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NADPH-CYTOCHROME C REDUCTASES OF TRYPANOSOMA CRUZI

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NADPH-dependent reduction of cytochrome c is catalyzed both by microsomes and the cytosolic fraction isolated from Trypanosoma cruzi homogenates. About one-third of the activity is microsomal and two-thirds is cytosolic. The microsomal activity is increased by Lubrol and sodium cholate, but pretreatment with phenobarbital has negligible effect. On the other hand, detergents do not affect the cytosolic activity but it is increased by phenobarbital. From these observations, it is concluded that the NADPH-dependent reduction of cytochrome c by microsomes and the cytosol corresponds to two distinct enzymes. The cytosolic enzyme has been purified to a single SDS-PAGE band of about 53,000 da and partially characterized.

Trypanosoma cruzi, the causative agent of Chagas' disease, is resistant to known antiprotozoal drugs effective against other trypanosomiases. Permeability factors do not seem to play a role in resistance, and the possible involvement of detoxifying enzymes(known to be operational in vivo in this organism) appears to be a more plausible explanation of the phenomenon. To substantiate this view from the biochemical standpoint, we have undertaken the solubilization and purification of the components of the cytochrome P-450 system in culture forms of the organism. In this communication, we report the purification and some properties of a cytosolic NADPH-cytochrome c reductase from T. cruzi.

METHODS

Cells - Cultivation and collection of T. cruzi epimastigotes(Brazil strain) were done as previously reported(1,2). The cells were suspended in 0.25M sucrose, 10mM KH_2PO_4, pH 7.7, 30µg leupeptin/ml(Buffer I) and homogenized at 1,000 lb/sq. in. in a French Press cell.

Subcellular fractions - These were obtained by differential centrifugation at 900g x 15min(nuclei), 12,000g x 15min(mitochondria), and 105,000g x 90min (microsomes). The supernatant fluid remaining after the 105,000g centrifugation was carefully decanted(cytosol) and the "fluffy layer" on top of the microsomal pellet separated and saved.

Enzyme purification - The cytosol, made 2µM with respect to FAD, was loaded onto a DE-53 column (1.5 x 20cm) previously equilibrated with 10mM KH_2PO_4, pH 7.7, 20% glycerol, and 0.1mM EDTA(Buffer II). After washing the column with Buffer II, elution was carried out with a 0.0 to

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0.3M NaCl gradient in Buffer II containing 2μM FAD. The reductase which eluted at about 0.1M NaCl was then directly loaded onto a 2',5'-ADP-agarose column (1.5 x 6cm) equilibrated with Buffer II + 2μM FAD. The column was washed with the same buffer and then eluted with 5mM 2'-AMP in the same buffer. The fractions with the highest reductase activity were pooled and loaded onto a small DE-53 column (1.5 x 3cm) equilibrated with Buffer II. After washing the column with Buffer II, the enzyme was eluted with 0.2M NaCl in Buffer II. The fractions containing the highest reductase activity were pooled and finally concentrated by ultrafiltration through an Amicon PM-10 filter.

Analytical - SDS-PAGE was made using 7.5% acrylamide slab gels according to Laemmli(3). NADPH-cytochrome c reductase was followed as previously described(4)(1 unit = 1μmole of cytochrome c reduced/min).

RESULTS AND DISCUSSION

Table I shows the subcellular distribution of the NADPH-cytochrome c reductase activity of T. cruzi. Most of the activity is localized in the microsomal and cytosolic fractions in a ratio of approximately 1:3. This localization does not seem to be due to artifacts occurring during centrifugation or homogenization. Thus, various biochemical markers (succinate-cytochrome c reductase, acid phosphatase, NADH-cytochrome c reductase and carboxylesterase) showed the usual distribution pattern, i.e., succinate-cytochrome c reductase in the mitochondria, NADH-cytochrome c reductase in the microsomal pellet and acid phosphatase in the cytosol.

Since preliminary evidence suggested that the cytosol and the microsomal reductase activities may correspond to distinct enzymes and not just to NADPH-cytochrome c reductase released from the microsomal fraction into the cytosol during homogenization, the cytosol enzyme was purified about 95-fold by the procedure described under "Methods". The enzyme eluted with 2'-AMP showed two electrophoretic bands with molecular weights of 53,000 and 35,000 da. The reductase corresponded to the 53,000 da band since the lower molecular weight contaminant band was removed by the second DE-53 column chromatography step. Paper chromatography (5% Na_2HPO_4) of an enzyme extract showed the presence of only FAD (Rf = 0.33) without

Table 1. Subcellular distribution of NADPH-cytochrome c reductase activity in T. cruzi (relative distribution, %)

Fraction	Total Protein, mg.	Reductase Activity, total units	Specific Activity, units · mg^{-1}
Homogenate	89.1 (100)	3.713 (100)	0.0418
Nuclei	7.04(7.9)	0.135 (3.6)	0.0192
Mitochondria	13.8(15.5)	0.236 (6.3)	0.0171
Microsomes	23.6(26.5)	0.636(17.1)	0.0269
"Fluffy Layer"	2.6 (2.9)	0.099 (2.6)	0.0381
Cytosol	31.6(35.5)	1.884(50.7)	0.0596

any trace of FMN (Rf = 0.49). The enzyme showed a spectrum characteristic of flavoproteins, with major bands in the visible range at 450-460nm and 370nm and a pronounced band in the uv range at 276nm. From the absorbance at 450nm as well as by spectrofluorometry(5), ratios of mol FAD/mol of enzyme subunit (which varied from 0.3 to 0.86) were obtained. This suggested that the prosthetic group may not be tightly bound to the apoprotein. Thus, the enzyme lost about 10% of its activity daily when stored at 2-4°. However, the activity could be restored by the addition of 8μM FAD but not by FMN (up to 50μM). Freshly-isolated reductase with a specific activity of about 8 units/mg protein and a FAD to enzyme subunit ratio of about 0.3 could be reactivated by FAD to specific activities of up to 24 units/mg protein. The specific reactivation by FAD as well as the three-fold increase in activity not only substantiates that the cytosolic reductase is a FAD-enzyme but that the enzyme may have a ratio of mol FAD to mol of enzyme subunit of close to unity.

Table II summarizes some of the properties of the purified reductase. The K_m values for cytochrome c and NADPH are of the same order of magnitude as those reported for the mammalian microsomal NADPH-cytochrome c reductase and the corresponding housefly enzyme (6). It should be recalled that the K_m for NADPH reported for liver DT-diaphorase (7) is one order of magnitude higher, which, coupled to the inability of the DT-diaphorase to reduce cytochrome c, clearly differentiates it from the T. cruzi reductase. The T. cruzi enzyme is inhibited competitively with respect to NADPH by 2'-AMP and $NADP^+$; whereas, the inhibition is noncompetitive with respect to

Table II. Properties of the cytosolic T. cruzi NADPH-cytochrome c reductase

Compound	K_m(μM)	V_{max} (units/min/mg)	Turnover No. (units/min/ μmol enzyme)	K_i Variable Substrate	
				cytochrome c	NADPH
Cytochrome c	62.89	8.9	476(1,417)[a]		
NADPH	3.03	3.1	165(491)[a]		
2'-AMP				2.2 (nc)[b]	1.0(c)[c]
5'-AMP				0.0	0.0
5'-ADP				6.0 (nc)	---
$NADP^+$				0.62(nc)	0.076(c)

[a]Turnover Number based on FAD content as a measure of reductase present and on a subunit molecular weight of 53,200 da.

[b]Noncompetitive

[c]Competitive

cytochrome c. However, although the K_i values for $NADP^+$ are similar to those reported for mammalian microsomal NADPH-cytochrome c reductases (8), K_i values for 2'-AMP are much higher. 5'-AMP is not inhibitory while 5'-ADP shows slight inhibition with a K_i of at least 6.0mM.

Kinetic analysis of the NADPH-cytochrome c reductase indicates that the enzyme follows a Bi Bi ping-pong mechanism, as it has been reported for the porcine microsomal reductase (9). The enzyme was essentially NADPH-dependent, since NADH was 100-fold less active than NADPH in the reduction of cytochrome c. The enzyme showed a similar pattern of diaphorase activity to the mammalian microsomal NADPH-cytochrome c reductase (8), although adrenalin was not reduced at all and the activity with menadione was low.

The subcellular localization and the FAD-nature of the enzyme differentiates it clearly from the microsomal cytochrome P-450 reductase and, as expected, the cytosolic reductase appears to be unable to donate electrons to cytochrome P-450. The kinetic properties as well as the pyridine nucleotide specificity distinguish the enzyme from the DT-diaphorase and other cytosol diaphorases. However, the function of the T. cruzi enzyme is not understood at present. Whether the cytosolic reductase is involved or not in the generation of active oxygen intermediate species, as it has been reported for the mammalian P-450 reductase, remains to be established(10).

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