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EVIDENCE FOR THE FUNCTIONING OF CYTOCHROME *o* IN KINETOPLASTIDA

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SUMMARY

Action spectra for the photochemical relief of CO-inhibition of respiration have provided evidence for two functional terminal oxidases, cytochrome a_3 and cytochrome *o* in several Kinetoplastida genera. These include: *Trypanosoma mega*, *Blastocrithidia culicis* and *Leishmania tarentolae*. No action spectral evidence for cytochrome *o* was found in *Crithidia oncopelti*. No correlation was observed between the CN-insensitivity of the respiration of the organisms and the amount of the cytochrome *o* observed.

INTRODUCTION

Trypanosomatids have been found to alter their respiratory biochemistry when they adapt to the various environments of their life cycle [1]. In laboratory cultures, *Crithidia fasciculata*, *C. oncopelti*, *Leptomonas sp.*, *Blastocrithidia culicis*, *Herpetomonas muscarum*, *Leishmania tarentolae*, *Trypanosoma lewisi*, *T. conorhini*, *T. cruzi*, and *T. mega* exhibit spectral evidence in CO difference spectra for both cytochrome a_3 and cytochrome *o* [2–4]. Understanding the role of cytochrome *o* may be important to unraveling the complicated respiration of these protozoa.

One important problem in the respiration of trypanosomatids is the persistence of oxygen uptake in the presence of high concentrations of cyanide in culture forms which contain cytochrome a_3 [1, 4]. If cytochrome *o* functions in trypanosomatids, it might be the terminal oxidase in the cyanide-resistant pathway. The various possibilities of a branched electron transport system have been discussed [2, 4]. However, no previous reports have provided action spectral evidence for the functioning of cytochrome *o* in Kinetoplastida.

Not all CO-binding pigments with the spectra of cytochrome *o* in its various states of oxidation and complexation react directly with oxygen. Of two isolated from *Vitreoscilla*, only one is autooxidizable [5]. Such a pigment also is observed in the absorption spectrum of *Ascaris* mitochondria, but its spectral features do not appear in the photochemical action spectrum for relief of CO inhibition, which shows only cytochrome a_3 [6].

Spectral evidence for cytochrome *o* appears during an earlier stage of growth than cytochrome a_3 in cultures of *C. oncopelti* [3]. If cytochrome a_3 reacts with oxygen faster than cytochrome *o*, then its presence and function may be the result of an adaptation to hypoxic conditions. Cytochrome a_3 may function when the reaction of cytochrome *o* with oxygen is rate-limiting. To investigate the conditions under which these two cytochromes function as oxidases in trypanosomatids, we have determined action spectra for the photochemical relief of CO-inhibition of respiration in several species in moderately low oxygen and extreme hypoxia. Our results demonstrate action spectral evidence for cytochrome *o* in several members of the Kinetoplastida.

MATERIALS AND METHODS

Culture of the organisms

B. culicis, *C. oncopelti* and *L. tarentolae* were grown with glucose as the carbon source in a medium previously described [7]. *T. mega* was grown in a medium described by Ray and Cross [8].

Optical absorption spectra

Difference spectra were determined as previously described using a Chance split-beam spectrophotometer [9].

Action spectra

For these measurements, a drop of cell suspension was retained by its own surface tension in a small ring and in contact with the tip of a 2-mm diameter polarographic oxygen electrode. This assembly was inside a small chamber at 25 °C, through which a prepared O_2–CO mixture could be flushed [10]. A window admitted condensed light from a monochromator with a 9-nm bandwidth, illuminated by a 450-W xenon lamp through a 2-cm water filter and louvre for varying the light intensity. As the wavelength was varied, the light intensity was kept constant. At each wavelength the sample was illuminated, and the derivative of the changing oxygen-electrode current was recorded. The level of oxygen in the dark was estimated by the level of the oxygen electrode signal in the dark or by integrating the derivative signal over the period when the oxygen consumption rate was constant during illumination.

RESULTS

T. mega

Fig. 1 shows the absorption spectra of the reduced cytochromes of *T. mega*, with the large contribution of cytochrome *b* (560 and 428 nm) and the smaller peaks from cytochromes aa_3 (602 nm). The CO-difference spectrum ($R_{Succ}+CO-R_{Succ}$) is dominated by spectral evidence for cytochrome *o*, with peaks (and relative intensities) at 418 nm [1], 538 nm (0.07), and 570 nm (0.08). Because of the preponderance of cytochrome *o*, the peaks of cytochrome a_3-CO (430 nm, 550 nm and 595 nm) are difficult to detect.

These cells are similar to those previously studied in which the cyanide-resistant pathway (capable of carrying 50–90 % of the respiration) was shown to be sensitive to salicylhydroxamic acid but resistant to 0.3 atm carbon monoxide [8]. Even at 0.9 atm carbon monoxide, inhibition was not complete in the dark, but the steady

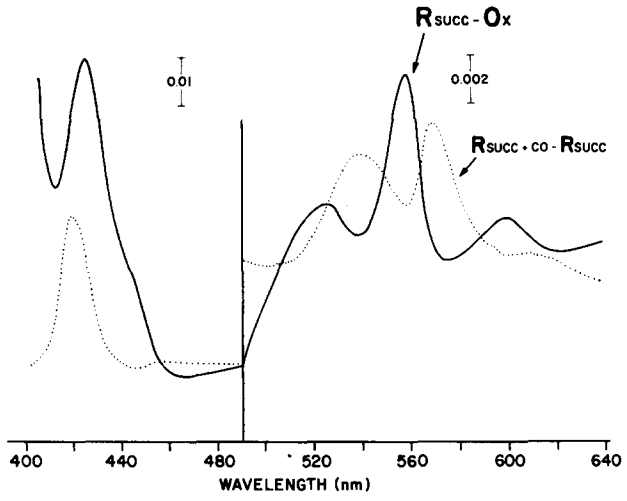

Fig. 1. Difference spectra of the crude mitochondrial fraction from *T. mega*. Cytochrome reduced by 10.0 mM succinate minus mitochondria with the cytochromes oxidized (———). Substrate reduction of cytochrome was complete after 5 min. The protein concentration was 3.5 mg/ml. Cytochromes reduced with 11.0 mM succinate and then saturated with CO for 10 min minus mitochondria reduced with 10.0 mM succinate (· · · · ·).

state oxygen level was high enough in our photolysis experiments so that transient respiration rates could be measured when the sample was illuminated.

In the 9 : 1 or 95 : 5 CO–O_2 mixtures used for the action spectra, the cells respired in the dark, maintaining the oxygen concentration in the drop of suspension

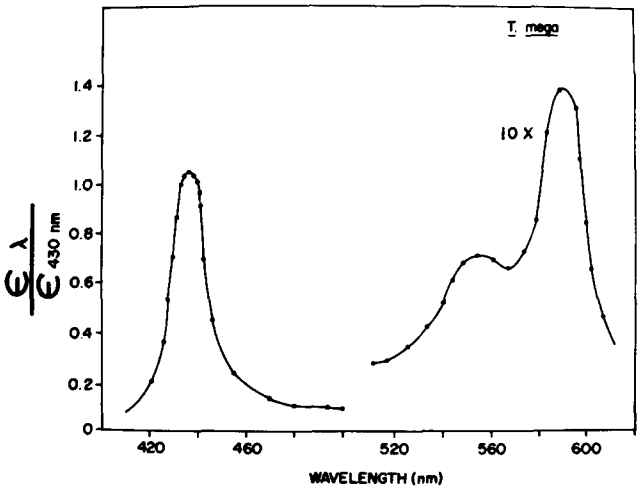

Fig. 2. Photochemical action spectrum for the relief of carbon monoxide inhibition of the endogenous respiration of *T. mega*. The oxygen level in the cell suspension was less than 1 torr. The cells were suspended in a buffer of 0.3 M sucrose and 0.25 mM Tris–HCl (pH 7.4). The spectrum was obtained at a low light intensity (15 A). The data points in this figure are plotted from the respiration data after correction for the light intensity and photon energy used. The intensity-corrected points are thus relative absorption coefficients, $\varepsilon_\lambda/\varepsilon_{430nm}$.

at a low level. The reading of the oxygen electrode when the drop was mounted in the dark indicated that this level was less than 1 torr. The very hypoxic suspensions showed no evidence of cytochrome *o* in their CO action spectra (Fig. 2). Both in wavelength and relative intensity, the bands are identical to those of cytochrome a_3-CO which we obtained from similar experiments on cytochrome aa_3 isolated from pigeon-breast muscle by the method of Yonetani [11]. This is true whether the cells had only endogenous substrate or whether this was supplemented by large amounts of added succinate. Salicylhydroxamic acid, which inhibits only the cyanide-resistant part of the respiration, had no effect on the action spectrum of these cells. In a more dilute cell suspension, levels of oxygen greater than 1 torr could be maintained in the dark steady state. In such a suspension of cells harvested during their early stationary phase, the action spectrum is still predominantly cytochrome a_3, but a small shoulder appears at 418 nm. The shoulder is also evident when a higher light intensity is used, bringing the system closer to light saturation (Fig. 3).

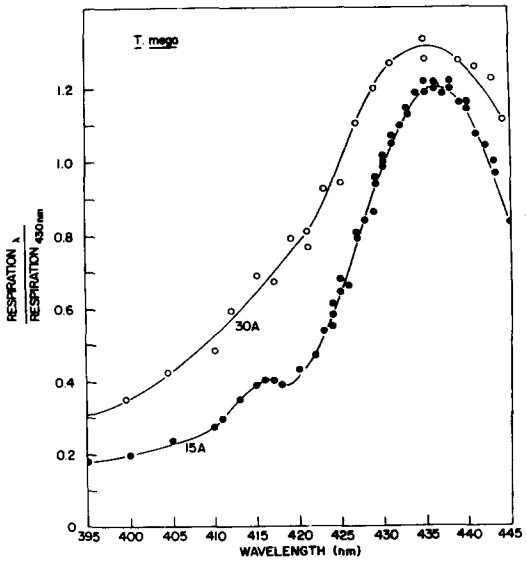

Fig. 3. Photochemical action spectra for *T. mega*. The oxygen level in the cell suspension was greater than 1 torr. In addition, the spectra were obtained at two light intensities differing by a factor of 4.

Blastocrithidia culicis

Respiration in *B. culicis* was inhibited 85 % by 0.001 M KCN, and 98 % by cyanide plus salicylhydroxamic acid [2]. The reduced minus oxidized difference spectrum was similar to that recorded for *T. mega* with both cytochromes a_3 and *o* evident. The shoulder at 418 nm in the action spectrum (Fig. 4) shows a small contribution of cytochrome *o* to the respiration, less than 10 % with the present low oxygen concentration.

C. oncopelti

Respiration in this species is very sensitive to cyanide. The reduced plus CO minus reduced difference spectrum is very similar to the other trypanosomatids

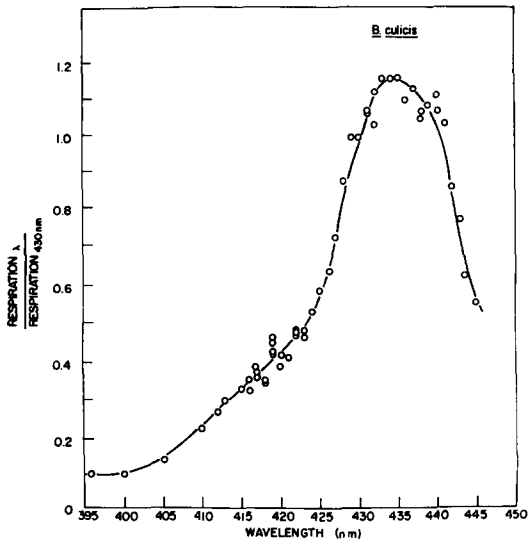

Fig. 4. Photochemical action spectrum of *B. culicis*. The oxygen concentration was less than 1 torr and the spectrum was obtained at the lower light intensity (15 A).

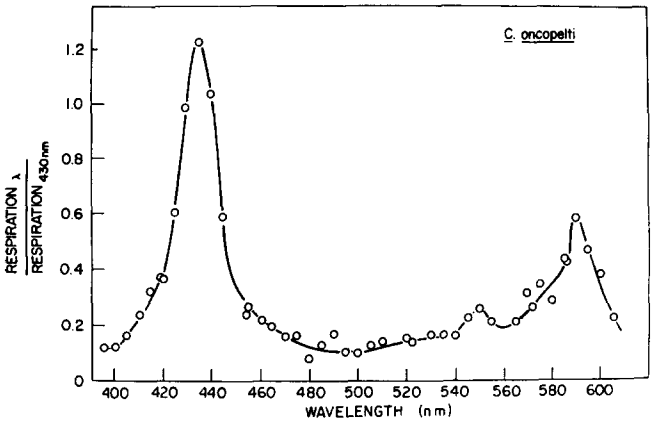

Fig. 5. Photochemical action spectrum of *C. oncopelti*. The oxygen concentration was greater than 1 torr and the spectrum was obtained at the higher light intensity (30 A).

studied. However, the action spectrum even at oxygen pressure above 1 torr provided evidence for only cytochrome a_3 (Fig. 5).

L. tarentolae

The respiration of *L. tarentolae* is inhibited 80 % by 0.001 M KCN [2]. Again, a suggestion of cytochrome *o* is found in CO-difference spectra. In concentrated suspensions of cells harvested during their stationary growth phase, no evidence for cytochrome *o* activity was evident (Fig. 6). When the level of oxygen was raised above 1 torr, early log cells had a clear spectral feature at 418 nm amounting to a separate peak attributable to cytochrome *o* (Fig. 7).

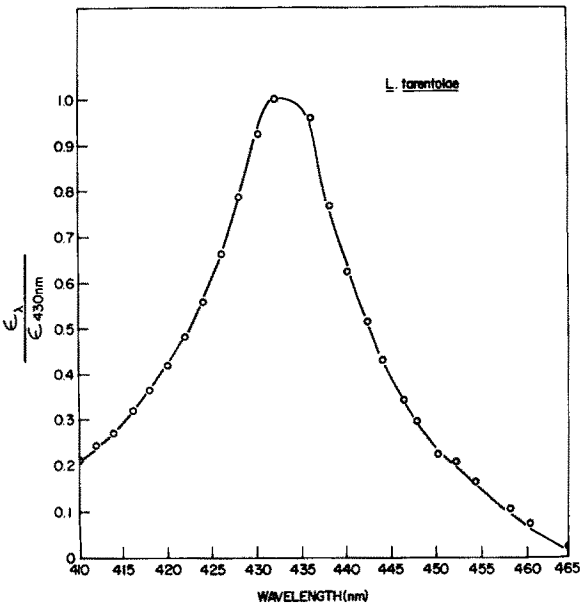

Fig. 6. Photochemical action spectra of *L. tarentolae*. The oxygen concentration in the cell suspension was less than 1 torr. The spectrum was obtained at the lower light intensity (15 A). The data points in this figure are plotted from the respiration data after correction for the light intensity and photon energy used. The intensity-corrected points are thus relative absorption coefficients, $\varepsilon_\lambda/\varepsilon_{430\,nm}$.

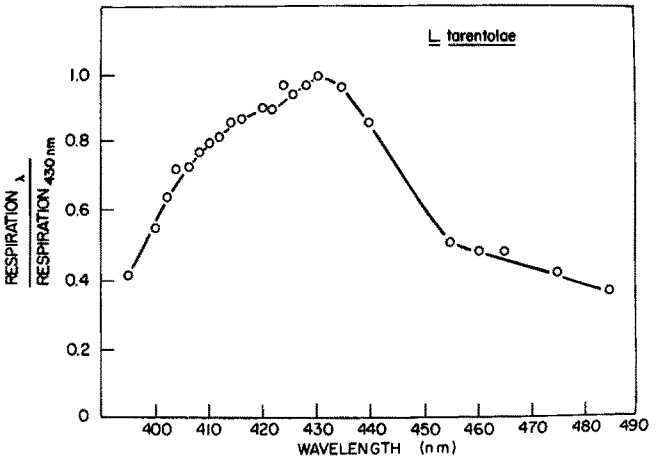

Fig. 7. Photochemical action spectrum of *L. tarentolae*. The oxygen concentration in the cell suspension was greater than 1 torr. The spectrum was obtained at the lower light intensity (15 Å).

DISCUSSION

The CO-absorption spectra of *T. mega* in the stationary stage of growth showed that their ratios of cytochrome *o* to a_3 were at least 3 : 1, and that the cytochrome *o* pigment reacted with the mitochondrial respiratory chain (Fig. 1). The respiratory transients used to determine the CO-action spectra in *T. mega* and the other organisms

were obtained under two conditions of oxygenation: (a) P_{O_2} above 1 torr and (b) P_{O_2} well below 1 torr. These two conditions are referred to as high-oxygen and low-oxygen conditions respectively. In the low-oxygen experiments of Figs 2 and 6, the small amounts of cytochrome oxidase were enough to support nearly all the respiration. These low contributions of cytochrome *o* under the extremely hypoxic conditions could be due to cytochrome *o* having a low affinity for oxygen.

As previous studies indicate, cytochrome *o* binds CO and cyanide more weakly than does cytochrome a_3. It is reasonable to expect that its affinity for oxygen is less than for cytochrome a_3, so that the respiration of trypanosomes in hypoxia is primarily mediated by cytochrome a_3. This possibility also exists for *Ascaris*, in which cytochrome a_3 dominates the action spectrum [6]. The experiments on *L. tarentolae* (Fig. 7) were performed at the limits of the experimental sensitivity, with very few cells in the sample drop. Here the oxygen tension was greater than 1 torr. Other experiments at high oxygen pressure, on *T. mega* (Fig. 3), and on *B. culicis* (Fig. 4) demonstrated that in these organisms cytochrome *o* is functioning.

In the action spectrum experiments, if R is the respiratory rate at a given wavelength under light intensity w, and $w^x_{\frac{1}{2}}$ is the light intensity needed to relieve by one-half the CO-inhibition of cytochrome x, then the ratio of respiration due independently to cytochrome a_3 in comparison to that for cytochrome *o* is:

$$Y/(1-Y) = (R_{436}/R_{418})\,(w+w^a_{\frac{1}{2}})/(w+w^o_{\frac{1}{2}})$$

Studies of cytochrome *o* in bacteria showed that $w^a_{\frac{1}{2}}$ was about the same as $w^o_{\frac{1}{2}}$ [12]. If we are in fact dealing with a very similar hemoprotein, then the large ratio of R_{436}/R_{418} which we found in hypoxia must be due to a large value of $Y/(1-Y)$.

No evidence for cytochrome *o* activity was found in *C. oncopelti*, either at high- or low-oxygen. The cells used here were harvested in the stationary phase. Since there is reported to be spectral evidence for cytochrome *o* in the log and stationary phases of growth [3], we would expect cytochrome *o* activity in such cells. Action spectral evidence for cytochrome *o* has also not been observed in *C. fasciculata* [13, 14].

The respiration of *C. oncopelti*, which depends on cytochrome a_3 rather than *o*, is very sensitive to cyanide ($\sim$ 98 %). *L. tarentolae*, whose respiration is 80 % cyanide sensitive, could respire through cytochrome *o*. *T. mega*, 40-90 % resistant to cyanide, respired nearly entirely through cytochrome a_3, even in the more oxygenated suspension. Clearly, there seems to be no correlation of cyanide resistance among organisms and the relative dependence on cytochrome *o* for respiration.

We have shown that the CO-binding pigment, cytochrome *o*, can function as an electron-transfer cytochrome in trypanosomatids, on a pathway to oxygen which does not pass through cytochrome aa_3. These experiments do not prove that it is a terminal oxidase, an inference which depends on our knowledge of the chemistry of hemoproteins which bind oxygen interchangeably with CO, NO, etc. We are interested in electron transfer to oxygen, however, which is not observed directly here. The possibility remains that CO inhibits the cytochrome *o* on binding to it by changing its redox potential or its specific interaction with a "cytochrome *o*-oxidase", rather than by competition with oxygen. If the effects of the inhibitor salicylhydroxamic acid on the action spectrum of cytochrome *o* is to be determined, experiments on these species must be done with instrumentation that will permit obtaining action spectra in the presence of high oxygen concentrations.

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