins produced in *E. coli* from constructs which contain *C. fasciculata* mt DNA segments. This approach and the application of mass spectrometry should give us the possibility to identify the products of mt mRNAs in the near future.

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