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Characterization of a Protein Fraction Containing Cytochromes *b* and c_1 from Mitochondria of *Leishmania tarentolae*

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SHAW, J. M., AND SIMPSON, L. 1989. Characterization of a protein fraction containing cytochromes *b* and c_1 from mitochondria of *Leishmania tarentolae*. *Experimental Parasitology* 68, 443–449. A soluble red band fraction was obtained from *Leishmania tarentolae* cells by sucrose gradient sedimentation of a Triton X-100 lysate. Spectral analysis indicated that cytochrome *b* was present in the red band: the reduced minus oxidized difference spectra revealed absorption maxima at 562, 527, and 431 nm at room temperature and 562, 530, and 422 nm at 77K. In addition, a 28-kDa protein was identified in this fraction which retained heme-associated peroxidase activity even after denaturation on SDS–polyacrylamide gels. The amino acid composition of this protein showed a strong similarity to cytochrome c_1 of both bovine and yeast. © 1989 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania tarentolae*; Kinetoplastid; Mitochondria; Cytochrome *b*; Cytochrome c_1; Heme protein; Sodium dodecyl sulfate (SDS); Phenylmethylsulfonyl fluoride (PMSF); 3,3′,5,5′-Tetramethylbenzidine (TMBZ); Hydrogen peroxide (H_2O_2); Phenylisothiocyanate (PITC); Nanometers (nm); Deoxyribonucleic acid (DNA); Ribonucleic acid (RNA).

INTRODUCTION

The kinetoplastid protozoa are a group of lower eukaryotes that contain a single, large mitochondrion in each cell. In all of the kinetoplastids, except for the blood stream African trypanosomes, the mitochondrion appears to have a fully functional respiratory system (Hill 1976; Martin and Mukkada 1979; Hart *et al.* 1981). Evidence for the presence of cytochrome *b*, cytochrome aa_3, and possibly cytochrome *o* has been obtained by spectral analysis (Hill and Cross 1973; Hill 1976; Martin and Mukkada 1979). The complete (or partial) amino acid sequence of cytochrome c_{555} has been determined for six kinetoplastids representing four different genera (Hill *et al.* 1971a,b; Pettigrew 1972; Rassam and Dawood 1986), and a cDNA encoding the apoprotein was recently cloned from *Trypanasoma brucei* and was partially sequenced (Torri and Hajduk 1988). To date, however, cytochrome *c* is the only respiratory protein that has been purified and biochemically characterized from these organisms.

This paper describes the properties of cytochromes *b* and c_1 found in a mitochondrial protein fraction of the kinetoplastid *Leishmania tarentolae*. In addition, a 28-kDa heme-binding protein has been isolated from this fraction and identified by amino acid composition analysis as the nuclear-encoded cytochrome c_1.

MATERIALS AND METHODS

Cell culture. L. tarentolae (UC strain) was grown in Difco brain–heart infusion medium supplemented with 10–20 μg/ml hemin at 27°C as described previously (Simpson and Braly 1970). Cells were grown to midlog phase and were used immediately for the isolation of mitochondria.

Mitochondrial isolation. Mitochondria were isolated from midlog phase cells by the Renografin flotation method (Simpson and Braly 1970; Simpson and Simpson 1978). Mitochondria were either used immediately or stored as a pellet at −70°C prior to solubilization.

Preparation of mitochondrial lysate. Mitochondria were solubilized by homogenization at 0°C in 0.2 *M* Tris–Cl, pH 8.0, 0.4 *M* KCl, 0.2 *M* sucrose, 35 m*M*

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$MgCl_2$, 25 m*M* EGTA, 2% Triton X-100, 1 m*M* PMSF, and 1.5 m*M* 2-mercaptoethanol. Subsequent fractionation over a 35–45% sucrose gradient for 24 hr at 60,000 rpm yielded two protein fractions which were visualized as a lower red band and an upper green band. These bands were recovered by dripping.

Gel electrophoresis. Protein samples were electrophoresed on 10–20% acrylamide gradient gels using a buffer system described by Laemmli (1970) or on 15% acrylamide/8 *M* urea/SDS gels as described by Ching and Attardi (1982).

Spectral analysis. The dithionite-reduced minus ferricyanide-oxidized difference spectra of sonicated, whole mitochondria and the red band fraction were measured with a Cary Model 219 double beam spectrophotometer at room temperature. The red band fraction was air-oxidized and ferricyanide was omitted from this sample. Whole mitochondria were sonicated in a buffer containing 0.25 *M* sucrose, 20 m*M* Tris-Cl, pH 7.9, and 2.0 m*M* EDTA. The scan rate was 1 nm/sec between 400 and 700 nm and the scale sensitivity was adjusted according to the absorbance.

TMBZ staining of polyacrylamide gels. The red band protein fraction was separated on 10–20% polyacrylamide gels prepared as described by Laemmli (1970). After electrophoresis, the gel was briefly rinsed in distilled water and stained with TMBZ for peroxidase activity using a procedure described by Thomas *et al.* (1976). A 6.3 m*M* TMBZ (Aldrich Chemical Co.) solution was freshly prepared in methanol. Immediately before use, three parts of the TMBZ solution were mixed with seven parts of 0.25 m*M* sodium acetate, pH 5.0. The gel was immersed in this solution for 1–2 hr in the dark with constant agitation. H_2O_2 was added to a final concentration of 30 m*M*. After the stain had developed (30 min to 1 hr), the gel was immersed in isopropanol:0.25 *M* sodium acetate, pH 5.0, at a ratio of 3:7 to clear the gel background and enhance the staining intensity. The gel was then photographed and stained for protein with Coomasie brilliant blue to facilitate gel isolation of the heme-binding protein.

Amino acid analysis of the 28-kDa heme-binding protein. After staining with Coomassie blue, the 28-kDa heme-containing protein band was excised from the gel, was soaked in several changes of distilled water, and the protein was eluted by electrophoresis into dialysis tubing using a buffer system described by Hunkapiller *et al.* (1983). After dialysis in 0.02% sodium dodecyl sulfate, 0.01 *M* NH_4HCO_3, the sample was dried and used for amino acid analysis. The use of a glycine buffer in this gel system results in slightly elevated values for glycine in the subsequent amino acid analysis.

The amino acid composition of the eluted protein was determined after 18, 24, and 65 hr of hydrolysis in 6 *N* HCl at 110°C using the Waters Picotag system and precolumn derivatization with PITC. Duplicate analyses were performed on each hydrolysate.

Results

Spectral analysis of whole mitochondrial particles. The dithionite-reduced minus ferricyanide-oxidized difference spectra of whole, sonicated mitochondria from *L. tarentolae* are shown in Fig. 1. The following components of the mitochondrial respiratory chain are represented in the spectrum: cytochrome aa_3 with α absorption maximum at 606 nm, a large amount of cytochrome *b* with α band at 560 nm, the β band at 530 nm, and the Soret γ band at 430 nm, and the flavins which appear as a trough in the 460 nm region. As shown for several other kinetoplastids (Hill and Cross 1973; Hill 1976; Martin and Mukkada 1979), the α absorption maxima (555–558 nm) of cytochrome *c* are obscured by the relatively large amount of cytochrome *b* present in the organelle.

Preparation and spectral analysis of the mitochondrial red band fraction. A mitochondrial fraction was obtained from *L.*

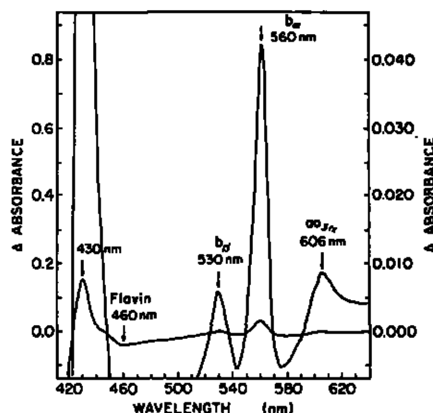

FIG. 1. Difference spectrum of *L. tarentolae* mitochondria at room temperature. The dithionite-reduced minus ferricyanide-oxidized difference spectrum of sonicated mitochondria was recorded in 0.25 *M* sucrose, 20 m*M* Tris–Cl, pH 7.9, and 2.0 m*M* EDTA on a scale of 1.0 for Δ absorbance (*y*-axis at left). The Δ absorbance for the amplified tracing is indicated on the *y*-axis at the right.

tarentolae using the Renografin flotation method (Simpson and Braly 1970; Simpson and Simpson 1978) after hypotonic cell lysis. Sedimentation of a Triton-solubilized lysate through a 35–45% sucrose gradient yielded red and green protein bands (Fig. 2A). The Coomassie blue-stained profiles shown in Fig. 2B indicate that the red (lane 2) and green (lane 3) bands are enriched for different protein species, which represent complex subsets of the proteins present in whole mitochondria (lane 1).

Figure 3 shows the dithionite-reduced minus air-oxidized difference spectrum of the red band fraction. The α and β absorption maxima at 562 and 527 nm respectively, indicate that this fraction contains cytochrome *b*. After fractionation through the sucrose gradient, these bands were shifted 2–3 nm toward the red end of the spectrum. Spectral analysis at low temperature was used to show that multiple *b* cytochromes were not present in the red band. At 77K, the difference spectra revealed absorption maxima at 562 nm (α), 530 nm (β), and 422 nm (γ), again confirming that this fraction contained cytochrome *b* (unpublished results). In other organisms, nuclear-encoded cytochrome *b* was shown to produce two α peaks (557 and 550 nm) in a low temperature spectrum which was not detected in the red band fraction (Davis *et al.* 1972, 1973; von Jagow and Sebald 1980). The α absorption maxima of the *c* cytochrome(s) are obscured by the large amount of cytochrome *b* in this fraction, as indicated above in the spectral analysis of whole mitochondrial particles. A low level of the α absorption maximum for cytochrome aa_3 was also detected at 77K, suggesting that additional cytochromes may be present in this fraction. In fact, we have shown that the mitochondrial-encoded cytochrome oxidase subunit II polypeptide can be detected in this sample using a peptide antibody specific for the

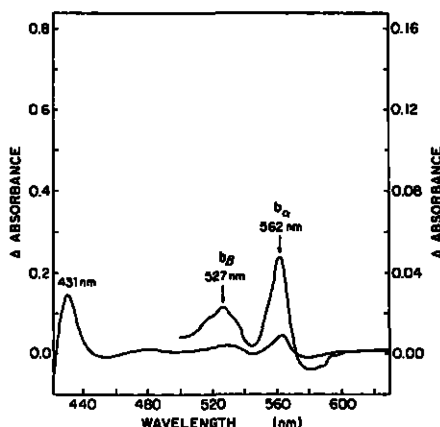

FIG. 3. Difference spectrum of the mitochondrial red band fraction at room temperature. The dithionite-reduced minus air-oxidized difference spectrum of the red band fraction was measured using a scale of 1.0 for Δ absorbance (y-axis at left). The red band sample in the solubilization buffer was collected directly from the gradient. An amplified tracing of the cytochrome *b* α and β peaks is also shown (absorbance scale at right).

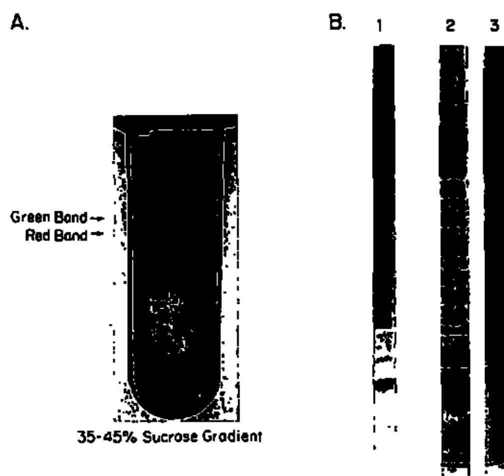

FIG. 2. (A) Mitochondria from *L. tarentolae* were solubilized in a buffer containing 2% Triton X-100 (see under Materials and Methods) and fractionated by band sedimentation through a 35–45% sucrose gradient. Two fractions (marked by arrows) were visualized as red and green bands in the gradient. (B) Coomassie blue-stained protein profiles of fractions separated on a 15% acrylamide/8 *M* urea/SDS gel: lane 1, total mitochondrial protein; lane 2, red band fraction; lane 3, green band fraction.

carboxy terminus of the predicted protein (J. Shaw and L. Simpson, unpublished results).

Identification of cytochrome c_1 in the red band fraction. In other cells, the nuclear-encoded cytochrome c_1 is often released in a complex with cytochrome *b* when solubilization of mitochondria is performed using a nonionic detergent such as Triton X-100. The heme group of cytochrome c_1 is covalently linked to the apoprotein and is not separated from the polypeptide during electrophoresis on denaturing gels. To determine whether cytochrome c_1 is present in the red band fraction, the heme-associated peroxidase activity was assayed after denaturation and separation of proteins on a 10–20% SDS–polyacrylamide gel. When

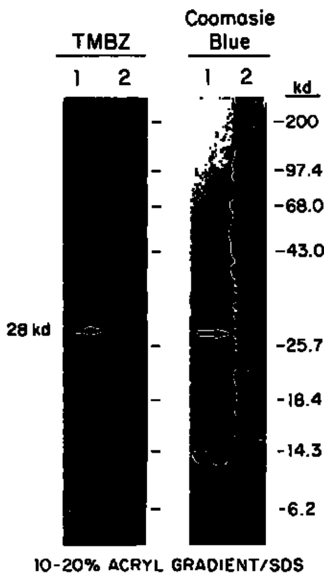

FIG. 4. TMBZ–H_2O_2 staining of a 28-kDa heme protein in the mitochondrial red band fraction. Protein samples were separated on a 10–20% acrylamide/SDS gradient gel and stained for heme-associated peroxidase activity with TMBZ–H_2O_2 (panel at left): lane 1, 0.01 μg horse heart cytochrome *c*; lane 2, 120 μg red band fraction. The arrow in lane 2 marks the 28-kDa heme protein identified as cytochrome c_1. The right panel shows the protein profiles of the same samples after staining with Coomassie blue. The arrow in lane 2 marks the 28-kDa heme protein identified by the heme staining in panel 1.

the gel was treated with TMBZ–H_2O_2, a 28-kDa protein from the red band fraction was strained (Fig. 4, TMBZ, lane 2, arrow), suggesting that this sample contained cytochrome *c*. The horse heart cytochrome *c* control was also stained very darkly (TMBZ, lane 1). Cytochrome *b* could not be identified after SDS gel electrophoresis because its heme group is not covalently linked with its polypeptide. Coomassie blue staining of the red band fraction after TMBZ–H_2O_2 staining identified a 28-kDa protein band (Fig. 4, Coomassie blue, lane 2, arrow) that comigrated with the 28-kDa heme peroxidase-labeled band. The molecular weight of this heme-binding protein is identical to that of bovine and yeast cytochrome c_1. Moreover, cytochrome *c*, which also contains a covalently bound heme group, is not present in this fraction, as shown by the lack of a 13-kDa heme-staining band.

Amino acid analysis of the 28-kDa cytochrome c_1 protein. The amino acid composition of the 28-kDa heme-binding protein was determined after gel isolation, and was compared to the amino acid compositions of cytochrome c_1 for both bovine and yeast; the bovine composition is from the c_1 amino acid sequence (Wakabayashi *et al.* 1980) and the yeast data are derived from the gene sequence (Sadler *et al.* 1984), omitting the residues in the cleaved presequence. The results in Table I show that 14 of the 15 amino acid values determined for the *L. tarentolae* heme-binding protein are very similar to those obtained for the bovine and yeast cytochrome c_1 proteins. The high value for isoleucine is inconclusive since lower values (e.g., 11) were obtained in some hydrolysates and the higher value shown here may represent an artifact of this particular analysis (unpublished results). Cysteine and tryptophan, which are known to be labile under the hydrolysis conditions used, and proline, which was obscured by NH_3 in the sample, could not be determined.

TABLE I
Amino Acid Composition Analysis

Amino acid	*L. tarentolae* 28-kDa heme protein	Bovine cytochrome c_1	Yeast cytochrome c_1
Aspartic acid	21	19	24
Glutamic acid	24	21	22
Serine	21	16	13
Glycine	25	17	18
Histidine	9	9	8
Arginine	15	15	14
Threonine	13	7	12
Alanine	19	18	24
Proline	—	22	22
Tyrosine	12	15	12
Valine	21	15	14
Methionine	4	10	5
Isoleucine	35	4	9
Leucine	15	25	18
Phenylalanine	10	8	10
Lysine	16	12	14
Tryptophan	ND[a]	3	5
Cysteine	ND[a]	5	4
Total residues		241	248
M_r	28 kDa	27.9 kDa	27.4 kDa

[a] Not determined.

Discussion

The mitochondrial difference spectrum reported here is characteristic of that found with other kinetoplastids (Hill and Cross 1973; Hill 1976; Martin and Mukkada 1979). Cytochrome aa_3 is present at a low level, while cytochrome *b* dominates the spectrum and completely masks the spectral contribution of the c_{555} cytochromes. The *L. tarentolae* cytochrome aa_3 α band has a maximum at 606 nm.

Solubilization of the *L. tarentolae* mitochondria in Triton X-100 followed by sucrose gradient centrifugation of the lysate yielded two fractions visualized as red and green bands (Fig. 2A). The red band contained cytochrome *b* as shown by spectral analysis of this fraction (Fig. 3). The composition of the green band was not investigated in this study, although preliminary results indicate the presence of flavoprotein (J. Shaw and L. Simpson, unpublished results). Coomassie blue-stained protein gel profiles (Fig. 2B) indicate the presence of a complex subset of mitochondrial proteins in the red band fraction.

Although our results indicated that absorption maxima for the nuclear-encoded *b* cytochromes were not present in the red band, we do not yet know whether the cytochrome *b* characterized in this fraction is encoded by the nuclear or mitochondrial genome of these cells. A transcriptionally active gene for cytochrome *b* has been found in the mitochondrial DNA of *L. tarentolae* but was shown to lack a 5′ AUG codon for the initiation of translation (de la Cruz *et al.* 1984). We recently showed that an AUG codon is created in the 5′ ends of the cytochrome *b* transcripts by an unusual form of RNA processing called RNA editing (Feagin *et al.* 1988; Shaw *et al.* 1988). An N-terminal amino acid sequence is required to determine the genomic origin of the mitochondrially localized cytochrome *b*

protein and this data will also indicate whether or not edited mitochondrial cytochrome *b* RNAs are translated. The red band fraction described in this report should provide a starting point for the further purification of the cytochrome *b* protein.

There is a limited amount of indirect evidence in the literature suggesting that cytochrome c_1 is a component of the electron transport chain in kinetoplastids (Hill and White 1968; Kusel and Storey 1973). In this study, an absorption shoulder between 556 and 558 nm provided suggestive evidence that cytochrome c_1 was present in the red band fraction. A putative cytochrome c_1 protein was identified as a 28-kDa band in an SDS acrylamide gel which exhibited a heme-associated peroxidase activity. The amino acid composition analysis of this protein showed a strong similarity to the amino acid composition of cytochrome c_1 from both bovine and yeast. These results are consistent with the identification of the 28-kDa protein as cytochrome c_1 in *L. tarentolae*. It is likely, although not yet demonstrated, that cytochrome c_1 is present in a complex with cytochrome *b* as in other mitochondria.

Acknowledgments

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