BBA 45586

PREPARATION OF RED AND GREEN ELECTRON TRANSPORT PARTICLES FROM *AZOTOBACTER VINELANDII*

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(Received April 20th, 1967)

SUMMARY

1. A method for the preparation of red and green electron transport particles from *Azotobacter vinelandii* using sodium deoxycholate and high salt concentrations is described.

2. The red particles contained high levels of flavoprotein, Q-8, non-haem iron and cytochromes $c_4 + c_5$, b_1 and o. These particles catalysed the oxidation of succinate and of ascorbate *via* a variety of electron mediators.

3. The green particles contained high levels of cytochrome a_2, together with substantial amounts of cytochromes b_1 and a_1, Q-8 and non-haem iron. The oxidase activity of these particles was low.

4. Electron micrographs have been presented to show the structures of the parent and derivative particles.

5. The results have been discussed in relation to the presence in *A. vinelandii* of a branched respiratory chain.

INTRODUCTION

Although it has been possible to isolate and purify some of the individual electron carriers of particulate bacterial respiratory systems, *e.g.* quinones[1], cytochromes[2–4] and non-haem iron proteins[5], little work has so far been reported on the preparation of enzymically active sub-units derived from these systems. The constitution and behaviour of such subunits of the mitochondrial electron transport system has been described by GREEN AND BRIERLEY[6] and has helped to confirm the general sequence of electron carriers in the intact respiratory chain as determined by kinetic and inhibitor studies. With a similar object in view, this paper describes the preparation of two distinct types of respiratory particles from *Azotobacter vinelandii* by methods based on those developed for the fractionation of the mitochondrial respiratory chain[7].

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate; TMPD, tetramethyl-*p*-phenylenediamine.

METHODS

General

A. vinelandii (N.C.I.B. 8660) was cultured, harvested and fractionated to yield small particles by the method of JONES AND REDFEARN[8]. Assays of electron carriers, enzyme activities and the effect of inhibitors were carried out by the standard procedures described previously[8,13]. V_{max} values were calculated where indicated for ascorbate–2,6-dichlorophenolindophenol(DCIP) oxidase by the conventional Lineweaver–Burk plot. Non-haem iron was estimated by the method of DOEG AND ZIEGLER[9].

Preparation of red and green electron transport particles

Batches of washed small particles of *A. vinelandii* were prepared and stored at −15°. When required the thawed particles were homogenized in 0.025 M sodium potassium phosphate buffer (pH 7.4), adjusted to approx. 30 mg protein per ml and placed in 3-ml vol. on centrifuge tubes containing a lower layer of 70% (w/v) sucrose (8 ml) and an upper layer of 30% (w/v) sucrose (27 ml). The contents were centrifuged at 43000 × *g* under swing out conditions in an MSE Superspeed 40 centrifuge for 2 h. This treatment successfully separated the ribosomes and very small oxidosomes (respiratory particles) from the bulk of the dark brown oxidosomes (Fig. 1a). This method of preparation of oxidosomes was a modification of the method of DE LEY[10].

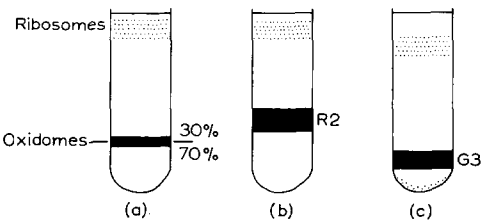

Fig. 1. The separation of derivative particles from *A. vinelandii* by density-layer and density-gradient centrifugation at 43000 × *g*. (a) Small particles for 2 h to yield oxidosomes; 30% + 70% sucrose layers (b) R1 for 2 h to yield R2; 0–60% sucrose gradient. (c) G2 for 30 min to yield G3; 0–80% sucrose gradient. Other details as described in METHODS.

The lower layer of oxidosomes was removed, dialysed overnight against 0.005 M sodium/potassium phosphate buffer (pH 7.4) to remove the sucrose and concentrated by centrifugation at 105000 × *g* for 90 min.

The oxidosomes were then suspended in KCl (72 mg/ml), adjusted to 10 mg protein/ml and treated with sodium deoxycholate (0.3 mg per mg protein) for 10 min at 0°. At the end of this period the slightly clarified suspension was adjusted to approx. 5 mg/ml with KCl solution and made 0.66 M with respect to sucrose. The resultant suspension was centrifuged at 105000 × *g* for 30 min in an angle head rotor to yield a clear orange–red supernatant and a hard-packed olive brown residue (G1).

The supernatant was dialysed overnight against 0.005 M sodium/potassium phosphate buffer (pH 7.4), with several changes of buffer, to remove the sucrose and deoxycholate. Removal of the latter caused a great increase in turbidity; the orange-red particles responsible (R1) were then readily sedimented by centrifugation at

105000 × *g* for 30 min. These particles were further purified by centrifugation at 43000 × *g* for 2 h on a 0–60 % (w/v) sucrose gradient of approx. 10 mg/ml initial concentration (Fig. 1b). The major pinkish-red band resulting from this treatment was removed from the 35–45 % sucrose region, then dialysed and sedimented to yield the final washed red particles (R2) as described above. An upper pale orange band was discarded.

The olive-green residue (G1) was further treated with sodium deoxycholate (0.5 mg per mg protein) under the same conditions as described previously for the treatment of the oxidosomes. The resultant red-brown clear supernatant was discarded; the olive-green particles (G2) were washed twice in 0.025 M sodium/potassium phosphate buffer (pH 7.4) to remove residual deoxycholate and then centrifuged at 43000 × *g* for 30 min under swing-out conditions in a 0–80 % (w/v) sucrose gradient. The resultant major green band (Fig. 1c) lying in the 70 % region was removed, dialysed and sedimented to yield the final green particles (G3). The light brown-green sediment on the base of the tube and a very pale yellow-green layer above the main band were both discarded.

The final red (R2) and green (G3) particles each represented 5–10 % of the original washed small particle protein.

Electron microscopy

Specimens of the various particulate fractions were prepared on carbon coated copper grids by negative contrast staining with 1.6 % dodecaphosphotungstate at pH 6.7 following fixation by brief (20 sec) exposure to 2 % osmic acid vapour. The preparations were examined in a Siemens Elmiskop I operating in the main at 80 kV and at an electron optical magnification of 40000.

RESULTS

The preparation of oxidosomes from small particles of *A. vinelandii* by sucrose density layer centrifugation was accompanied by a striking increase in the concentration of electron carriers and oxidase activities (Table I). A concomitant fall in the *A* 260/*A* 280 ratio was also observed, which suggested the removal of contaminating ribosomal material. The resultant oxidosomes (or gradient respiratory particles) were

TABLE I

THE DISTRIBUTION OF ELECTRON CARRIERS AND OXIDASE ACTIVITIES DURING THE PREPARATION OF *A. vinelandii* OXIDOSOMES

All assays of electron carrier concentrations and oxidase activities were carried out as described in the text.

Fraction	*Flavo-protein*	*Cytochromes (μmoles/g protein)*			*Q-8*	*A260/A280 ratio*	*Oxidase activity (μmoles/min per mg protein)*	
		$c_4 + c_5$	b_1	a_2			L-*Malate*	*Succinate*
Small particles	2.29	1.00	0.99	0.32	8.00	1.65	0.47	0.31
Ribosomal fraction		0.15	0.12	0.04	5.66	1.85	0.33	
Oxidosomes (gradient particles)	3.79	2.36	2.34	0.84	14.80	1.11	1.89	0.44

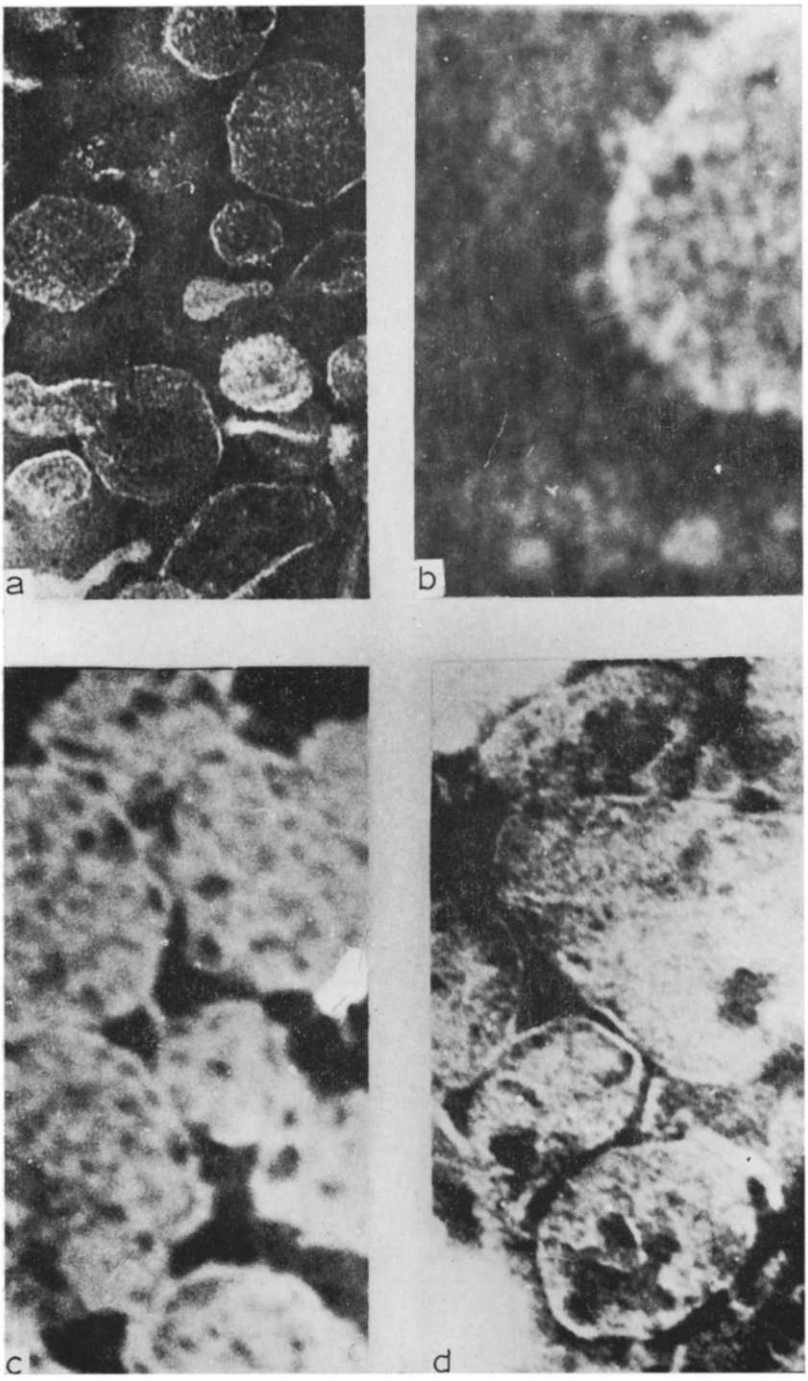

Fig. 2. Electron micrographs of various particulate preparations from *A. vinelandii*. Specimens prepared and stained as described in METHODS. (a) Oxidosomes; × 140000. (b) Elementary particles around the edge of an oxidosome; × 640000. (c) Red particles (R2) freshly prepared; × 140000. (d) Green particles (G3); × 140000.

a deep red-brown in colour. Examination of specimens by electron microscopy confirmed the absence of ribosomes; the oxidosomes appeared as flat, roughly circular discs varying in diameter from 70–120 mμ (Fig. 2a). In some preparations, small projections similar to the projections on mammalian mitochondria were visible along the edges of some membrane fragments and appeared to consist of a head and a short stalk (Fig. 2b). The heads were approx. 40–50 Å in diameter compared with 80–100 Å for those of mitochondria[11] and 65–85 Å for those present on the membranes of *Bacillus stearothermophilus*[12].

Red (R2) and green (G3) electron transport particles were prepared as described in METHODS. The striking colour differences observed between the small particles and the derivative particles were reflected in the cytochrome content of these particles as determined by visible region reduced *minus* oxidized (Fig. 3) and Soret region CO-reduced *minus* reduced (Fig. 4) difference spectra. The cytochrome components of the oxidosomes were similar to those observed previously for *A. vinelandii* small particles[8,13] and comprised cytochromes $c_4 + c_5$ (551 mμ), b_1 (560 mμ), a_1 (437 mμ) in Fig. 4. The red particles appeared to contain only low levels of a_1 and a_2 but were

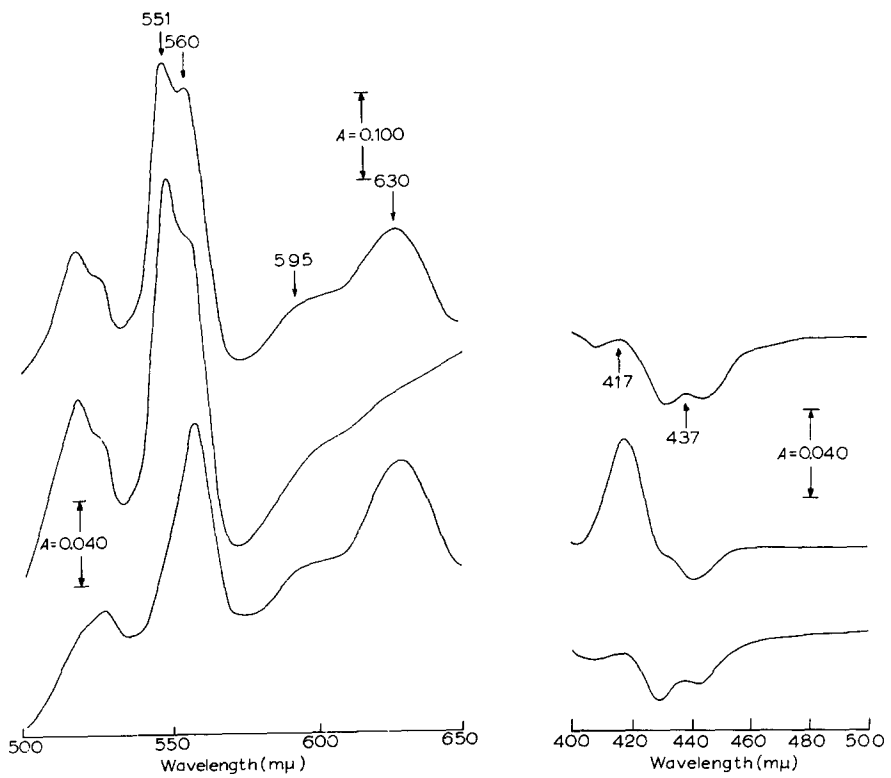

Fig. 3. Visible region reduced *minus* oxidized difference spectra of various particulate preparations from *A. vinelandii*. Upper curve: small particles, 9.40 mg protein/ml. Centre curve: red particles (R2), 2.19 mg protein/ml. Lower curve: green particles (G3), 3.06 mg protein/ml.

Fig. 4. Soret region CO-reduced *minus* reduced difference spectra of various particulate preparations from *A. vinelandii*. Upper curve: Small particles, 2.13 mg protein/ml. Centre curve: red particles (R2), 0.94 mg protein/ml. Lower curve: green particles (G3) 0.75 mg protein/ml. All spectra are corrected to a horizontal base line.

enriched in cytochromes $c_4 + c_5$, b_1 and also, from the CO spectrum, in cytochrome *o*. On the other hand, the green particles were rich in cytochromes a_1 and a_2, relatively poor in cytochromes $c_4 + c_5$ and *o* and yet still contained high levels of cytochrome b_1.

These qualitative cytochrome data together with those of two other electron transport components, Q-8 and non-haem iron are expressed quantitatively in Table II. Q-8 and non-haem iron appeared to be present predominantly in the red particles although the significant levels of these two carriers and cytochrome b_1 in the green particles suggested that all three components may be close together in the electron transport system of the intact oxidosomes and that deoxycholate causes a splitting of the chain in this region to yield the derivative particles. The ratio of the molar concentrations of cytochromes $c_4 + c_5$ to a_2 in the oxidosomes, red particles and green particles was 1.00:2.37:0.24.

The enzyme activities of the oxidosomes and derivative particles are summarized in Table III. Treatment with deoxycholate appeared to inactivate both malate and NADH oxidation; the derivative particles had little oxidase or dehydrogenase activity with either substrate and remixing of the particles did not result in reactivation. Deoxycholate treatment, however, did not appear to inactivate substantially the oxidation of either succinate alone or of ascorbate via DCIP, tetramethyl-*p*-phenylenediamine (TMPD) or *A. vinelandii* $c_4 + c_5$.

The red particles retained over 50% of the succinate oxidase activity of the oxidosomes, whilst succinate dehydrogenase and $c_4 + c_5$ reductase activities were

TABLE II

THE DISTRIBUTION OF ELECTRON CARRIERS DURING THE PREPARATION OF RED AND GREEN PARTICLES FROM *A. vinelandii* OXIDOSOMES

The concentrations of electron carriers were determined as described in the text.

Fraction	*Flavo-protein*	*Cytochromes (μmoles/g protein)*					*Q-8*	*Non-haem Fe*	*Molar ratio* c_4+c_5/a_2
		c_4+c_5	b_1	a_1	a_2	*o*			
Oxidosomes	2.01	1.95	1.89	++	0.53	++	15.1	8.53	3.68
Red particles (R2)	4.48	2.88	3.09	+	0.33	+++	23.3	16.4	8.73
Green particles (G3)	1.61	0.71	1.71	+++	0.82	+	14.9	7.8	0.87

TABLE III

THE ACTIVITY OF ELECTRON TRANSPORT SYSTEM ENZYMES IN PARTICLES DERIVED FROM *A. vinelandii*

Oxidase activity was assayed in a Clark electrode at 30°; dehydrogenase and $c_4 + c_5$ reductase activities were assayed at 21° as described in the text. V_{max} values were calculated for ascorbate–DCIP oxidase by a standard Lineweaver–Burk plot after correcting for oxidation of ascorbate in the absence of DCIP. Data are expressed in μmoles substrate oxidized/min per mg protein.

Fraction	*Succinate*			L-*Malate*		*NADH oxidase*	*Ascorbate–DCIP oxidase* (V_{max})	*Ascorbate–* $c_4 + c_5$ *oxidase*
	Oxidase	*Dehydrogenase*	$c_4 + c_5$ *reductase*	*Oxidase*	*Dehydrogenase*			
Oxidosomes	0.44	0.31	0.027	0.68	1.06	6.72	1.16	0.08
Red particles (R2)	0.24	0.49	0.054	0.04	Nil	0.11	10.00	0.23
Green particles (G3)	0.05	0.05	0.010	0.10	Nil	Nil	0.31	0.05

substantially increased. Very striking were the greatly increased activities of ascorbate–DCIP and ascorbate–$c_4 + c_5$ oxidases in the red particles compared with the oxidosomes. The sensitivity of ascorbate–DCIP oxidase to KCN and azide (Table IV) was similar to that of the oxidosomes and to that described in the previous paper on *A. vinelandii* small particles. Low concentrations of either inhibitor (40 μM KCN; 2 mM azide) reduced ascorbate–DCIP oxidase activity by approx. 90–95 % in both the small particles, oxidosomes and red particles. A similar sensitivity was exhibited by ascorbate–$c_4 + c_5$ oxidase.

TABLE IV

THE SENSITIVITY OF ASCORBATE–DCIP OXIDASE TO KCN AND AZIDE

Ascorbate–DCIP oxidase activity was measured in a Clark electrode at 30° using 0.04 mM DCIP for the red particles and 0.20 mM DCIP for the oxidosomes and green particles. 0.3–0.6 mg protein was used for the assays and the rate of oxygen consumption in the absence of DCIP was less than 5 % of the DCIP-mediated rate in all cases.

Fraction	*Ascorbate-DCIP oxidase (% activity)*		
	NIL inhibitor	*40 μM KCN*	*2 mM azide*
Oxidosomes	100	6.0	11.5
Red particles (R2)	100	3.7	5.2
Green particles (G_3)	100	23.1	30.3

The green particles, on the other hand, were incapable of oxidizing succinate at a significant rate using either oxygen, phenazine methosulphate (PMS)–DCIP or *A. vinelandii* $c_4 + c_5$ as electron acceptors, although they were capable of oxidizing ascorbate via TMPD, DCIP or *A. vinelandii* $c_4 + c_5$ even though the rates were considerably lower than those exhibited by either the red particles or the oxidosomes. The sensitivity of ascorbate oxidation to low concentrations of KCN or azide (Table IV) was strikingly lower than that exhibited by either the oxidosomes or red particles.

The dark sensitivity to CO of ascorbate–DCIP oxidation by the red particles was found to be similar to that already observed in the previous paper for the small particles. This inhibition was readily relieved by high intensity white light and blue light (Wratten filter 47b; 385–490 mμ) but relief by red light (Wratten filter 29; $>$610 mμ) was considerably less and much lower than expected by analogy with the small particles where relief by blue or red light was quantitatively similar.

The appearances of the red and green particles under the electron microscope were entirely different. Freshly prepared red particles (Fig. 2c) appeared as large, flat, rather net-like discoid structures varying from approx. 110–210 mμ in diameter. The particles appeared to gain in size on aging and after 48 h at 0° were up to 800 mμ in diameter. Such particles readily settle out on standing.

The green particles were morphologically very different from the red particles. They possessed none of the red particles net-like structure and their appearance as flat, somewhat granular discs of approx. 90–150 Å in diameter (Fig. 2d) was very similar to that of the parent oxidosomes.

DISCUSSION

The results presented in this paper indicate that the particulate respiratory chain of *A. vinelandii* was disrupted by the use of high concentrations of sodium deoxycholate and salt to yield two derivative particulate fractions which were readily distinguished from each other by colour, structure, electron carrier content and enzyme activity.

Although the enzymes catalysing the initial dehydrogenation of both NADH and L-malate were inactivated by this treatment, the succinate oxidase system appeared to be unaffected. Indeed, the red particles were capable of rapid succinate oxidation and also appeared to contain a highly active terminal oxidase system effective in oxidizing ascorbate via DCIP or *A. vinelandii* $c_4 + c_5$. The high levels of flavoprotein cytochromes $c_4 + c_5$ and b_1, Q-8 and non-haem iron exhibited by these particles suggested that they were derived predominantly from the components lying at the substrate end of the respiratory chain. The domination of the CO-reduced *minus* reduced difference spectrum by cytochrome *o* (contrast small particles) suggested that this cytochrome component was the terminal oxidase of these particles. This was supported by the sensitivity of the ascorbate–DCIP oxidase of these particles to low concentrations of KCN and azide, together with the more easily effected relief of CO-inhibited respiration by blue light compared with red light. Thus the electron carrier content and enzymic activity of the red particles supported the concept put forward in the previous paper that in the respiratory chain of *A. vinelandii* cytochromes $c_4 + c_5$ were oxidized by a cytochrome(s) other than a_2.

The green particles on the other hand were relatively rich in the electron carriers which comprised the terminal portion of the respiratory chain—*viz.* cytochromes b_1, a_1 and a_2. In spite of the relatively high concentrations of two terminal oxidases the enzymic activity of these particles was low and they failed to oxidize ascorbate *via* a variety of electron mediators at a rate comparable even with that of the small particles. However, the relatively high concentrations of cytochromes b_1 and a_2 in these particles, allied to the relatively low concentrations of cytochromes $c_4 + c_5$ and *o* lend further indirect support to the branched chain hypothesis.

The distribution of cytochrome a_1 in these red and green electron transport particles, and especially its presence with cytochrome a_2 in the green particles suggested that this component was functionally more closely linked to cytochrome a_2 than was previously thought. However, the marked failure of blue light substantially to relieve CO-inhibited NADH oxidase activity in the small particles (previous paper) strongly suggests that the oxidase activity of cytochrome a_1, if any, in the highly active b_1–a_2 pathway was very small. Obviously a more intensive study of the function of cytochrome a_1 in this organism is required.

Electron microscope studies of the particles were disappointing in that although obvious structural differences were apparent between the red and green particles, the observed structures of the particles threw little light on the possible origins of these derivative particles within the parent oxidosomes.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. A. J. ROWE for expert instruction in the techniques of electron microscopy and Miss J. A. CRONIN for technical assistance. This work was supported by the Science Research Council.

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