PHOTOCHEMICAL ACTION SPECTRA OF BACTERIAL *a*- AND *o*-TYPE OXIDASES USING A DYE LASER

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1. Introduction

Bacterial respiratory systems exhibit a diversity of components and of pathways of electron flow [1]. These may be terminated by any of a number of oxidases which include cytochromes *o*, *d*, a_1, or aa_3. The properties of these oxidases have been reviewed [2,3].

Although readily detected by difference spectra a role for an in vivo function has not been demonstrated for all bacterial oxidases. In particular, it has been difficult to characterize cytochrome a_1 as a terminal oxidase. Its definition has been made solely on the position of its α-band at 585–595 nm in CO-reduced difference spectra [4].

Identification of functional terminal oxidases has utilized the reversal of CO-inhibited respiration by light of differing wavelengths to produce a photochemical action spectrum. Maximum relief occurs at the λ_{max} of the oxidase [5–7]. However, the light intensity required for maximal relief of CO-inhibition can vary between different bacterial species and at different stages of growth of a single species [2]. This may be a critical factor for demonstrating a functional role for poorly characterized oxidases such as a_1.

Here we describe the application of a tunable dye laser to measure photochemical action spectra of bacterial cells from different phases of growth.

2. Experimental

All bacteria were grown at 30°C with vigorous aeration. Growth media for *Bacillus subtilis* [8], *Azotobacter vinelandii* [9] and *Escherichia coli* [10] were as described. *Pseudomonas putida* was grown on a simple salts medium which contained (g/l): KH_2PO_4, 7; K_2HPO_4, 7; $(NH_4)_2SO_4$, 1; $MgSO_4$, 0.2; $FeCl_3$, 0.5; yeast extract, 0.1.

Harvested cells for determination of difference spectra were washed once with a buffer that contained 50 mM Tris–HCl, 2 mM $MgCl_2$ and 1 mM EGTA (pH 7.4) and resuspended in the same buffer containing ethylene glycol (30%, v/v). Reduction of whole cell suspensions was by the addition of the growth carbon source: glucose for *B. subtilis*, mannitol for *A. vinelandii* and succinate for *E. coli* and *P. putida*, all to 50 mM. For CO-reduced difference spectra, suspensions were bubbled with CO for 5 min and then transferred to −20°C for a further 5 min. All spectra were recorded at 77 K maintained by a flow of cooled nitrogen over the cuvette in the sample compartment of the spectrophotometer.

Photochemical action spectra were determined directly on whole cell suspensions in growth medium. Monochromatic light generated by a Lexel model 95 argon ion laser was focused through a stream of either rhodamine 6G (for light at 580–610 nm) or rhodamine 560 (for light at 545–578 nm). The light beam was then tuned to the desired wavelength in a dye laser using a monochrometer. Light intensity was standardized prior to each measurement to 100 mW when using rhodamine 6G or 10 mW for rhodamine 560 using a photometer. Relief of CO-inhibited respiration was measured as a rate increase from the steady state respiration in the dark. All determinations were obtained by directing lased light on to the underside of the glass stage of a closed perspex chamber. A drop (~0.1 ml) or cell suspension was held within the chamber and held in the centre of the glass stage by a platinum microelectrode in a gas atmosphere of

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60:20, CO:O_2 ratio. Total gas flow rate through the chamber was maintained at a constant 80 ml/min using a gas flowmeter. All determinations were made at room temperature.

3. Results and discussion

A typical trace for the relief of CO-inhibited respiration by lased light is shown in fig.1 for *B. subtilis*. Light at 571 nm results in a relief of CO-inhibition detected as a rapid increase in respiration rate with time. When the light is switched off, CO binds back on to the oxidase and respiration is gradually inhibited, resulting in a gradual increase in the O_2 tension. Maximal relief of CO-inhibition is apparent here at 572 nm which corresponds to the λ_{max} for the cytochrome *o* of *B. subtilis* observed in difference spectra and action spectra.

Table 1 shows the *a* and *o*-type oxidases detected in the 4 bacterial species by CO-difference spectra.

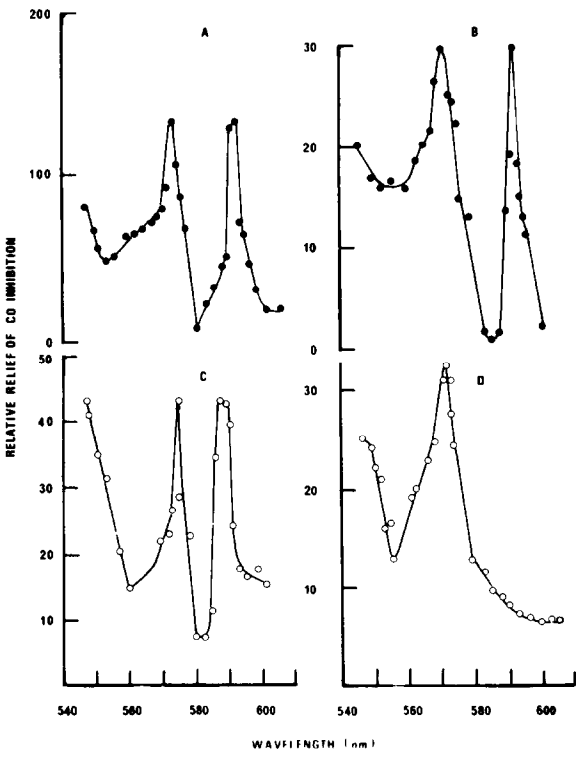

Fig.1. A typical trace for the relief of CO-inhibited respiration by light at different wavelengths. Here, is shown the relief of CO-inhibited respiration of exponential phase cells of *B. subtilis* by light at 571 nm, 572 nm and 573 nm.

Table 1
a- and *o*-Type oxidases present in CO-reduced minus reduced difference spectra

Organism	Oxidase(s)	
B. subtilis	aa_3	*o*
E. coli	a_1	*o*
A. vinelandii	a_1	*o*
P. putida	*o*	

Photochemical action spectra for the bacteria are presented in fig.2. Similar action spectra were obtained during all stages of growth for each of the bacterial species. Both cytochrome *o* and aa_3 function as terminal oxidases in *B. subtilis* with λ_{max} for each centred at 572 nm and 593 nm, respectively (fig.2A). In *E. coli*, both a_1 and *o* oxidases result in peaks in the action spectrum at 592 nm and 570 nm (fig.2B). Previous work has only demonstrated the presence of a functional *o* in *E. coli* and aa_3 in *B. subtilis* [7]. The finding that a_1 can function as an oxidase in *E. coli* is of considerable interest in view of the doubts expressed as to its ability to react with oxygen [4]. The results obtained for *A. vinelandii* confirm those in [11–13], namely, that cytochromes a_1 and *o* can function as oxidases in vivo. Maxima for relief of CO inhibition of a_1 and *o* are apparent at 589 nm and 573 nm, respectively (fig.2C). In *P. putida* only one peak at 571 nm is seen, confirming the sole presence of cytochrome *o* (fig.2D). Troughs in all the action spectra occur at 551 nm *(B. subtilis)*, 555 nm *(E. coli)*, 560 nm *(A. vinelandii)* and 555 nm *(P. putida)*, characteristic for an *o*-type oxidase. The peak in the region

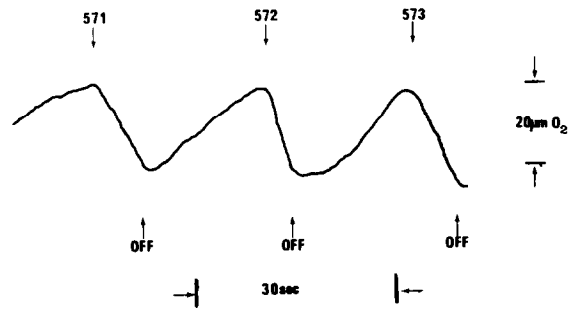

Fig.2. Photochemical action spectra for the relief of CO inhibition for: (A) *B. subtilis;* (B) *E. coli;* (C) *A. vinelandii;* (D) *P. putida.* The maximum relief of CO inhibition obtained at different wavelengths with rhodamine 6G (580–610 nm) is normalized to that obtained using rhodamine 560 (545–578 nm).

of 540–546 nm for this oxidase could not be resolved due to an acute falling off of laser light intensity. However, in all cases a progressive increase in relief of CO-inhibition occurs at wavelengths approaching this region (550–546 nm).

The method outlined here has been used to resolve the *o*- and *a*-type cytochrome oxidases of 4 species of bacteria. The increased light intensities that can be obtained using a dye laser result in a sharper resolution of the λ_{max} of oxidases and the identification of a_1 as functional oxidase in both *E. coli* and *A. vinelandii.*

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