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CYANIDE-RESISTANT RESPIRATION AND A BRANCHED CYTOCHROME SYSTEM IN *KINETOPLASTIDAE*

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SUMMARY

Two functional terminal oxidases, cytochrome aa_3 and cytochrome *o*, are present in the cytochrome system in cyanide-sensitive trypanosomatids. The organisms examined included *Crithidia fasciculata*, *Leptomonas* sp., *Blastocrithidia culicis*, *Herpetomonas muscarum*, *Leishmania tarentolae*, *Trypanosoma lewisi*, *Trypanosoma conorhini* and *Trypanosoma cruzi*. A CO-binding protein with the spectral properties of cytochrome *o* has been solubilized and partially purified from *C. fasciculata*. In *L. tarentolae*, *T. conorhini* and *C. fasciculata*, 10–20% of the respiration is cyanide-resistant but is inhibited by salicylhydroxamic acid. The various possibilities of a branched electron transport system are examined and discussed.

Characterization of the cytochrome system in *Kinetoplastidae* has received much attention recently. The cytochrome *c* is a mammalian type with unusual properties including 1–2 residues of ε-*N*-trimethyllysine, and α-peak in the reduced form between 555–558 nm, one residue of cysteine in the amino acid sequence and an isoelectric point of pH 8.8[1–5].

Spectral studies of *Crithidia fasciculata* have previously provided evidence for cytochromes *b*, *c*. aa_3 and *o*[1,6,7]. Similar spectra have been obtained for *Crithidia oncopelti* (ref. 8 and Spencer R., unpublished) and *Trypanosoma mega*[9]. We have extended our previous studies to determining whether all cyanide-sensitive trypanosomatids have both cytochrome aa_3 and *o*. In addition, we have attempted to purify cytochrome *o* and study the significance of two terminal oxidases in these organisms.

C. fasciculata, *Leptomonas* sp., *Blastocrithidia culicis*, *Herpetomonas muscarum* and *Leishmania tarentolae* were grown in an undefined medium with glucose or xylose as the carbon source[7]. The bloodstream forms of *Trypanosama lewisi* were obtained from rats using procedures outlined by Lanham[11]. *Trypanosoma cruzi* was grown in a medium described by Gutteridge *et al.*[12] and *Trypanosoma conorhini* in a medium described by Bacchi *et al.*[13].

A crude mitochondrial fraction was obtained with organisms following previously published procedures[1]. The final pellet contained 10–15% of the original cell protein and could oxidize several substrates including succinate, NADH, α-glycerophosphate and proline. O_2 consumption was measured polarographically.

The cytochromes reduced by the oxidation of these substrates were observed (Fig. 1) using a Chance split-beam spectrophotometer.

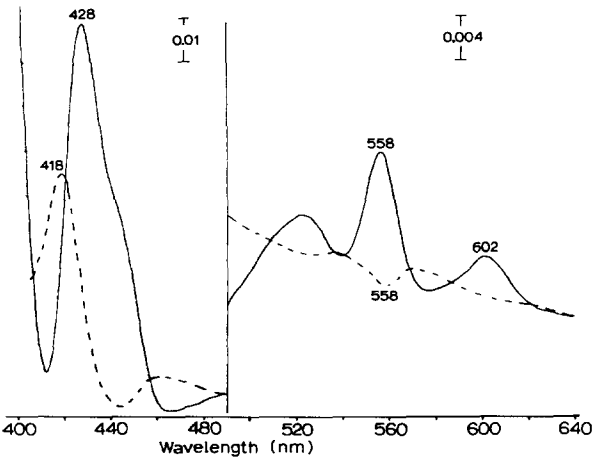

Fig. 1. Difference spectra of the crude mitochondrial fraction from *T. conorhini.* Cytochromes reduced by 1.0 mM NADH *minus* mitochondria with the cytochromes oxidized (———). Substrate reduction of cytochromes was complete after 6 min. The protein concentration was 4.0 mg/ml. Cytochromes reduced with 1.0 mM NADH and then saturated with CO for 10 min *minus* mitochondria reduced with 1.0 mM NADH (- - -).

No difference in the spectral appearance of cytochromes was observed whether intact cells or a crude mitochondrial pellet were examined or when different substrates were used to reduce the cytochromes. In the reduced *minus* oxidized difference spectrum of *T.conorhini* (Fig. 1), cytochrome aa_3 (602 nm) and cytochrome *b* (558 nm) are evident. A shoulder on the latter peak between 556 and 550 nm suggests the presence of cytochrome *c*. In low temperature difference spectra, a shoulder at 553 nm is quite distinct[1]. In the CO-reduced *minus* reduced difference spectrum (Fig. 1), two cytochromes which combine with CO are evident: cytochrome a_3 with a trough at 443 nm and cytochrome *o* with absorption peaks at 569, 538 and 418 nm. The presence of cytochrome *o* makes the detection of the cytochrome a_3–CO peaks at 590 and 432 nm sometimes difficult but these have been observed in previously published spectra[7].

All the organisms examined gave similar spectral evidence for the same cytochromes: cytochromes aa_3, *b*, *c* and *o*. *T. lewisi* was the only organism studied which had a greater proportion of cytochrome a_3 evident in contrast to cytochrome *o* (Hill, G. C. and Lanham, S., unpublished). Action spectra have provided evidence for the oxidase activity of cytochromes a_3 and *o* in *C. fasciculata*[14]. In addition, similar evidence has been obtained for *T. mega*, *L. tarentolae*, *B. culicis* and *C. oncopelti* (Kronick, P. and Hill, G., unpublished).

Attempts were made to purify cytochrome *o* from *C. fasciculata*. A CO-binding protein with a protoheme-containing prosthetic group was isolated from intact cells or the crude mitochondrial preparation. The cells or mitochondrial preparation were broken in 0.01 M sodium phosphate buffer, pH 8.0, by sonication or pressure (French press) at 20000 lb/inch^2. A soluble fraction containing cytochrome *c* and the CO-binding pigment was then obtained by centrifugation at $165000 \times g$ for 30 min.

The CO-binding pigment was separated from cytochrome *c* by passing the soluble protein through a cation resin (Amberlite IRC-50). The CO-binding pigment did not absorb on to the resin. Approximately 100 mg of CO-binding pigment protein was collected from 1 kg wet wt of intact cells.

The spectral properties of the partially purified CO-binding pigment were similar to the spectral properties of the substrate-reduced cytochrome *o* (Fig. 2).

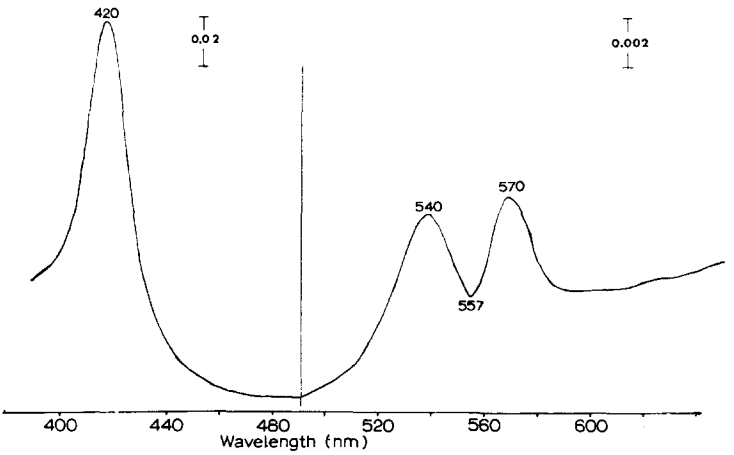

Fig. 2. Difference spectrum of the partially purified CO-binding pigment from *C. fasciculata*. The spectrum was obtained with the CO-binding pigment reduced with sodium dithionite and saturated with CO for 10 min *minus* the CO-binding pigment only reduced with sodium dithionite.

In addition, other spectral properties of the CO-binding pigment were observed (Table I). The α-peak of the reduced form had an absorption peak at 557 nm. It also had a pyridine hemochrome peak in the reduced form at 556 nm suggesting a protoheme prosthetic group. We have not observed any oxidase activity with this solubilized CO-binding pigment. Nevertheless, this CO-binding pigment may be cytochrome *o* or cytochrome *b* which have been released from the mitochondria.

TABLE I

SPECTRAL PROPERTIES OF THE CO-BINDING PIGMENT ISOLATED FROM *C. FASCICULATA*

Sample		*Peaks in reduced form (nm)*		
		α	β	γ
Dithionite treated − untreated		557	529	425
Pyridine hemochrome		556	523	420
Dithionite treated + CO − dithionite treated	(Peaks)	570	540	420
	(Trough)	557		

The presence of two functional terminal oxidases in trypanosomatids suggests a branched electron transport system. The respiration of *T. mega* varies between 40

and 90% cyanide-resistant. Ray and Cross[9,10] have provided evidence for both cytochromes aa_3 and *o* functioning in *T. mega* and have proposed a branched electron transport system for this trypanosomatid with cytochrome *o* functioning as the cyanide-resistant oxidase. The cyanide-resistant respiration in *T. mega* is inhibited by salicylhydroxamic acid[10], an inhibitor of cyanide-resistant respiration in yeast and plants. We have provided similar evidence for two functional terminal oxidases and a branched cytochrome system in *C. fasciculata*[15]. In *L. tarentolae*, *C. fasciculata* and *T. conorhini*, 10–20% of the respiration is cyanide-resistant but is sensitive to salicylhydroxamic acid (Table II). While it has been suggested that in trypanosomatids cytochrome *o* is the cyanide-resistant oxidase[9,15], cytochrome *o* also occurs in trypanosomatids which have less than 5% cyanide-resistant respiration (*e.g. H. muscarum*, *B. culicis*, *C. oncopelti*, *T. lewisi*). In addition, salicylhydroxamic acid inhibits cyanide-resistant respiration in yeast and plants which have no cytochrome *o* evident[16,17].

TABLE II

SENSITIVITY OF TRYPANOSOMATIDS AND SALICYLHYDROXAMIC ACID

The number in the parentheses represents the number of determinations.

Organism	*Percent of KCN-resistant NADH oxidase activity salicylhydroxamic acid* ($1 \cdot 10^{-3}$ *M*)		*Percent of NADH oxidase activity resistant to KCN* ($1 \cdot 10^{-3}$ *M*)	
L. tarentolae	98	(8)	19	(8)
C. fasciculata	96	(9)	10	(9)
T. conorhini	97	(8)	20	(8)

Studies have demonstrated cyanide-resistant respiration in culture forms which have undergone recent differentiation from bloodstream forms[18–21]. Spectral evidence for cytochrome aa_3 was not reported but spectral peaks for cytochrome *o* (ref. 21) and cytochrome a_2 (refs 19, 20) have been reported. Further studies with these differentiating trypanosome systems (*e.g. Trypanosoma brucei*, *Trypanosoma rhodesiense*) are needed to demonstrate whether the cyanide-resistant oxidase in the newly differentiated culture forms is cytochrome *o* or another cyanide-resistant oxidase. It is clear that cytochrome *o* is functioning in many insect trypanosomatids (*e.g. C. fasciculata*, *T. mega*) as one of the terminal oxidases.

Based on previously published spectral evidence[15], and the action of respiratory inhibitors on this system, the following possibilities exist for the branched electron transport system in *Kinetoplastidae* (Fig. 3). It seems clear that the cyanide-resistant pathway bifurcates before the antimycin-sensitive site. Antimycin or 2-*N*-heptyl-4-hydroxyquinoline-*N*-oxide have no effect on cyanide-resistant respiration or on cytochrome *o* reduction. Whether the branch occurs at cytochrome *b* (Scheme a) or at the flavin level (Scheme b) has not been determined. Neither rotenone nor sodium amytal are effective inhibitors in this system. In addition, neither pathway is affected by thiocyanate, α,α'-dipyridyl or 8-hydroxyquinoline, making it different from cyanide-resistant respiration observed in plant mitochondria[22]. It is possible that two separate

pathways are present (Scheme c). This seems unlikely because both systems appear to be localized in the mitochondria and no evidence for separate pathways has been demonstrated. Consideration must also be given to the cyanide-resistant oxidase not being cytochrome *o* but a cyanide-resistant oxidase similar to that found in yeast and plants[16,17,22]. The exact nature of this oxidase has not been identified.

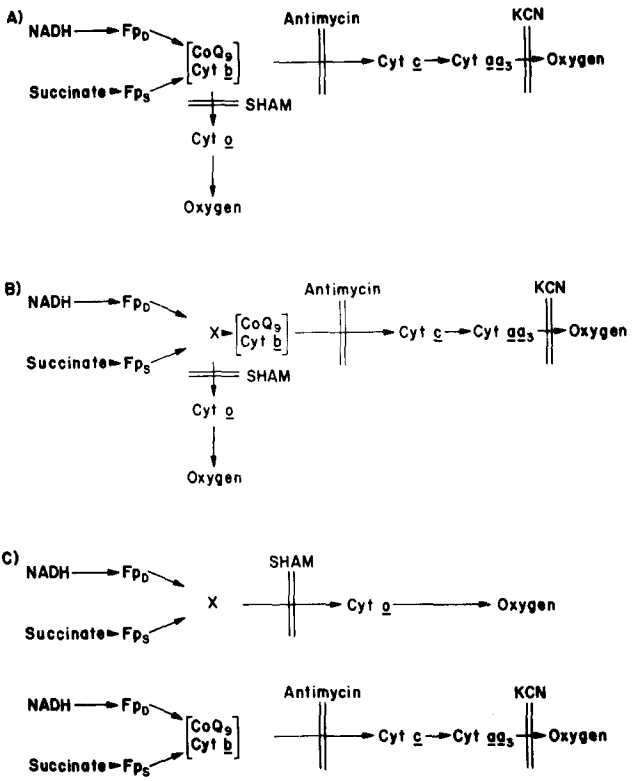

Fig. 3. Possible models for the electron transport system in *Kinetoplastidae*. Inhibition sites are indicated by the double line. X depicts an unspecified electron acceptor which could transfer electrons from the flavin region to cytochrome *o*. These proposed systems are similar to those proposed for cyanide-resistant respiration in yeast[23], plants[18] and *T. mega*[9,10]. Fp_D, NADH dehydrogenase; Fp_S, succinate dehydrogenase; SHAM, salicylhydroxamic acid.

We would suggest that the electron transport system branches at or before cytochrome *b* and that cytochrome *o* functions as a terminal oxidase in *Kinetoplastidae*, particularly during antimycin or cyanide-resistant respiration. The possibility does exist that cytochrome *o* is found outside the mitochondria as well and is reduced by substrate *via* a flavoprotein or is not mediated by other cytochromes. This possibility must be examined in light of the localization of cytochromes in bacterial membranes.

The differentiation which occurs during the development of trypanosomes in the insect vector and the vertebrate host has been reviewed by Vickerman[25]. The physiological function of the cyanide-resistant pathway and cytochrome *o* is not clear. Perhaps cytochrome *o* is necessary as an initial functional oxidase when

trypanosomes leave the bloodstream of vertebrates and develop in the less aerobic environment of the insect's digestive tract. Harrison[24] recently found that although the concentrations of cytochromes a_1 and a_2 were affected by varying the oxygen tension in the medium, the concentration of cytochrome *o* was not affected. A similar effect in the synthesis of cytochromes *o* and aa_3 may exist in trypanosomatids. There is no functional cytochrome system in the bloodstream forms of African trypanosomes. When the organisms are transmitted to the insect vector, the synthesis of cytochrome aa_3, in comparison to the synthesis of cytochrome *o*, may be prevented or inhibited due to the low oxygen tension in the insect's midgut. After the initial synthesis of cytochrome *o* as a cyanide-resistant oxidase, it is possible that as the trypanosome migrates to a location in the insect where the oxygen tension is greater (e.g. salivary glands), the synthesis of cytochrome aa_3 and the development of a cyanide-sensitive cytochrome system is stimulated. It is also possible that cytochrome *o* serves as an alternate oxidase when trypanosomes react with other oxidants besides oxygen during the development and function of their electron transport system in the insect vector.

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