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Characterization of mitochondrial electron-transfer in *Leishmania mexicana*

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Abstract

Some general features of the respiratory chain and respiratory control were characterized in coupled mitochondrial preparations from *Leishmania mexicana* promastigotes. O_2 uptake was sensitive to the electron-transfer inhibitors rotenone, flavone, malonate, 4,4,4-trifluoro-1-(2-thienyl) 1.3 butanedione (TTFA), antimycin A, 2*n*-nonyl-4-hydroxyquinoline-*N*-oxide (HQNO), myxothiazol, cyanide and azide. A high concentration of rotenone (60 μM) was required to inhibit O_2 uptake effectively. Difference spectra revealed the presence of cytochromes ($a + a_3$), *b* and *c*. Respiratory control was stimulated 2-fold by ADP with different exogenous oxidizable substrates. Calculated ADP/O ratios were consistent with the notion that ascorbate/*N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (TMPD)-linked and FAD-linked respiration proceeds, respectively, with one third and two thirds of the ATP producing capacity of NADH-linked respiration. State 3 was suppressed by the ATP synthase inhibitors oligomycin and aurovertin and by the adenine nucleotide translocator inhibitors atractyloside and carboxy atractyloside. The protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) provoked state 3u respiration. The mitochondrial preparation was capable of Ca^{2+} uptake and Ca^{2+} stimulated respiration. Data obtained suggests strongly that mitochondrial complexes I, II, III and IV are present in a major pathway of electron-transfer and that oxidative phosphorylation might proceed with high bioenergetic efficiency. © 1997 Elsevier Science B.V.

Keywords: Mitochondrial; Electron-transfer inhibitors; Difference spectra; Respiratory control; *Leishmania mexicana*

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HQNO, 2*n*-nonyl-4-hydroxyquinoline-*N*-oxide; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine; Tris, Tris(hydroxymethyl)aminomethane; TTFA, 4,4,4-trifluoro-1-(2-thienyl) 1.3 butanedione.

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1. Introduction

The Trypanosomatidae family to which the *Leishmania* genus belongs consists of unicellular flagellated protozoa, which display a single mitochondrion which convolutes and ramifies in situ and occupies around 12% of the cell volume [1–3].The ultrastructural complexity of this organelle and some basic features of its bioenergetic processes are not well understood. Studies on these aspects have been greatly hampered by difficulties in the isolation of intact mitochondria from trypanosomatids [4–6]. Isolation of this organelle requires breakage of cells under harsh conditions which are known to have adverse effects on mitochondrial membranes [6]. In fact, damage is difficult to avoid because of the large size and ramifications of the single mitochondrion and the presence of microtubule arrays adhering to the inner side of the cell membrane of trypanosomatids [8]. As a consequence, greater force is required to break the cells than that necessary to disrupt mitochondrial membranes [6–9]. To overcome these difficulties, digitonized cells have been useful as an experimental model [5,6,10–13]. Alternatively, several gentle cell rupture procedures and isolation methods have yielded well sealed phosphorylating vesicles (coupled mitochondrial preparations) resulting from rearrangement of mitochondrial fragments [9,14,17]. In spite of these efforts, information on the respiratory chain in *Leishmania* is incomplete and somewhat controversial. Martin and Mukada [7] reported evidence for the presence of complexes I, II, III and IV in the respiratory chain of *L. tropica*. In contrast, Santhama and Badhuri claimed recently [13] that complex I was absent from the respiratory chain of *L. donovani*. Also, these authors suggested a tentative scheme of electron-transfer in which a reversal of Krebs cycle enzymes occurred producing succinate that could be excreted or oxidized depending upon the energy demand of the cells. Nevertheless, a homologue of the 49 kDa subunit of complex I is coded for by the mitochondrial DNA of *L. tarentolae* [18]. Further, the effects of specific inhibitors, including rotenone, on the respiration of *L. braziliensis* submitochondrial particles [17] agrees with previous results in *L. tropica* [7].

Reports on energy coupling and oxidative phosphorylation in *Leishmania* are scarce and essentially restricted to the assay of ATPase activity and respiratory control [6,17] and to the solubilization and kinetic characterization of the F_1 portion of the ATP synthase complex [19]. In the present study, some general features of the respiratory chain of *L. mexicana* promastigotes have been characterized.

2. Materials and methods

2.1. Chemicals

Biochemicals were purchased from Sigma (St. Louis, MO). All other chemicals were of the highest purity available.

2.2. Organisms

The *Leishmania mexicana*, strain (NR) used in this study was isolated from a human patient [20]. Promastigotes were grown at 22°C in a liquid medium (LIT), supplemented with 7% inactivated fetal bovine serum (pH 7.2). Growth rate was estimated from turbidimetric measurements at 560 nm. At the late logarithmic phase of growth (3–3.5×10^7 cells ml^{-1}) cells were harvested and washed by centrifugation at $2600 \times g$ for 10 min, at 4°C with 140 mM NaCl, 11 mM KCl, 75 mM Tris–HCl and 1 mM EGTA, pH 7.2 (buffer A).

2.3. Isolation of mitochondrial membranes

The washed cell pellet (1.5×10^{10} cells) was suspended in 60 ml of buffer B (500 mM imidazol (p*K*a 6.95), 1 mM magnessium acetate, 0.15% (w/v) fatty acid free bovine serum albumin, 0.15 mg ml^{-1} soybean trypsin inhibitor, pH adjusted to 8.0, with acetic acid), to give $\approx 2 \times 10^8$ cells ml^{-1}. Clumping of cells was prevented by agitation of the cell suspension with a magnetic stirrer. After 15 min of swelling and hypertonic lysis, the suspension was forced through a syringe with a No. 26 gauge needle [7] into 300 ml of cold buffer C (410 mM mannitol, 10 mM Hepes, 1 mM magnessium acetate, 1 mM EGTA, 0.30% (w/v)

Table 1
Succinate-cytochrome *c* reductase, acid phosphate and respiratory activities in *L. mexicana* whole cell homogenates and mitochondrial preparations

Sample	Respiratory activity (nmol O min^{-1} (mg $protein^{-1}$))	Succinate-cytochrome *c* reductase activity (nmol min^{-1} (mg $protein^{-1}$))	Acid phosphatase activity (nmol min^{-1} (mg $protein^{-1}$))
Homogenate	1.9 ± 0.3 ($n = 5$)	0.7 ± 0.2 ($n = 4$)	9.7 ± 1.1 ($n = 3$)
Mitochondrial preparation	11.6 ± 1.3 ($n = 4$)	4.5 ± 0.7 ($n = 4$)	2.6 ± 0.5 ($n = 3$)
Sonicated mitochondrial preparation	10.8 ± 1.4 ($n = 3$)	4.6 ± 0.7 ($n = 3$)	2.2 ± 0.4 ($n = 3$)

Respiratory activity was measured polarographically using succinate (15 mM) as electron donor. Succinate-cytochrome *c* reductase and acid phosphatase activities were measured as described in Section 2. Homogenate was obtained by sonicating whole cells suspended in buffer D, pH 7.2 (succinate-cytochrome *c* reductase assays) or in 50 mM sodium acetate, pH 5.0 (acid phosphatase assays). For assays, the mitochondrial pellet was resuspended in buffer D; alternatively, in some assays the mitochondrial pellet was resuspended in buffer D or in 50 mM sodium acetate and sonicated; further details are described in Section 2; *n* refers to the number of independent experiments conducted with separate mitochondrial preparations and homogenates. Results are mean $\pm$ SD. Differences between the specific activities of homogenates and mitochondrial preparations were statistically significant.

fatty acid free bovine serum albumin, 0.15 mg ml^{-1} soybean trypsin inhibitor, 0.02 mg ml^{-1} leupeptine, pH adjusted to 7.2 with KOH). The suspension was monitored microscopically for cell breakage and centrifuged at $9200 \times g$ for 20 min at 4°C and the pellet was gently resuspended in 30 ml of buffer C. Ghosts and large cell debris were removed by centrifugation at $1100 \times g$ for 10 min at 4°C. Centrifugation of the supernatant at $8700 \times g$ for 20 min yielded a pellet which was washed with 30 ml of buffer C. The pellet was resuspended in 16 ml of buffer C and centrifuged at $8700 \times g$ for 20 min. The pellet obtained was carefully resuspended in 16 ml of buffer D (380 mM mannitol, 10 mM Hepes, 1.5 mM magnesium acetate, 10 mM KH_2 PO_4, 1mM EGTA, 0.15 mg ml^{-1} soybean trypsin inhibitor, 0.02 mg ml^{-1} leupeptin, pH adjusted to 7.2 with KOH) yielding the mitochondrial preparation directly used for succinate-cytochrome *c* reductase and respiratory activities assays. The protein concentration of this preparation was 1.6–2.1 mg protein ml^{-1} (for difference spectra assays, the mitochondrial pellet was resuspended in 7–8 ml of glycerol and buffer D and the protein concentration was higher). Phase contrast microscopy and negative staining of this material showed the presence of large vesicles, flagellar pieces and some small particles of unknown nature.

2.4. Determination of oxygen consumption

O_2 uptake was measured polarographically in a closed system by using a Clark type electrode and YSI oxygen monitor in a total volume of 3 ml at 22°C. The electrode was calibrated with air saturated water at 22°C, adopting an oxygen solubility as described by Reynafarje et al. [21]. Rates of O_2 uptake, respiratory control and ADP/O ratios were calculated according to Estabrook [22]. Respiratory states are defined according to Chance and Williams [23]. The protein concentration in the closed reaction vessel was 1.6–2.1 mg protein ml^{-1} (buffer D).

2.5. Enzyme assays

Succinate-cytochrome *c* reductase activity was measured at pH 7.2 using horse heart cytochrome *c*, according to Tysdale [24] at a protein concentration of 0.8–1.0 mg ml^{-1}. For some assays (Table 1), the mitochondrial preparation was sonicated (3 pulses, 20 s each) with a Brown Sonic 2000 sonifier. To detect succinate-cytochrome *c* reductase activity in homogenates, cells were washed, the pellet was resuspended in buffer D to a final density of 2×10^8 cell ml^{-1} and the cell suspension sonicated as described above at a protein concentration of 1.0–1.3 mg protein ml^{-1}.

Table 2
Substrate oxidation and phosphorylation of *L. mexicana* mitochondrial preparations

Substrate (mM)	Respiratory control ratio	ADP/O	State-3 rate	State-4 rate	*n*
Pyruvate (7.5)+L-malate (7.5)	2.08 ± 0.18	2.40 ± 0.17	21.2 ± 2.3	10.2 ± 1.6	5
Succinate (15)[a]	2.22 ± 0.15	1.61 ± 0.12	25.1 ± 2.4	11.3 ± 1.2	9
Ascorbate (3)[b]+TMPD (0.10)	1.87 ± 0.15	0.70 ± 0.06	27.8 ± 3.2	14.9 ± 1.7	3

Experiments were performed at 22°C as described in Section 2. Respiratory control and ADP/O ratios were calculated according to Estabrook [22]. O_2 uptake rates are given in nmol O min^{-1} (mg protein)$^{-1}$; *n* refers to the number of independent experiments conducted with separate mitochondrial preparations. Results are mean $\pm$ SD. Differences between ADP/O ratios and between state-3 and state-4 rates were statistically significant.
[a] In the presence of rotenone (60 μM).
[b] In the presence of antimycin A (2 μg ml^{-1}).

Acid phosphatase activity was detected according to Gottlieb and Dwyer [25]. For these assays, the mitochondrial pellet was resuspended in 2 ml of 50 mM sodium acetate, pH 5 (buffer E). Aliquots of this suspension were assayed in 50 mM sodium acetate, pH 5, 5 mM *p*-nitrophenyl phosphate, at 42°C [25] at a protein concentration of 3.6–4.0 mg protein ml^{-1}. To detect acid phosphatase activity in cell homogenates, cells were washed and the pellet was resuspended in buffer E to a final density of 2×10^8 cells ml^{-1}. The cell suspension was sonicated (3 pulses, 20 s each) and assayed as indicated above. The protein concentration was 1.0–1.3 mg protein ml^{-1}. Protein was determined according to Lowry et al. [26], using bovine serum albumin as standard.

2.6. Difference spectra

Absorption spectra were measured at room temperature in 1 cm optical path cells, using the Aminco DW-2A UV/VIS spectrophotometer in the split beam mode, (band width 2 nm). The mitochondrial pellet was suspended in glycerol:buffer D (30:70 v/v), pH 7.2. Both cuvettes contained the mitochondrial preparation (3–4 mg protein ml^{-1}) and the reference cuvette also contained 5 mM potassium ferricyanide. Cytochrome molar absorption coefficients given by Chance [27] were used: 16 for cytochrome *a*-band; 91 for cytochrome a_3-band; 22 for cytochrome *b*-band; and 19 for cytochrome *c*-band. The cytochrome content (nmol (mg protein)$^{-1}$) was estimated from different spectra generated by the optical density change measured at absorption maxima (peaks) and minima (troughs) which were respectively: 595 and 630 nm for cytochrome *a*; 445 and 465 nm for cytochrome a_3; 561 and 575 nm for cytochrome *b*; 554 and 540 nm for cytochrome *c* [27,7].

2.7. Measurement of calcium transport

Ionic calcium movements were followed essentially as described by Benaim et al. [17] by measuring the changes in the absorbance spectrum of the metallochromic indicator arsenazo III using an AMINCO DW-2A dual wave length spectrophotometer. In these assays, the mitochondrial pellet was resuspended in a modified buffer C (without EGTA).

2.8. Statistics

All results in Tables 1–3 are expressed as means $\pm$ SD for *n* different experiments. Statistical significance was determined by Student's *t*-test. Significance was considered for $P < 0.05$.

3. Results and discussion

3.1. Enzymatic and respiratory properties

The mitochondrial preparation (sonicated and not sonicated) used in the present work was near 6-fold enriched in succinate-cytochrome *c* reductase activity when compared with homogenates

Table 3
Effects of electron-transfer blockers on the O_2 uptake of *L. mexicana* mitochondrial preparation[a]

Exogenous substrate (mM)	Uninhibited respiratory rate	Inhibitor	% Inhibition of O_2 uptake (nmol O min^{-1} (mg protein)$^{-1}$)	*n*
Pyruvate (7.5)+L-malate (7.5)	10.4 ± 0.6	Rotenone (45 μM)	51.0	3
Pyruvate (7.5)+L-malate (7.5)	10.1 ± 0.6	Rotenone (60 μM)	71.3	4
None	5.6 ± 0.4	Rotenone (60 μM)	73.2	3
Succinate (15)	11.1 ± 0.5	Malonate (2 mM)	73.9	4
Succinate (15)	11.1 ± 0.6	TTFA (2 mM)	75.7	3
Pyruvate (7.5)+L-malate (7.5)	10.2 ± 0.5	Antimycin A (2 μg ml^{-1})	100	5
Succinate (15)	10.4 ± 0.5	Antimycin A (1 μg ml^{-1})	79.8	3
Succinate (15)	10.9 ± 0.6	Antimycin A (2 μg ml^{-1})	100	5
Succinate (15)	11.4 ± 0.7	Myxothiazol (90 μM)	39.5	4
Pyruvate (7.5)+L-malate (7.5)	10.2 ± 0.4	HQNO (20 μM)	63.7	3
Succinate (15)	11.6 ± 0.7	HQNO (20 μM)	56.9	3
Succinate (15)	11.1 ± 0.6	NaN_3 (8 mM)	65.8	3
Pyruvate (7.5)+L-malate (7.5)	10.0 ± 0.6	KCN (1 mM)	93.0	3
Succinate (15)	11.0 ± 0.6	KCN (1 mM)	91.8	5
Ascorbate (3)+TMPD (0.1)	15.3 ± 0.9[b]	KCN (1 mM)	91.5	4
None	6.2 ± 0.5	KCN (1 mM)	95.2	3

[a] O_2 uptake was measured polarographically at 22°C, as described in Section 2. Inhibitors were added after oxidation reached steady states; upon preincubating 3 min with each inhibitor, traces of the remaining respiratory rates were registered; *n* refers to the number of independent experiments conducted with separate mitochondrial preparations. Results are mean $\pm$ SD. Differences between the remaining respiratory rates and the initial uninhibited respiratory rates were statistically significant.
[b] In the presence of antimycin A (2 μg ml^{-1}).

obtained by sonic disruption of whole cells and exhibited a specific respiratory rate proportional to the enrichment of the mitochondrial marker enzyme (Table 1). Conversely, the activity of acid phosphatase, a plasma membrane marker enzyme in *Leishmania* [20,28,29], was low. Similar to mitochondrial vesicles from *C. fasciculata* [14,15], *L. tropica* [7] and *L. braziliensis* [17], but at variance with a mitochondrial preparation from *T. cruzi* [16], respiration in the absence of added oxidizable substrates was apparent (5.9 ± 0.5 nmol O min^{-1} (mg protein)$^{-1}$; $n = 6$) indicating the oxidation of endogenous substrates. The preparation yielded acceptable indexes of respiratory control (Table 2) in comparison with previous results in coupled mitochondrial preparations from other trypanosomatids [9,14–17]. Calculated ADP/O ratios were consistent with the notion that ascorbate/N,N,N',N'-tetramethylphenylenediamine (TMPD)-linked respiration and FAD-linked respiration proceeds respectively, with one third and two thirds of the ATP producing capacity of NADH-linked respiration. Besides the presence of respiratory control, one of the criteria used to assess the structural functionality of the inner mitochondrial membrane is the differential inhibition of succinate oxidation by its structural analogues oxaloacetate and malonate [14,16,30]. Accordingly, succinate oxidation in *L. mexicana* mitochondrial preparations was sensitive to 2 mM malonate and insensitive to 0.5 mM oxaloacetate, whereas in the sonicated mitochondrial preparation succinate oxidation was oxaloacetate-sensitive (data not shown). Also, AMP had no effect on respiration suggesting loss of the mitochondrial outer membrane and adenylate kinase from the inter membrane space during the isolation procedure. Alternatively, the result could also suggest the absence of adenylate kinase in these parasites.

3.2. Oxygen uptake inhibition by electron-transfer blocking agents

Several electron-transfer inhibitors were tested (Table 3). NADH-linked respiration was sensitive to the complex I inhibitor, rotenone, at relatively high concentrations and to the structurally related compound, flavone, which inhibits specifically complex I [31,32]. In succinate-sustained respiration the inhibitory effects of malonate and 4,4,4-trifluoro-1-(2-thienyl) 1.3 butanedione (TTFA), an agent that blocks electron transfer between Fe/S clusters and ubiquinone in mammalian mitochondria [33], suggest the following electron-transfer sequence:

$$\text{Succinate} \rightarrow \text{FAD(Fe/S)} \rightarrow \text{Q}$$

The effectiveness of the complex III inhibitors, 2*n*-nonyl-4-hydroxyquinoline-*N*-oxide (HQNO) and antimycin, was in contrast to the limited effect of myxothiazol, assayed at 90 μM, a relatively high concentration. In mammalian mitochondria, myxothiazol acts on a mechanistic site distinct from the antimycin binding site [34,35]. Inhibition with KCN and azide were essentially comparable to those reported previously in *L. tropica* [7] and *L. braziliensis*. [17].

Along with the results of Table 3, sequential assays of selective inhibition and reinstatement of respiratory activity (Fig. 1) were consistent with the presence of complexes I, II, III and IV in the respiratory chain. Although rotenone is a well-characterized inhibitor of complex I activity [36–38] it has been reported in mammalian mitochondria that rotenone, at high concentrations, also could affect electron-transfer at the regions of the electron-transfer chain [39]. However, Fig. 1A shows that the rotenone-inhibited respiratory rate was restored upon succinate addition. In fact, the respiratory rate observed is slightly lower than the mean values for succinate sustained respiration in the absence (Tables 1 and 3) and in the presence (Table 2) of rotenone. After the inhibition of complex III by antimycin, the respiratory rate was restored upon ascorbate/TMPD addition, with rotenone still present in the vessel. In fact, the respiratory rate observed is essentially equal to the mean values registered in the presence of ascorbate/TMPD, when rotenone was absent (Tables 2 and 3). Therefore, the magnitude of the inhibitory effect provoked by rotenone in *L. mexicana* cannot be ascribed to the hypothetical binding of rotenone at sites located in complex II, complexes II–III and/or complex IV. This point is critical, since the controversy around the presence or absence of a functional complex I is not restricted to *Leishmania*. When 2 μM rotenone was used in *T. brucei* [40] and in *T. cruzi* [41], electron-transfer activities were unaffected. In contrast, when 30 μM rotenone [42] and 100 μM rotenone [43] were used in *T. brucei*, rotenone-sensitivity was reported. In a wider

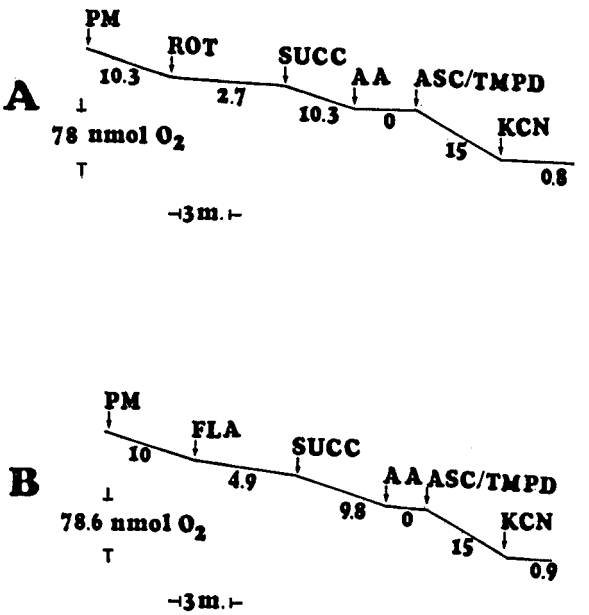

Fig. 1. Selective inhibition and restoration of O_2 uptake in *L. mexicana* mitochondrial preparations. In A and B, the reaction vessel contained 1.7 mg protein ml^{-1} in buffer D with 7.5 mM pyruvate + 7.5 mM malate (PM) as exogenous substrates, 60 μM rotenone (ROT) and 250 μM flavone (FLA) were added, respectively. Subsequent additions were as follows: 15 mM succinate (SUCC); 2 μg ml^{-1} antimycin A (AA); 3 mM ascorbate + 0.10 mM TMPD (ASC/TMPD); 1 mM KCN. Respiratory activities adjacent to the electrode traces are expressed as nmol O consumed min^{-1} (mg protein)$^{-1}$.

scope, 30 μM rotenone was required to inhibit the NDH-1 segment of NADH dehydrogenase isolated from the bacterium *T.thermophilus* [44] and an even higher concentration was required to half the control electron-transfer activity (I_{50}) of the NDH-1 segment of *E.coli* [38]. Finally, the high-affinity NADH dehydrogenase of plant mitochondria is much less sensitive to inhibition by rotenone compared with the mammalian enzyme [38]. Therefore, higher concentrations of this inhibitor were required to reach I_{50} [38].

To clarify this point in trypanosomatids, assays should not be restricted to the use of rotenone. A long and increasing list of complex I inhibitors is available [32,37,38]. Further, some experimental approaches could be useful as complementary alternatives. The presence of edited transcripts for several of the mitochondrially-encoded subunits of complex I in the mitochondrion of *T. brucei* [45] suggest that the subunits of this complex may indeed be synthesized and are functional in this organism.

3.3. Spectral absorption data

Difference spectrum in the presence of sodium dithionite (Fig. 2A) allowed the preliminary identification of respiratory chain redox components. In this reduced state, the predominance of cytochrome *b* is apparent, with an intense γ band with a maximum towards 429 nm, a β shoulder at 529–530 nm and an α-peak at 560–561 nm. The predominance of cytochrome *b* has been reported previously in *L. tropica* [7]. The cytochrome ($a + a_3$) complex was expressed by a weak γ-peak at 443–444 nm (cytochrome a_3) and an α-peak at 595 nm attributable to cytochrome *a*, based on analogy with previous data from *Crithidia* and *Leishmania* which yielded a cytochrome *a* α-peak at 594 nm [7,8]. In contrast, mammalian [27,30] and *T. cruzi* [46] cytochrome *a* has an α-peak at 600–605 nm. Cytochrome *c* was scarcely apparent

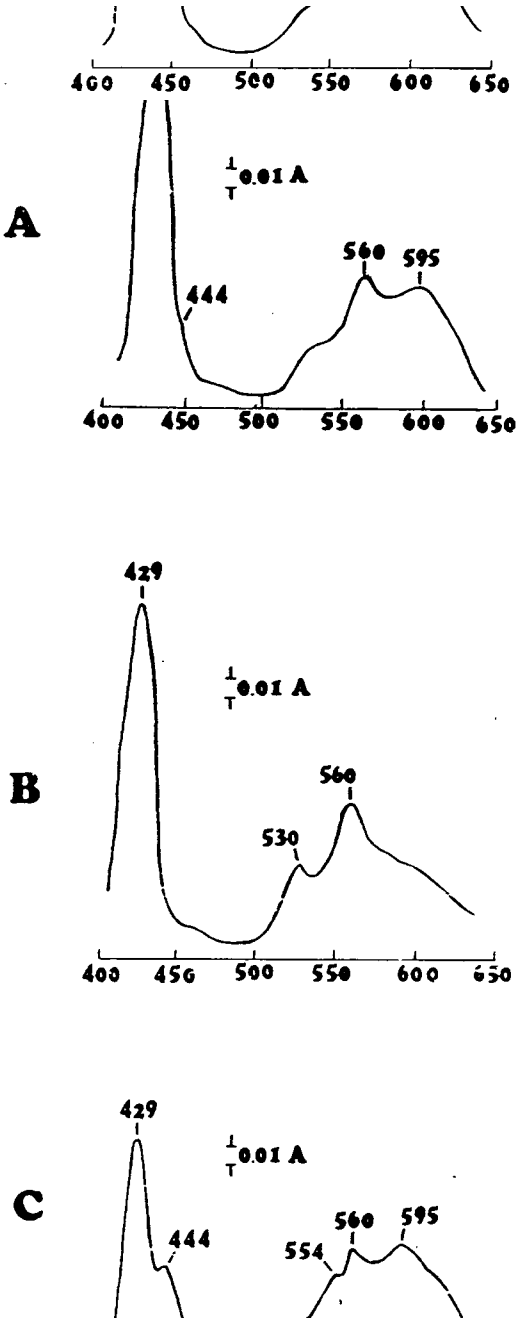

Fig. 2. Difference spectra of a *L. mexicana* mitochondrial preparation. Difference spectra after addition of 5 mg of solid sodium dithionite to the cuvette in A, 2 μg ml^{-1} antimycin in B and 3 mM ascorbate + 0.15 mM TMPD in C. Potassium ferricyanide (5 mM) was added to the reference cuvette and both experimental and reference cuvettes contained 4 mg protein ml^{-1}. Further details are described in Section 2.

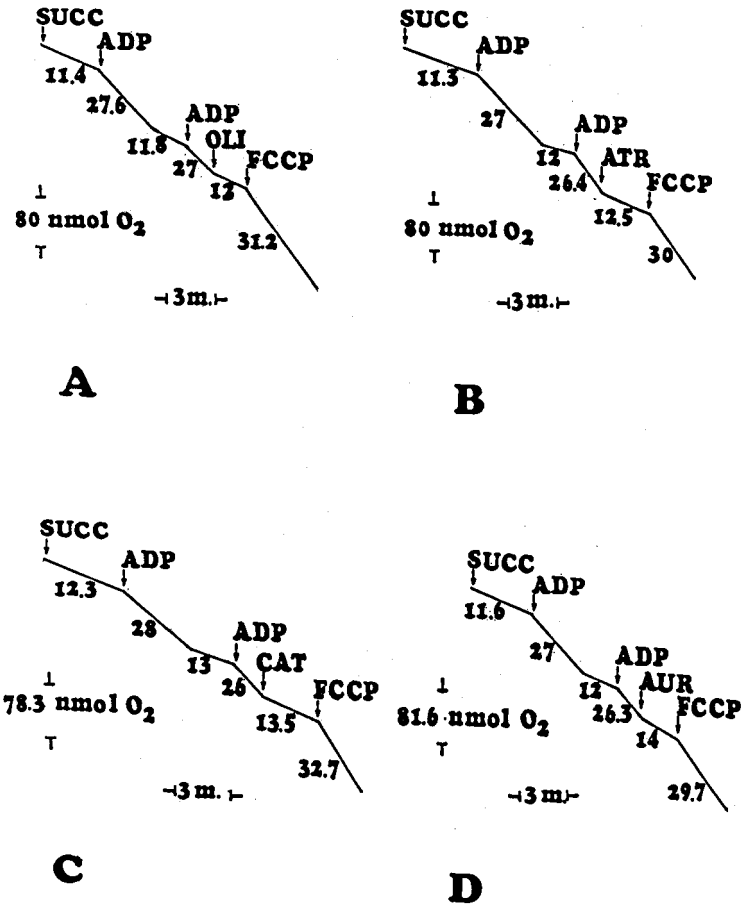

Fig. 3. Effect of different oxidative phosphorylation inhibitors on the rate of O_2 uptake by *L. mexicana* mitochondrial preparations. The reaction contained 1.8 mg protein ml^{-1} in buffer D. Additions, trace A: 15 mM succinate (SUCC); 100 μM ADP; 150 μM ADP; 2 μg ml^{-1} oligomycin (OLI); 1 μM FCCP. Additions, trace B: 15 mM succinate (SUCC); 100 μM ADP; 150 μM ADP; 150 μM atractyloside (ATR); 1 μM FCCP. Additions, trace C: 15 mM succinate (SUCC); 100 μM ADP; 150 μM ADP; 100 μM carboxyatractyloside (CAT); 1 μM FCCP. Additions, trace D: 15 mM succinate (SUCC), 100 μM ADP; 150 μM ADP; 10 μg ml^{-1} aurovertin (AUR), 1 μM FCCP. Respiratory activities adjacent to the electrode traces are expressed as nmol O consumed min^{-1} (mg protein)$^{-1}$.

in trypanosomatids due to the predominance of cytochrome *b* [4,7].

Difference spectrum in the presence of antimycin (Fig. 2B) yielded only peaks at 429 nm, 529–530 nm and 560–561 nm, evoking classical observations in the mammalian respiratory chain [27] and these peaks can be ascribed unequivocally to cytochrome *b*. The addition of ascorbate + TMPD (Fig. 2C) yielded a cytochrome *c* α-peak at 554 nm. In trypanosomatids cytochrome *c* is atypical because of its α-peak towards 553–558 nm [4,7,47]. Using the cytochrome molar absorption coefficients and the procedure described by Chance [27], the concentration of individual cytochromes was calculated. The cytochrome content (nmol (mg protein)$^{-1}$) was 0.16 (a_3), 0.18 (a), 0.10 (c) and 0.28 (b). Under the experimental conditions employed, cytochrome c_1 was not apparent, although its presence could be inferred from the effects of electron-transfer inhibitors (Table 3). Low temperature liquid nitrogen spectra might be more effective in revealing

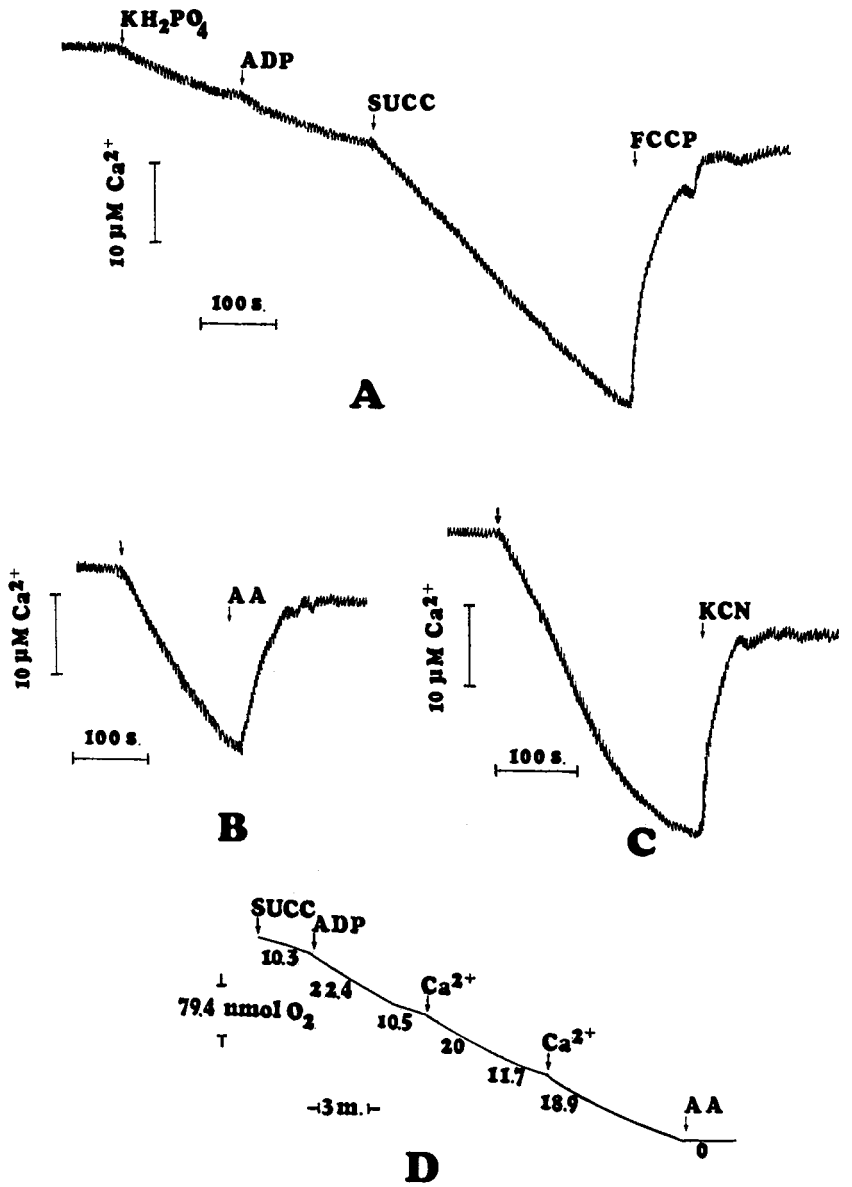

Fig. 4. Ca^{2+} uptake and release from *L. mexicana* mitochondrial preparations. In A, B and C changes in the free calcium concentration were followed using the metallochromic indicator arsenazo III (50 μM) and the wavelength pair 675–685 nm, as described in Section 2. Protein concentration was 1.5 mg ml^{-1}. Additions in A: 10 mM KH_2PO_4; 500 μM ADP; 15 mM succinate (SUCC); 1 μM FCCP. In B and C, 15 mM succinate was added (arrow) followed by 2 μg ml^{-1} antimycin A (AA) and 1 mM KCN, respectively. In D, O_2 uptake was followed polarographically as described in Section 2. Protein concentration was 1.6 mg ml^{-1} in buffer D without EGTA. Additions: 15 mM succinate (SUCC); 100 μM ADP; 200 μM $CaCl_2$ (Ca^{2+}), twice; 2 μg ml^{-1} antimycin A (AA).

this redox component. Also, carbon monoxide difference spectra might be necessary, besides other approaches, to establish the hypothetical presence of o and/or d-like pigments as redox components of a minor alternative pathway of electron transfer, a possibility which is not precluded by the present results.

Results, from difference spectra, O_2 uptake in-

hibition and selective inhibition and restoration of respiratory activity, are in agreement. Taken together, they suggest that *L. mexicana* displays a major pathway of electron-transfer to the terminal oxidase ($a + a_3$) according to the classical scheme firmly established in mammalian mitochondria. Also, the calculated ADP/O ratios indicate a high bioenergetic efficiency for NADH-linked substrates. Although this major pathway seems classical in its general features, several quantitative differences with respect to the mammalian respiratory chain are described. These differences might result from comparative differences at the molecular level and demand further research on their significance and importance.

3.4. Effect of ATP synthase and adenine nucleotide translocator inhibitors

The addition of ADP to these mitochondrial preparations caused an increase in O_2 uptake, which was inhibited by oligomycin (Fig. 3A). Subsequent addition of the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) provoked 3u respiration. Atractyloside, a reversible inhibitor of the mammalian adenine nucleotide translocator [40], abolished the stimulation of O_2 uptake by ADP (Fig. 3B). Again, subsequent addition of FCCP caused more than a 2-fold increase in respiration. A similar effect was obtained with carboxyatractiloside (Fig. 3C), a quasi irreversible inhibitor [48]. The observed effects of atractyloside and carboxyatractiloside are indirect evidence for the presence of an adenine nucleotide translocator in the inner mitochondrial membrane of *L. mexicana*. Aurovertin, a specific inhibitor of the mammalian ATP synthase which binds to the β subunit of the F_1 portion of the enzyme [49], also suppressed state-3 respiratory rate (Fig. 4D), but only at a relatively high concentration (10 μg ml^{-1}). The insensitivity of trypanosomatid ATP synthase to aurovertin was also observed in *C. luciliae* by Opperdoes et al. [9]. These authors favoured the explanation of evolutionary divergence between the ATP synthase of the trypanosomatids and animal/fungi.

3.5. Calcium transport assays

The capacity of the mitochondrial preparation for Ca^{2+} transport was determined with arsenazo III as an indicator of ionic calcium. As observed in Fig. 4A, P_i and ADP induced Ca^{2+} uptake, as reported for other mitochondrial transport systems, even in the absence of added oxidizable substrates. Upon succinate addition, Ca^{2+} uptake increased and FCCP induced a Ca^{2+} efflux (Fig. 4A). Antimycin (Fig. 4B) and KCN (Fig. 4C) also induced rapid Ca^{2+} efflux. The addition of 200 μM Ca^{2+} stimulated the respiratory activity of the coupled mitochondrial preparation (Fig. 4D), as observed previously in mammalian mitochondria [50] and digitonized cells of *T. cruzi* [12]. Previous studies in *Leishmania* indicate that the mitochondrial compartment is involved in calcium ion homeostasis [17,51], a concept that is supported by the results of the present study.

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