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THE CYTOCHROME SYSTEM OF *AZOTOBACTER VINELANDII*

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SUMMARY

1. Difference spectra and carbon monoxide action spectra of *Azotobacter vinelandii* small particles indicate the presence of cytochromes $c_4 + c_5$, b_1, a_1, a_2 and o, of which a_1, a_2 and possibly o may be acting as terminal oxidases.

2. The aerobic steady-state reduction of cytochrome b_1 was unexpectedly found to be much lower than that of cytochromes $c_4 + c_5$, a result which was incompatible with redox potential and anaerobic state data.

3. 50 μM KCN and 2 mM azide were found to maximally inhibit ascorbate-2,6-dichlorophenolindophenol oxidase whereas NADH oxidase was not significantly inhibited. These results, together with the ability of these inhibitors to increase greatly the aerobic steady-state reduction of cytochromes $c_4 + c_5$ but not of b_1 suggested the presence of a branched cytochrome system, each branch linked to a functionally separate oxidase system.

4. The concept of a branched cytochrome system was supported by the pattern of light relief of CO inhibition exhibited by the terminal oxidases.

INTRODUCTION

The electron transport system of *Azotobacter vinelandii* has long been recognized as being one of the most active yet encountered in living tissues. The respiratory particles contain a rich and complex complement of electron carriers[1] and an apparent multiplicity of terminal oxidases[2]. However, in spite of a number of investigations into subcellular enzyme distribution[3,4] coupled oxidative phosphorylation[5–10] and the purification of individual cytochrome components[11–14], little is known of the actual pathways of electron transport within these particles and even less of the nature of the highly active cytochrome oxidase system. Such studies have been rendered far more difficult than in mitochondria due to the relative insensitivity of bacterial electron-transport systems to common respiratory inhibitors. This difficulty has been further accentuated in previous studies[15] on the cytochrome oxidase of Azotobacter by the observation that although readily inhibited by CO, the relief of this inhibition by light was not readily achieved. Thus investigations into the cytochrome oxidase system were severely hampered by this apparent insensitivity to light. The position was greatly improved by the introduction by CASTOR AND CHANCE[16,2] of a new

Abbreviations: AHQO, 2-*n*-alkyl-4-hydroxyquinoline-*N*-oxide; DCIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulphate.

method of detecting CO action spectra in a wide range of living tissues. These workers were able to implicate cytochromes *o* and a_1 as terminal oxidases in Azotobacter but were unable to implicate cytochrome a_2 on technical grounds.

This paper describes investigations into the nature of the terminal cytochrome system of *A. vinelandii* particles using such inhibitors as are available, together with measurements of cytochrome oxidation–reduction kinetics by dual-wavelength spectrophotometry and photochemical techniques.

MATERIALS AND METHODS

Chemicals

CO was obtained in lecture bottles from the Matheson Gas Corporation, U.S.A. via Cambrian Chemicals Ltd., London. NADH was obtained from C. F. Boehringer und Soehne, Mannheim, Germany. All other chemicals were obtained from British Drug Houses Ltd., Poole, England, and were of the finest grade available. Glass double-distilled water was used throughout this work.

General methods

Cultures of *A. vinelandii* (N.C.I.B. 8660) were grown on nitrogen-free medium (using N_2 in air as the sole nitrogen source) as described previously[1]. The harvesting and fractionation of the cells to yield washed small particles, the determinations of cytochrome levels from reduced *minus* oxidized difference spectra and the measurement of oxidase activities were all carried out as described by JONES AND REDFEARN[1]. CO reduced *minus* reduced difference spectra were measured after bubbling CO through a dithionite reduced preparation for approx. 1 min. All room temperature spectra were measured on a Unicam SP 800 recording spectrophotometer, the traces being expanded as required on to a slave recorder.

Low temperature and CO action spectra

Difference spectra were recorded at the temperature of liquid N_2 in a split-beam spectrophotometer[17] by Dr. D. F. WILSON, and the CO action spectra in a specially designed apparatus by Mr. T. HYDE at the Johnson Research Foundation, University of Pennsylvania, U.S.A.

Dual-wavelength spectrophotometry

The reduction kinetics of the individual cytochrome components of *A. vinelandii* small particles were determined using an Aminco-Chance dual wavelength spectrophotometer. The following wavelength pairs were employed: cytochromes $c_4 + c_5$, 551–544 mμ; b_1, 560–568 mμ; a_2, 630–619 mμ. All measurements were carried out at 20–22° in the 2–5 % transmission range in a final vol. of 3 ml. The reaction mixture contained 100 μmoles Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) 1.5–5.5 mg small particle protein and glass double-distilled water to 2.97 ml. The reaction was initiated by the addition of 9 μmoles of substrate, or in the case of ascorbate–2,6-dichlorophenol-indophenol (DCIP), 9 μmoles of ascorbate + 0.5 μmoles DCIP in a vol. of 0.03 ml.

CO inhibition and light relief

CO inhibition of oxidase activity and the relief of this inhibition by light was

measured with a Clark electrode at 20–22°. Varying ratios of O_2:CO were obtained by mixing varying volumes of air and CO satd. 25 mM phosphate buffer (pH 7.4).

High intensity illumination of the clear Perspex reaction chamber was provided by a 1000 W spot lamp. A 31-inch round bottom flask filled with water was used as a heat filter and focussing device between the lamp and the electrode, the centre of the flask being 39 cm from the lamp and 16 cm from the centre of the electrode reaction chamber. The latter was kept at a constant temperature using a thermostatically controlled water-jacket. Coloured light was obtained using Wratten filters number 47b (blue, 385–490 mμ) or 29 (red > 610 mμ). Low intensity illumination was provided by two 150 W spot lamps under similar conditions. Unless otherwise stated, high-intensity light was used for all illumination experiments.

RESULTS

The room temperature reduced *minus* oxidized difference spectrum of *A. vinelandii* respiratory particles has previously been described by many workers (see refs. 1 and 18 as examples). The complete cytochrome complement appeared to consist of *c*-type cytochrome (522, 551 mμ), b_1 (427, 529, 560 mμ), a_1 (440 shoulder, 593 mμ) and a_2 (628–630 mμ). The CO-binding pigment cytochrome *o* could not be detected as a spectroscopically distinct entity in such a spectrum. The *c*-type cytochrome has been purified[11,12] and shown to consist of 2 separate components, cytochromes c_4 and c_5 having similar redox potentials and with α-bands at 551 and 555 mμ respec-

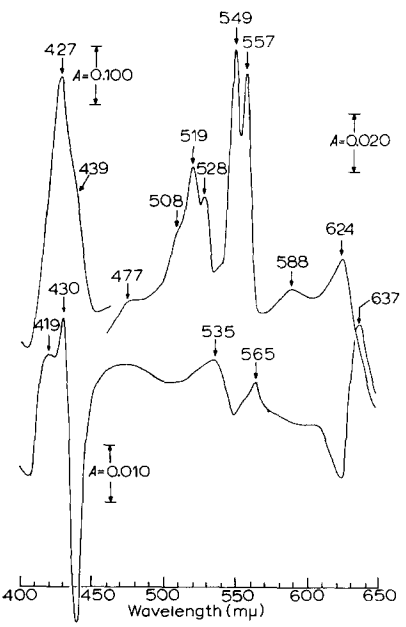

Fig. 1. Low temperature (77° K) difference spectra of *A. vinelandii* respiratory particles. Above: dithionite reduced *minus* oxidized (4.02 mg protein/ml); below: CO-dithionite reduced *minus* dithionite reduced (3.24 mg/ml). Where required, CO was added as a steady stream for approx. 1 min.

tively which fuse to give the peak seen at 551 mμ in room temperature difference spectra.

A low temperature (77° K) reduced *minus* oxidized difference spectrum of *A. vinelandii* particles is shown in Fig. 1. Although all the bands were considerably sharper than in the room temperature spectra, and shifted several mμ towards the blue, no further splitting of the bands to show the presence of two *c*-type cytochromes or cytochrome *o* could be observed although new shoulders did appear at 477 and 508 mμ. Extraction of whole cells for cytochromes $c_4 + c_5$ by the method of TISSIÈRES[11] yielded only cytochrome c_4. This and the spectral evidence suggest that the levels of cytochrome c_5 may be very low in this strain of *A. vinelandii*.

The low temperature CO-reduced *minus* reduced difference spectrum is also shown in Fig. 1. This indicates the presence of cytochromes a_1 (430 mμ, little absorption in the 590-mμ region), a_2 (637 mμ) and *o* (strong shoulder at 419, 535 and 565 mμ). The characteristic W-shaped Soret band, previously observed in *P. vulgaris*[19] and attributed to a mixture of these two cytochrome components, is no longer visible when the spectrum is recorded at low temperature on a split-beam spectrophotometer, but reappears when recorded at room temperature on a Unicam SP 800.

TABLE I

THE CYTOCHROME CONTENT OF *A. vinelandii* RESPIRATORY PARTICLES

Cytochrome concn. are expressed as μmoles per g protein and are typical values. The concentration of electron carriers in these particles show only small variations in successive preparations and are calculated by the methods described by JONES AND REDFEARN[1] and using the millimolar extinction coefficient of 80 quoted for the Soret band of O-CO by TABER AND MORRISON[20].

Nitrogen source	*Cytochromes*					$c_4 + c_5/b_1$ *ratio*
	$c_4 + c_5$	b_1	a_1	a_2	*o*	
N_2 (air)	1.62	1.54	++	0.67	0.08	1.05

The concentrations of the individual cytochrome components are shown in Table I. These values indicate that the particles are rich in cytochromes, a fact compatible with the extremely high oxidase activities exhibited by these particles[1]. The concentration of cytochrome *o* (0.08 μmoles per g protein) may be slightly inaccurate since cytochrome *o* appears only as a shoulder on the main a_1-CO band. Nevertheless, the low concentration of cytochrome *o* compared with cytochrome a_2 is very striking. The value of 1.05 for the ratio of the concentrations of $c_4 + c_5 : b_1$ in the particles was remarkably consistent for many separate particle preparations.

The action spectrum for relief of CO inhibition of whole cells oxidizing endogenous substrate is shown in Fig. 2. The spectrum was measured by a modification of the method of CASTOR AND CHANCE[16] and is not corrected for changes in light intensity over the wavelength range employed. The spectrum is especially noteworthy for the very large contribution of cytochrome a_2; cytochrome a_1 is also clearly defined but the relief by light in the 520–550 mμ region is neither consistently reproducible nor entirely compatible with the known spectral characteristics of cytochrome *o*. The Soret region (not shown) was characterized by a broad band with a maximum

at 430–436 mμ, with weak shoulders at approx. 410 and 470 mμ; absorbance in the Soret region was lower than expected.

Substrate reduction of individual cytochrome components of the particulate electron transport system of *A. vinelandii* is shown in Fig. 3. On the addition of substrate an aerobic steady-state reduction is attained which persists until all the O_2 dissolved in the reaction mixture has been consumed; at this point the cytochrome becomes further reduced and passes into the anaerobic reduced state. The addition of dithionite enables the total cytochrome content to be determined.

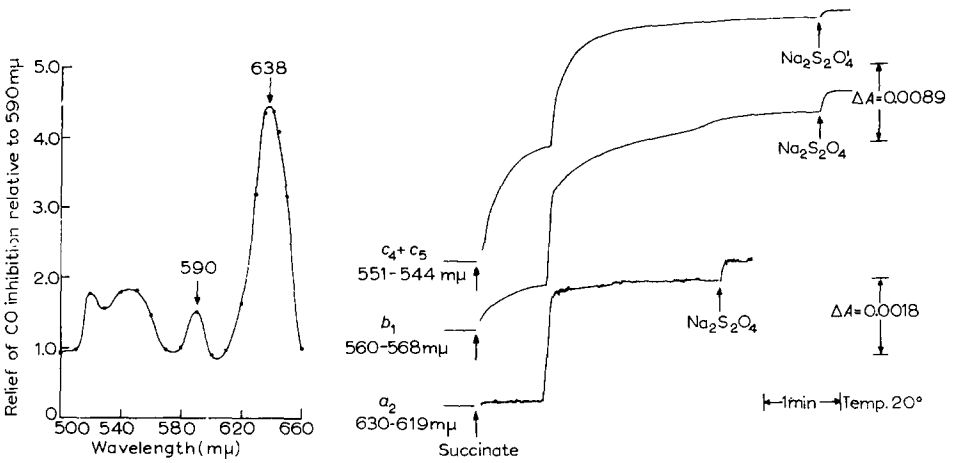

Fig. 2. CO action spectrum of *A. vinelandii* whole cells oxidizing endogenous substrate. The extent of this relief is plotted with 580 mμ irradiation equalling unity.

Fig. 3. Substrate reduction of the cytochrome components of *A. vinelandii* respiratory particles as determined by dual wavelength spectrophotometry. An upward deflection of the trace indicates reduction.

Cytochromes $c_4 + c_5$, b_1 and a_2 are the only components the reduction of which can be followed with any degree of accuracy. The further reduction of cytochrome b_1 from the aerobic steady state to the anaerobic state differs from that of $c_4 + c_5$ and a_2 in that this progressive increase in absorbance is not smooth, but characterized in most preparations by a second much smaller increase some time after the initial large change, as if a second component absorbing in this region is being reduced either after the b_1 or at a slower rate than the b_1. However, simultaneous measurements of O_2 utilization and absorbance changes indicate that this component is neither cytochrome a_2 nor $c_4 + c_5$ since these pass rapidly into the anaerobic state at the exact point when the O_2 tension reaches zero.

The aerobic steady-state and anaerobic state reductions of the cytochromes by different substrates are shown in Table II. The natural substrates, succinate, L-malate and NADH, all of which are oxidized at maximal rates by unsupplemented particles, alone are capable of almost complete reduction of all the cytochrome components in the anaerobic state. With all three substrates, however, unexpected results are obtained in that the aerobic steady-state reduction of cytochromes $c_4 + c_5$ is much greater than cytochrome b_1; as expected both are much

TABLE II

SUBSTRATE REDUCTION OF *A. vinelandii* CYTOCHROME SYSTEM AS DETERMINED BY DUAL WAVELENGTH SPECTROPHOTOMETRY

The system consisted of 100 μmoles Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) 1.5–5.5 mg small particle protein and glass distilled water to give a final vol. of 3.0 ml. The reaction was started by the rapid addition of 9 μmoles L-malate, succinate or NADH; 9 μmoles ascorbate *plus* 0.5 μmole DCIP. The following wavelength pairs were used in these experiments: $c_4 + c_5$ 551–544 mμ, b_1 560–568 mμ and a_2 630–619 mμ; temperature 20–22°. Oxygen consumption was measured on a Clark electrode at 30°. The numbers in brackets refer to the number of aerobic steady-state determinations made on successive particle preparations.

Substrate	*Cytochrome*						*Ratio cytochrome* $c_4 + c_5$: b_1 *aerobic steady-states*	*μmoles substrate oxidized per min/mg protein*
	$c_4 + c_5$		b_1		a_2			
	Aerobic steady-state (%)	*Anaerobic state* (%)	*Aerobic steady-state* (%)	*Anaerobic state* (%)	*Aerobic steady-state* (%)	*Anaerobic state* (%)		
L-Malate	40 (12)	90	22 (11)	90	0	84–97	1.83	1.37
Succinate	41 (30)	90	19 (22)	90	0	85–100	2.18	0.42
NADH	36 (29)	90	21 (26)	90	0	82–100	1.76	2.86
Ascorbate–DCIP	79 (9)	83 (9)	36 (9)	56 (9)	0	79–95		0.63

greater than cytochrome a_2. It is also of interest to note that the ratio of the aerobic steady-state reductions of $c_4 + c_5 : b_1$ calculated from a large number of separate and coupled determinations, is inversely proportional to the oxidase activity. This is true both for different substrates (as shown in Table II) and for a single substrate, *e.g.* succinate, in the presence of increasing amounts of inhibitor, *e.g.* malonate (not shown).

In the case of the artificial electron donor system ascorbate–DCIP, the aerobic steady-state reduction of cytochromes $c_4 + c_5$ is extremely high, thus suggesting that the system donates electrons predominantly at the level of the *c*-type cytochrome. The aerobic steady-state reduction of cytochrome a_2 is not increased by this relatively positive potential system, and the low anaerobic state reduction of cytochrome b_1 suggests that the redox potential of this carrier is too negative for the ascorbate–DCIP system to effect complete reduction.

A. vinelandii respiratory particles readily oxidize ascorbate only in the presence of intermediate electron carriers such as DCIP (K_m = 0.105 mM; V_{max} = 1.07 μmoles per min per mg protein), exogenous *A. vinelandii* $c_4 + c_5$ (K_m = 0.084 mM; V_{max} = 0.50 μmoles/min per mg protein) or exogenous mammalian cytochrome *c* ($K_m = 0.20 \times 10^{-4}$ M; V_{max} = 0.02 μmoles/min per mg protein). The rate of oxidation of ascorbate *via* these intermediate carriers is proportional to particle concentration over the range 0–3.0 mg protein. Unmediated oxidation of ascorbate by the particles is slow and never attains a rate greater than 10 % of the ascorbate–DCIP oxidase activity.

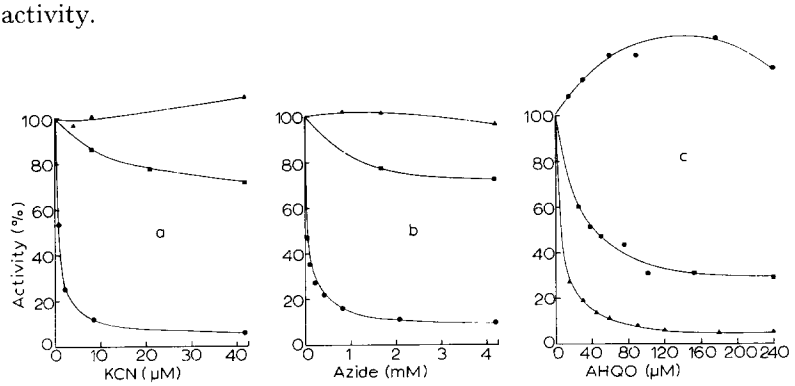

Fig. 4. The sensitivity of NADH (▲—▲), succinate (■—■) and ascorbate DCIP (●—●) oxidase of *A. vinelandii* respiratory particles to (a) KCN (b) Azide and (c) AHQO. In all cases the particles (0.14–1.02 mg protein) were incubated for 2 min with the inhibitor immediately prior to the addition of substrate. Oxidase activities were assayed using a Clark electrode at 30° as described in MATERIALS AND METHODS. For measuring AHQO inhibition, the same amount of protein (1.02 mg protein) was used for all three substrates, thus keeping the concentration of inhibitor per mg protein the same.

The action of low concentrations of KCN and azide, the classical inhibitors of cytochrome oxidase, on the activities of particulate *A. vinelandii* NADH succinate and ascorbate–DCIP oxidases are shown in Fig. 4. Low concentrations of either inhibitor (40 μM KCN, 4 mM azide) almost maximally depress ascorbate–DCIP oxidase, (also ascorbate–*A. vinelandii* $c_4 + c_5$ oxidase and reduced *A. vinelandii* $c_4 + c_5$ oxidase), whilst causing from nil to partial inhibition of succinate oxidase and little or no inhibition of NADH oxidase. High concentrations of KCN (2 mM) completely inhibit all three oxidases, whereas azide (0.1 M) inhibits succinate oxidase

by 49 % and NADH oxidase by 57 % (not shown in Fig. 4). The relative insensitivity to these inhibitors of NADH oxidase compared with ascorbate–DCIP oxidase is unexpected in view of the much higher activity of NADH oxidase (see Table II) and argues against a terminal oxidase system common to both substrates.

The effect of 2-*n*-alkyl-4-hydroxyquinoline-*N*-oxide (AHQO) is shown in Fig. 4c. High concentrations of this inhibitor (200 μM) inhibit NADH oxidase by 90–95 %, whilst the percentage inhibition of succinate oxidase is considerably less and ascorbate–DCIP oxidase is slightly stimulated. In all the assays carried out the residual NADH oxidase activity is comparable, on the basis of specific activity, with the residual succinate oxidase activity. However, as AHQO of this concentration also substantially inhibits succinate dehydrogenase (phenazine methosulphate (PMS)–DCIP as acceptors) and is in approx. 300-fold molar excess over cytochrome b_1, its traditionally accepted site of action, these results must be evaluated with caution.

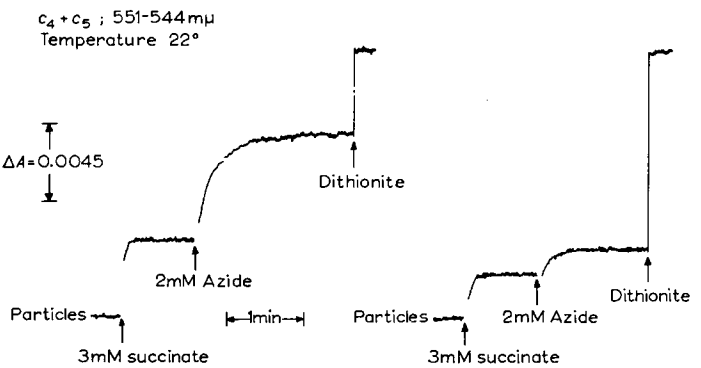

Fig. 5. The effect of 2 mM azide on the aerobic steady state of cytochromes $c_4 + c_5$ and b_1 of *A. vinelandii* particles oxidizing succinate as measured on a dual wavelength spectrophotometer. An upward deflection of the trace indicates reduction.

Fig. 5 shows the effect of low concentrations of azide on the aerobic steady-state reduction of cytochromes $c_4 + c_5$ and b_1 of *A. vinelandii* respiratory particles oxidizing succinate. 2 mM azide, a concentration sufficient to maximally inhibit DCIP mediated oxidation of ascorbate, causes a large increase in the aerobic steady state reduction of cytochrome $c_4 + c_5$, whilst increasing that of cytochrome b_1 only marginally. A similar result (not shown) was obtained with 50 μM KCN.

The effects of electron-transport inhibitors on the aerobic steady-state reduction of cytochromes $c_4 + c_5$ and b_1 are extended and summarized in Table III. For succinate, these results confirm those shown graphically in Fig. 4. Low concentrations of KCN or azide greatly increase the aerobic steady-state reduction of cytochrome $c_4 + c_5$ whilst increasing that of cytochrome b_1 by only a small amount; the effect on cytochromes $c_4 + c_5$ can be further potentiated by aging the particles. High concentrations of KCN (5 mM) are required to cause a similar increase in the aerobic steady-state reduction of cytochrome b_1, using either succinate or NADH as substrate. On the other hand high concentrations of azide (200 mM) only marginally increase the aerobic steady-state reduction of cytochrome b_1 with succinate as substrate, but substantially increase this reduction when NADH is the source of electrons.

TABLE III

THE EFFECT OF ELECTRON TRANSPORT INHIBITORS ON THE AEROBIC STEADY-STATE REDUCTION OF CYTOCHROMES $c_4 + c_5$ AND b_1 IN *A. vinelandii* RESPIRATORY PARTICLES

The assays were carried out on a dual wavelength spectrophotometer at 20–22° using approx. 2 mg particle protein as described in MATERIALS AND METHODS. The particles were incubated in the reaction cuvette with the appropriate inhibitor for 1 min immediately prior to the addition of substrate. The degree of inhibition was calculated from the time taken to reach anaerobic conditions. In the case of CO, 1 ml of CO-saturated buffer was mixed with 2 ml air-saturated buffer containing the respiratory particles.

Additions	*Concn.*	*Succinate*				*NADH*			
		Expt. No.	*Aerobic steady-state*		*Activity (%)*	*Expt. No.*	*Aerobic steady-state*		*Activity (%)*
			c_4+c_5	b_1			c_4+c_5	b_1	
—	—	I	32	18	100	II	29	20	100
Malonate	0.33 mM		29	10	14				
Malonate	3.3 mM		22	4	very low				
KCN	50 μM		74	31	84		35	21	111
KCN	5 mM		89	71	10		90	82	10
Azide	2 mM		61	26	96		37	28	97
Azide	50 mM		73	29	42		65	41	72
Azide	200 mM		73	31	25		86	56	46
Malonate + KCN	0.33 mM 5 mM		91	67	very low				
Malonate + KCN	3.3 mM 5 mM		86	32	very low				
—	—	III	48	20	100	III	41	20	100
AHQO	200 μM		52	30	49		46	31	34
—	—	IV	26	16		V	30	22	
CO			40	43			53	49	

This latter observation is probably a reflection of the large difference in the specific activities of NADH and succinate oxidases; in the former case the cytochrome oxidase needs to be only partially inhibited for it to become rate limiting whereas with succinate the overall oxidase activity is much slower such that a very much greater inhibition by azide is required before cytochrome oxidase again becomes the rate-limiting step.

The effect of KCN on the aerobic steady-states in the presence of malonate is especially interesting. Malonate alone substantially inhibits succinate oxidase activity and in doing so also decreases the aerobic steady-state reduction of cytochromes $c_4 + c_5$ and b_1; this latter effect being marginally greater on the *b*-type cytochrome. The addition of a high concentration of KCN (5 mM) together with the malonate (0.33 or 3.3 mM) brings about the expected large increase in the aerobic steady-state reduction of cytochromes $c_4 + c_5$, but at the higher malonate concentration does not bring about the expected large increase in the aerobic steady-state reduction of cytochrome b_1 which can be effected by 5 mM KCN alone. Thus it is again apparent that by decreasing the electron flux from the dehydrogenase to a low rate by the use of malonate, it is no longer possible to increase the aerobic steady-state reduction

of cytochrome b_1 by inhibition of the terminal oxidase, although a large increase in that of cytochrome $c_4 + c_5$ is easily attained, even at low concentrations of KCN (50 μM; not shown in Table III). These results suggest that cytochromes $c_4 + c_5$ and b_1 may be functionally attached to separate cytochrome oxidases of differing activity and different sensitivity to inhibitors.

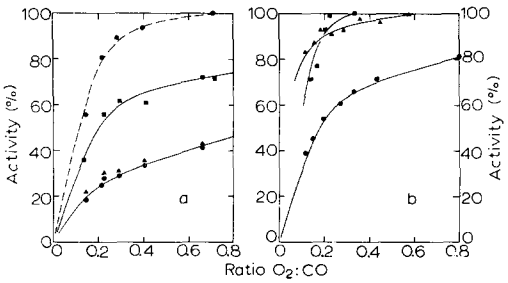

Fig. 6. CO inhibition of (a) NADH oxidase and (b) ascorbate-DCIP oxidase of *A. vinelandii*. particles under various conditions. Illumination was provided by a 1000-W lamp as described in METHODS AND MATERIALS. White light (●---●), dark (●—●), blue light (▲—▲) and red light (■—■). Dark conditions were attained using Wratten filter 88A or by switching the light off. Blue light (Wratten filter 47b; 385–490 mμ) confined the absorption of light to the Soret bands of cytochromes *o* and a_1; red light (Wratten filter 29; above 610 mμ) confined the absorption to the visible region band of cytochrome a_2.

AHQO causes a small increase in the aerobic steady-state reduction of both cytochromes $c_4 + c_5$ and b_1; the increase being marginally greater in the latter component. CO in fairly low concentration also causes increases in the aerobic steady-state reduction of both cytochromes and again the increase is somewhat greater for cytochrome b_1. AHQO and CO exert these effects with either succinate or NADH as substrate.

The inhibition of NADH and ascorbate–DCIP oxidases by CO, together with the relief of this inhibition by light, is summarized in Fig. 6 and Table IV. NADH

TABLE IV

THE EFFECT OF KCN ON THE RELIEF BY LIGHT OF NADH AND ASCORBATE–DCIP OXIDASES

Experimental details as described in MATERIALS AND METHODS. The particles were incubated in the reaction mixture with KCN for 1 min prior to the addition of substrate. Temperature 21.8°.

Substrate	*O_2:CO ratio*	*Further additions*	*Activity (%)*			
			Dark	*White light*	*Blue light*	*Red light*
NADH		—	100	100		
	0.23	—	20	89	29	54
		50 μM KCN	102	103		
	0.24	50 μM KCN	18	93	27	58
		2 mM KCN	5			
	0.25	2 mM KCN	4	7	5	5
Ascorbate–DCIP		—	100	107		
	0.22	—	56	97	81	94
		50 μM KCN	Nil	Nil		
	0.21	50 μM KCN	Nil	Nil		

oxidase is strongly inhibited by CO and this inhibition is relieved by light only with difficulty; white light of low intensity has little effect whereas considerable relief can be obtained with much higher intensity white light. Ascorbate–DCIP oxidase is less sensitive to CO but the resultant inhibition is very sensitive to light; considerable relief is obtained with white light of either low or high intensity (Table IV).

Control experiments show that the 2 to 3-fold difference in particle protein concentration in the assays of these oxidases is not responsible for the different sensitivities towards CO. Also substitution of argon for CO has little inhibitory effect; the rate of oxidation of either substrate appears to be linear down to low concentrations of O_2. In the absence of CO, light has no stimulatory effect on either oxidase activity.

Since light sensitivity is greatest at those wavelengths where the CO-complexes formed by the individual oxidases absorb most strongly, the effect of blue (385–490 mμ) and red (610 mμ) light on the CO-inhibited oxidases is most revealing (Fig. 6). Red light causes considerable relief of CO-inhibited NADH oxidase, whereas relief by blue light is confined to the lower O_2:CO ratios and even there is very weak. On the other hand CO inhibition of ascorbate–DCIP oxidase appears to be easily and almost equally relieved by either blue or red light.

The ability of KCN to abolish light relief of the CO-inhibited oxidase is shown in Table IV. A low concentration of KCN (50 μM), which alone maximally inhibits ascorbate–DCIP oxidase (Fig. 4a) completely abolishes the relief of CO inhibition of this oxidase by either white, red or blue light. On the other hand the same concentration of KCN neither inhibits NADH oxidase activity (Fig. 4a) nor significantly alters the pattern of light relief of the CO-inhibited oxidase. High concentrations of KCN (2 mM) are required for both these effects.

DISCUSSION

Difference and action spectra of *A. vinelandii* particles and whole cells indicate the presence of cytochromes c_4 ($+c_5$?), b_1, a_1, a_2 and o of which cytochromes a_1, a_2 and possibly o are demonstrated to be acting as terminal oxidases. The action spectrum is striking for the large contribution of cytochrome a_2 relative to the other oxidases. The poor contribution of cytochrome o confirms a similar observation by CASTOR AND CHANCE[2] but these workers because of technical difficulties were unable to detect cytochrome a_2. It is interesting to note that the relative contributions of cytochromes a_2 and o observed in the action spectra described in this paper are entirely different to those observed by CASTOR AND CHANCE[2] for *Escherichia coli* (a_2, o) or *P. vulgaris* (a_1, a_2, o). In these latter two organisms the contribution of cytochrome a_2 is relatively small and the action spectrum is dominated by cytochrome o. Although the interpretation of action spectra in relation to oxidase activity is fraught with danger it seems likely that in *A. vinelandii* the major respiratory pathway may be mediated by cytochrome a_2 whereas cytochrome o appears to fulfil this function in *E. coli* and *P. vulgaris*.

Incubation of the particles with a variety of substrates causes, on anaerobiosis, the rapid and virtually complete reduction of all the cytochrome components. Cytochrome b_1 is slightly peculiar in this respect in that a small portion appears to be reduced after the majority of the cytochrome, pointing to the possibility of a small

pool of b_1 separate from the main fast reacting pool and which is reduced at a slower rate.

The substrate reduction experiments yield results which, on the surface, are incompatible. Aerobic steady-state reduction values of the individual cytochrome components suggest a cytochrome sequence $c_4 + c_5 \rightarrow b_1 \rightarrow$ oxidases, whereas the ability of ascorbate–DCIP under anaerobic conditions almost to reduce completely $c_4 + c_5$ and yet only reduce partially b_1 supports the sequence $b_1 \rightarrow c_4 + c_5 \rightarrow$ oxidases. This latter sequence would satisfy the standard redox potentials measured for *A. vinelandii* $c_4 + c_5$ by TISSIÈRES[11] and for *E. coli* b_1 by DEEB AND HAGER[21], and might be the expected sequence of cytochromes by analogy with mammalian and other bacterial systems. Since the light sensitivity of CO-inhibited terminal oxidases is greatest at the absorption maxima of the cytochrome–CO complex, some idea of the relative activities of the individual cytochrome oxidase components can be obtained by studying the pattern of light relief of CO inhibition. Thus illumination with red light confines the absorption to cytochome a_2 whilst blue light only affects the CO complexes of a_1 and *o*. The CO complex of a_2 has been reported to have little or no absorption in the Soret region[19]. NEGELEIN AND GERISCHER[15] observed that the CO-inhibited cytochrome oxidase of *A. chroococcum* cells was relatively intensitive to light. YAMAGUTCHI[22] extended this property to a wide range of bacteria and later CASTOR AND CHANCE[2] indicated that cytochrome a_2 was probably the pigment responsible in these organisms for the low sensitivity to light. On the other hand, the ready light reversal of the CO inhibition of cytochromes a_1 and *o* is well established[2].

Recent work on the cytochrome system of Achromobacter[23,24] has indicated that cytochrome a_2 is relatively insensitive to cyanide and azide, whereas cytochrome *o* (and probably a_1) is very sensitive to both these inhibitors. Similarly cytochrome a_2 from *E. coli*, *A. aerogenes* and *Pseudomonas pseudomalei* are all relatively insensitive to cyanide[24].

Thus the relative insensitivity of CO-inhibited NADH oxidase to white light, the ability of high intensity red light but not blue light to relieve substantially this inhibition, the failure of low concentrations of KCN to abolish this light relief and the relative insensitivity of the NADH oxidase system to both KCN and azide, all point to the presence of cytochrome a_2 as the major, if not the sole terminal oxidase of this pathway. The inability of red light (*cf.* white light) to relieve completely CO-inhibited NADH oxidase is probably due to the unavoidably lower intensity of the red light owing to the use of filters, rather than to the existence of yet another terminal oxidase whose CO-complex does not absorb in either the blue or red regions of the spectrum covered by these filters.

The low aerobic steady-state reduction of cytochrome b_1, together with the inability of low concentrations of KCN or azide to increase significantly this reduction, points to the presence of a highly active $b_1 \rightarrow a_2 \rightarrow$ oxygen pathway which carries most of the electron flux from potent electron donors such as NADH dehydrogenase.

The high sensitivity of CO-inhibited ascorbate–DCIP oxidase to white light, the abolition of this relief by low concentrations of KCN, together with the high sensitivity of this oxidase system to low concentrations of KCN and azide, all suggest that cytochromes *o* and/or a_1 comprise the terminal oxidase of this pathway. The ready relief of CO inhibition by blue light supports this concept but the relief by red light does not, and *per se* suggests the involvement of cytochrome a_2. If this were so, then

it might be expected that the oxidation of ascorbate *via* DCIP would be somewhat less sensitive to KCN than is actually observed, since a_2 would offer an alternative route to oxygen for electrons in the presence of low concentrations of KCN. Since this is not observed and because red relief is completely abolished by low concentrations of KCN, it is unlikely that cytochrome a_2 is involved in this pathway. The explanation of this red relief is more probably due to an intermolecular transfer of energy in such a manner that the red light absorbed by cytochrome a_2 can be passed on to dissociate the CO complex of the functional oxidase[2]; *viz.* cytochromes *o* and/or a_1.

Aerobic steady-state reduction values indicate that ascorbate–DCIP donates electrons at the level of cytochromes $c_4 + c_5$. The relatively high aerobic steady-state reduction of the cytochromes $c_4 + c_5$ in the presence of natural substrates such as succinate and NADH, together with the considerable increase in this reduction on the addition of low concentrations of KCN or azide support the concept of a $c_4 + c_5 \rightarrow o/a_1 \rightarrow$ oxygen terminal oxidation pathway.

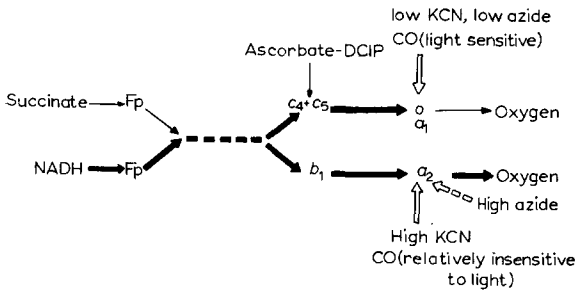

Fig. 7. The branched electron transport system of *A. vinelandii*. Solid lines indicate pathways of electron flow; open lines represent the sites of action of electron-transport inhibitors. Fp, flavoprotein.

The particulate electron transport system of *A. vinelandii* is summarized diagramatically in Fig. 7. The branched cytochrome system proposed in this scheme is capable of explaining all the observations reported in this paper and is in part supported by the work of REPASKE AND JOSTEN[25]. These workers obtained by differential centrifugation of a cell free extract of *A. vinelandii* a partially purified particulate NADH oxidase, the purification being accompanied by a much greater intensification of cytochromes b_1 and a_2 compared with cytochromes $c_4 + c_5$ and a_1, thus suggesting only a casual relationship of these latter cytochromes to NADH oxidase. This work supports the concept expressed in this paper of a highly active $b_1 \rightarrow a_2 \rightarrow$ oxygen pathway capable of catalysing the majority of the NADH oxidation of the particles. The presence of a branched cytochrome system as part of the particulate respiratory chain of *A. vinelandii* is not inconsistent with the stopped flow oxidation–reduction kinetics of the individual cytochrome components as measured by B. CHANCE (unpublished work).

The advantages to the organism of having a branched cytochrome system of this type are not immediately apparent, especially as one branch of the chain is apparently able to catalyse maximally NADH oxidation when the terminal oxidase(s) of the other branch are fully inhibited. Obviously a great deal more work is required before the function of such a branched system can be fully evaluated.

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