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THE EFFECT OF COMBINED-NITROGEN SOURCES ON THE SYNTHESIS AND FUNCTION OF THE ELECTRON TRANSPORT SYSTEM OF *AZOTOBACTER VINELANDII*

CHRISTOPHER J. KNOWLES* AND ERIC R. REDFEARN**

Department of Biochemistry, University of Leicester, Leicester (Great Britain)

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SUMMARY

1. *Azotobacter vinelandii* was grown on a synthetic medium containing no combined nitrogen source (*i.e.*, under nitrogen-fixing conditions) and on media containing urea, glutamate or ammonium sulphate as nitrogen sources and the electron transport system examined.

2. Electron transport particles derived from cells grown under nitrogen-fixing conditions and on glutamate or ammonium medium contained high concentrations of ubiquinone, flavoprotein and cytochromes $c_4 + c_5$, b_1, a_1 and a_2. Cytochrome a_2 was present in low concentrations only in particles from cells grown on urea medium.

3. There was only little variation of the succinate, L-malate or reduced nicotinamide adenine dinucleotide oxidase activities under all growth conditions.

4. The aerobic steady-state reduction of cytochromes $c_4 + c_5$ was greater than cytochrome b_1 for all growth conditions. Cytochromes $c_4 + c_5$, b_1 and a_2 were 84–100 % reduced in the anaerobic state of particles derived from cells grown on glutamate or nitrogen-free media, but in particles from cells grown on urea medium the cytochrome b_1 was only 55–70 % reduced in the anaerobic state.

5. In the particles from nitrogen-free growth L-malate, succinate or NADH reduced 35–40 % of the ubiquinone in the aerobic steady-state and 70–80 % in the anaerobic state, with no increased reduction by addition of multiple substrates. There was no reduction of ubiquinone by ascorbate-2,6-dichlorophenolindophenol. The ubiquinone reductase rates were only 35–60 % of the oxidase rates. In the particles from growth on urea medium the ubiquinone was less reduced in the anaerobic state.

INTRODUCTION

Variation of carbon sources is known to affect the electron transport systems of bacteria[1], but very little is known about the effects of different assimilatory

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; nitrogen-free, glutamate, ammonium and urea particles refer to the small particulate fraction derived from cells grown in media containing no combined nitrogen source and glutamate, ammonium sulphate or urea as nitrogen sources respectively.

* Present address: Biochemistry Department, Dartmouth Medical School, Hanover, New Hampshire, U.S.A.

** Deceased.

nitrogen sources on the synthesis and composition of the electron transport system.

It was shown by WILSON, HULL AND BURRIS[14] that *Azotobacter vinelandii* is capable of utilizing certain sources of combined nitrogen such as ammonium salts and urea to the virtual exclusion of molecular nitrogen, but other nitrogen sources, like the amino acids aspartate or glutamate, are utilized to only a minor extent and have little inhibitory effect on the fixation of atmospheric nitrogen. A study has therefore been made of the affects of urea and other combined-nitrogen sources on the synthesis and composition of the electron transport system of *A. vinelandii*. Previous work[2,3] has shown that this organism contains high concentrations of flavoprotein, ubiquinone (Q-8), and cytochromes $c_4 + c_5$, b_1, a_1, a_2 and *o* when grown under nitrogen fixing conditions on a medium containing no combined nitrogen.

MATERIALS AND METHODS

Chemicals

NADH was obtained from C. F. Boehringer and 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.

Growth and fractionation of bacteria

Cultures of *A. vinelandii* (N.C.I.B. 8660) were grown on the nitrogen-free medium described by JONES AND REDFEARN[2]. For growth of the bacterium with combined nitrogen sources $NaMoO_4$ was omitted from the medium and the following nitrogen sources used; 2.4 g $(NH_4)_2SO_4$, 1.0 g urea, or 4.8 g sodium glutamate per l. With $(NH_4)_2SO_4$ as nitrogen source double the normal quantity of phosphate buffer and 0.5 g $CaCO_3$ per l were used in an attempt to stabilize the pH.

Cultures of *Escherichia coli* (strain W) were grown on the minimal salts medium described by ASHWORTH AND KORNBERG[4] using 25 mM sodium DL-lactate as carbon source and 25 mM $(NH_4)_2SO_4$ *plus* 25 mM urea, 50 mM $(NH_4)_2SO_4$ or 50 mM urea alone as nitrogen sources.

The bacteria were grown to the mid-log phase, then harvested, ruptured and fractionated as described by JONES AND REDFEARN[2]. In all experiments the washed small particulate fraction was used.

Concentrations of the electron carriers

The concentrations of the individual cytochromes and the total flavoprotein were determined from the $Na_2S_2O_4$-reduced *minus* oxidized difference spectra as previously described[2]. The concentration and redox state of ubiquinone were determined by the chemical extraction method of PUMPHREY AND REDFEARN[5]. 1 ml of bacterial particles (6–10 mg protein) was incubated at 30° for 10 min. The reaction was started by addition of 9 μmoles succinate or L-malate, 3 μmoles of NADH, or 0.5 μmole 2,6-dichlorophenolindophenol (DCIP) *plus* 9 μmoles sodium ascorbate in 0.1 ml. The reaction was terminated after a suitable time interval by addition of 4 ml of redistilled methanol containing 1 mg per ml of pyrogallol at −60°. Ubiquinone was then estimated by the method of PUMPHREY AND REDFEARN[5], but due to the lack of interfering lipid the partition step with methanol was omitted. The time taken for

oxygen depletion was measured in a parallel experiment at the same temperature, using an oxygen electrode. The reduction level of ubiquinone in the anaerobic state was measured by addition of 3 mM KCN to the initial incubation mixture, a concentration that gave 100 % inhibition of all oxidase activities.

Dual-wavelength spectrophotometry

The redox states of the cytochromes were determined with an Aminco Chance dual-wavelength spectrophotometer using the following wavelength pairs: cytochromes $c_4 + c_5$, 551–544 mμ; cytochrome b_1, 560–568 mμ; cytochrome a_2, 630–619 mμ. All determinations were carried out at 20–22° in a reaction mixture containing 100 μmoles Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) in a volume of 2.97 ml. Reactions were started by the addition of 9 μmoles of succinate or L-malate, or 3 μmoles of NADH in a volume of 0.03 ml.

Oxidase activities

Oxidase activities were measured polarographically by the methods previously described[2].

RESULTS

Concentration of electron carriers

The initial stable metabolite of nitrogen fixation is probably glutamate, formed by an energy dependent process involving an ammonium intermediate[7]. *A. vinelandii* was therefore grown on media containing no combined-nitrogen source, $(NH_4)_2SO_4$ and glutamate. Growth on $(NH_4)_2SO_4$ medium, even on addition of 0.5 g $CaCO_3$ per l and double the normal quantity of phosphate buffer, resulted in a drastic drop in medium pH and low overall growth yields. When urea was used alternatively as a source of ammonium-nitrogen the pH remained fairly stable and normal growth yields were obtained.

The concentration of the electron carriers obtained under the different growth conditions are shown in Table I. The values obtained for growth under nitrogen-free conditions and in the glutamate medium were very similar. The concentrations of the electron carriers for growth in $(NH_4)_2SO_4$ medium were somewhat lower, though

TABLE I

THE CYTOCHROME AND UBIQUINONE CONCENTRATIONS OF *A. vinelandii* GROWN WITH VARIOUS SOURCES OF NITROGEN

The cells were grown to the mid-log phase on media containing the nitrogen sources indicated. Concentrations of carriers are expressed in μmoles per g small particulate fraction protein. Data for cytochrome concentrations as described by JONES AND REDFEARN[2]; the molar extinction coefficient of cytochrome a_1 is unknown. The values given are each the average of 5 experiments.

Growth condition	*Cytochromes* $c_4 + c_5$	*Cytochrome* b_1	*Cytochrome* a_2	*Cytochrome* a_1	*Flavoprotein*	*Ubiquinone (Q-8)*	*Ratio* $(Q\text{-}8)/b_1$
Nitrogen-free	1.63	1.53	0.62	++	2.0	9.2	6.1
Glutamate	1.49	1.29	0.41	++	1.7	8.9	6.9
Urea	1.28	1.12	0.08	+	1.6	7.4	6.6

their relative concentrations were similar to those of the nitrogen-free and glutamate particles. There was remarkably little variation in the concentrations of electron carriers from many samples with growth on nitrogen-free, glutamate or urea medium, but in the case of growth on $(NH_4)_2SO_4$ medium there was much more variability in concentrations and the small particulate fraction proved more difficult to isolate. The difficulty of isolation of the small particulate fraction under these conditions implies a structural change in the bacteria with growth in the $(NH_4)_2SO_4$ medium.

In the case of the particles from cells grown on urea medium there was a drastic 5 to 8 fold decrease in the concentration of cytochrome a_2. There was also a small but definite increase in the cytochromes $c_4 + c_5$ to cytochrome b_1 ratio, which may reflect a loss of some *o*-type cytochrome. Cytochrome *o* having previously been shown to contribute to the cytochrome b_1 α-peak[10]. There was a distinct difference in the color of the small particulate fraction of the bacteria grown on urea medium compared with that from bacteria grown on nitrogen-free medium; in the former case the particles were much redder and were similar in appearance to the red fraction of the nitrogen-free particles isolated after fractionation with detergents[8]. This difference is reflected in the spectra obtained (Fig. 1), where the ratio of the *b* and *c*-type cytochromes, giving the red color, to the *a*-type cytochromes is much higher in urea particles than in nitrogen-free particles.

Urea does not act as a general repressor of a_2-type cytochromes, as growth of *E. coli* on media containing urea, $(NH_4)_2SO_4$ or a combination of the two showed no variation in the cytochrome a_2 content.

In *A. vinelandii* a ubiquinone to cytochrome b_1 ratio of 6–7 was found for all growth conditions tried. This constant ratio agrees with the findings of WHITE[9] using *Hemophilus parainfluenzae*, in which there was a constant naphthoquinone to cytochrome b_1 ratio independent of growth conditions.

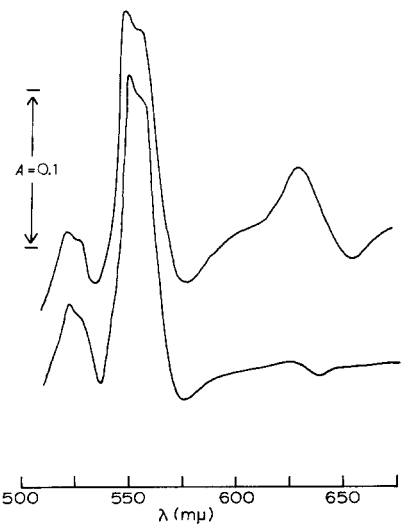


Fig. 1. $Na_2S_2O_4$-reduced *minus* oxidized difference spectra of *A. vinelandii* small particles. Particles suspended in 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 7.4). Upper curve, 6.8 mg protein per ml of nitrogen-free particles; lower curve, 10.7 mg protein per ml of urea particles. Light path: 1 cm. Cytochrome α-peaks shown are: 630 mμ, cytochrome a_2; 595–605 mμ, cytochrome a_1; 560 mμ, cytochrome b_1; 551 mμ, cytochromes $c_4 + c_5$. For both particles there was a Soret peak at 428 mμ.

Oxidase activities

Oxidase activities of particles derived from the bacterium grown under the different conditions are shown in Table II. The values of the oxidase activities of the glutamate particles are very similar to those of the nitrogen-free particles. In view of the low concentration of cytochrome a_2, a possible cytochrome oxidase in the urea particles, it was thought possible that the oxidase activities of these particles would be greatly reduced. However, this was not so, though there was some reduction of activity, especially in the case of the NADH oxidase. Ascorbate-DCIP acts as a high potential electron donor at the cytochrome $c_4 + c_5$ level in *A. vinelandii*[10] and might thus also be expected to show a reduced activity in the urea particles. Results for all growth conditions were somewhat variable from sample to sample (0.2 to 0.8 μatom oxygen uptake per min per mg protein), but a large decrease in the ascorbate-DCIP oxidase activity was not found in the urea particles.

Dual-wavelength spectrophotometry

Previous work from this laboratory has shown that in *A. vinelandii* grown on nitrogen-free medium, the steady-state reduction of cytochromes $c_4 + c_5$ was greater than that of cytochrome b_1 (ref. 10) and in contrast with the situation found in mammalian systems[6]. The redox states of the cytochromes were therefore measured for each growth condition in order to see the effect of variation of the composition of the electron transport system on the function of the individual cytochromes.

Growth with glutamate as nitrogen source gave values essentially the same as those previously reported for nitrogen-free growth[10]; that is, 85–100 % reduction of cytochromes $c_4 + c_5$, b_1 and a_2 in the anaerobic state and in the aerobic steady-state 37–41 % reduction of cytochromes $c_4 + c_5$, 18–24 % reduction of cytochrome b_1 and less than 5 % reduction of cytochrome a_2, with succinate, L-malate or NADH as substrates.

With growth in urea medium, the cytochrome a_2 content of the particles was too low for measurement of its redox state. Cytochrome b_1 in the urea particles appears to behave differently as it was only 55–70 % reduced in the anaerobic state; this was particularly noticeable with L-malate as substrate with which it was never

TABLE II

THE OXIDASE ACTIVITIES OF THE SMALL PARTICULATE FRACTION OF *A. vinelandii* GROWN ON MEDIA CONTAINING DIFFERENT NITROGEN SOURCES

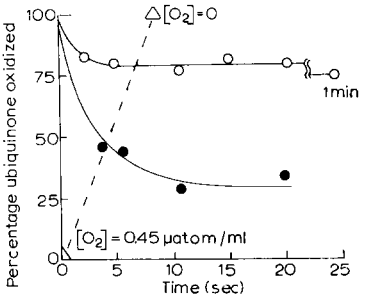
The cells were grown to the mid-log phase on media containing the nitrogen sources indicated and the oxidase activities measured at 30° with the washed small particulate fraction. O_2 uptake was measured with a Clark electrode with a reaction mixture of 2.4 ml consisting of 200 μmoles KH_2PO_4–Na_2HPO_4 buffer (pH 7.4), a suitable concentration of small particulate fraction protein and 30 μmoles of substrate (2.25 μmoles of NADH). Oxidase activities expressed in μatoms oxygen uptake per min per mg protein and are each the average of 6 determinations.

Nitrogen source	*Substrate*		
	NADH	L-*Malate*	*Succinate*
N_2 (air)	3.6	0.60	0.12
Glutamate	3.8	0.46	0.14
Urea	1.7	0.32	0.08

more than 60 % reduced. No more than 75 % reduction of this cytochrome was noticed with several additive substrates. In the aerobic state the cytochrome b_1 was more reduced (27–34 %) in the urea particles than in the other particles. Cytochromes $c_4 + c_5$ exhibited aerobic and anaerobic redox states similar to those found for the nitrogen-free particles.

Kinetics of ubiquinone reactions

Under all growth conditions tested ubiquinone was present in high concentrations and in a 6 to 7 fold excess over the cytochromes (Table I), this is similar to the excess found in mammalian systems.



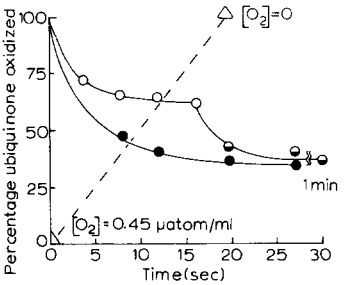


Fig. 2. The L-malate-ubiquinone reductase of *A. vinelandii* nitrogen-free small particles. Aerobic steady-state (○—○); anaerobic reduced state, with 3 mM cyanide (●—●). Assay at 30° by the extraction technique described in the text. The oxygen content (△—△) of the uninhibited solution was measured at the same temperature with an oxygen electrode.

Fig. 3. The L-malate-ubiquinone reductase of *A. vinelandii* urea small particles. Aerobic steady-state (○—○); natural anaerobic state (◒—◒), and toxic anaerobic state with 3 mM cyanide (●—●). Assay at 30° by the extraction technique described in the text. The oxygen content (△—△) of the non-cyanide inhibited solution was measured at the same temperature with an oxygen electrode.

Assay of the L-malate–ubiquinone reductase in the nitrogen-free particles showed that when the oxidase activity was 100 % inhibited by KCN (3 mM), 72 % of the total quinone was reduced (Fig. 2). In the absence of cyanide inhibition no more than 37 % of the enzymically reducible quinone was found to be reduced, even after apparent anaerobiosis (as measured with an oxygen electrode). The reason for the lack of further reduction on apparent anaerobiosis is probably due to either oxygen diffusion into the system or its introduction by the reaction terminating mixture of methanol–pyrogallol. The values obtained in the absence of cyanide were therefore taken as the aerobic steady-state values.

In the urea particles the methanol–pyrogallol addition step was sufficiently fast to terminate the reaction and permit measurement of natural anaerobiosis (Fig. 3). At the measurement time of oxygen depletion from the medium (19 sec) a further reduction of the quinone occurred to a level in good agreement with the values for toxic anaerobiosis with 3 mM KCN. The aerobic steady-state value was taken as the reduction level found immediately before oxygen depletion.

In the nitrogen-free particles very little ubiquinone was reduced either by endogenous substrates (5 %) or by ascorbate-DCIP (8 %). Thus the site of action of ascorbate-DCIP is either on the oxygen side of the site of action of ubiquinone or,

alternatively, acting on another pathway of electron transport. The reduction level of ubiquinone in nitrogen-free particles in the aerobic steady-state was similar for the succinate, L-malate and NADH–ubiquinone reductase (*i.e.*, 35–40 % reduction). In the anaerobic state, ubiquinone was never more than 85 % reduced even with addition of several substrates together, and a proportion of the total ubiquinone appears therefore never to be active under these conditions. As found for cytochrome b_1, the ubiquinone was less reduced in the anaerobic state in the urea particles than in the nitrogen-free particles, with a maximum reduction of 69 %. In urea particles the ubiquinone was more reduced in the aerobic steady state (48–54 %).

Comparison of the rate of ubiquinone reduction directly into the anaerobic state inhibited with 3 mM cyanide with the uninhibited oxidase rate in the nitrogen-free particles for succinate, L-malate or NADH as substrate showed that the ubiquinone reductase rates were only 35–60 % of the oxidase rates and thus insufficient to place all the ubiquinone on the main pathway of electron transport. Similar lower rates of ubiquinone reductases have been reported for beef heart-muscle preparations[12,13]. The ubiquinone reductases showed first-order kinetics.

DISCUSSION

WILSON, HULL AND BURRIS[14] showed that *A. vinelandii* grown on a medium containing either urea or ammonium salts preferentially utilized the medium nitrogen, but continued to use atmospheric nitrogen when grown with glutamate as the medium nitrogen. Experiments described in this paper show that *A. vinelandii* synthesizes an electron transport system identical in composition and activity when grown on glutamate or nitrogen-free media.

As nitrogen fixation is an energy requiring process[7] it would be expected that the bacterium grown on media containing fixed sources of nitrogen might synthesize an electron transport system of either lower activity or altered composition. The use of ascorbate-DCIP as substrate, and inhibitor studies[10] have shown that there is a terminally branched electron transport system in *A. vinelandii* after growth on nitrogen-free medium; the major pathway is *via* cytochromes b_1 and a_2 and the minor pathway is *via* cytochromes $c_4 + c_5$, a_1 and *o*. This explains the apparently anomalous greater reduction of cytochromes $c_4 + c_5$ than cytochrome b_1 in the aerobic steady state. When the bacteria were grown on urea-containing medium, cytochrome a_2 was synthesized in a much lower concentration (Table I) and, furthermore, cytochrome b_1 was only partially reduced in the anaerobic state. It therefore appears that the cytochrome b_1–cytochrome a_2 pathway is of less importance in cells grown on urea medium. The NADH oxidase system, which in the nitrogen-free particles donates a greater proportion of electrons to the cytochrome b_1–cytochrome a_2 pathway than the L-malate or succinate oxidases[10], was more affected by the reduced capacity of this pathway of electron transport in the urea particles, and its oxidative capacity was affected more than that of the succinate or L-malate oxidases. Ascorbate-DCIP donates electrons to the cytochrome $c_4 + c_5$ pathway at the cytochrome $c_4 + c_5$ level and as the concentrations of the carriers in this pathway were largely unaffected in the urea particles, the ascorbate-DCIP oxidase activity was relatively unchanged in the particles from urea-grown cells.

It is possible that the major function of the cytochrome b_1–cytochrome a_2

pathway is to synthesize high-energy compounds for nitrogen-fixation and which are not required in cells grown on sources of combined nitrogen; sufficient energy being obtained *via* the cytochrome $c_4 + c_5$ pathway in these cells.

A. vinelandii is comparatively insensitive to most inhibitors of electron transport[2] and it is therefore difficult to ascertain the precise site of action of ubiquinone. However, the lack of reduction of ubiquinone with ascorbate-DCIP as substrate suggests that the ubiquinone acts on the substrate side of cytochromes $c_4 + c_5$. In the urea particles there was less anaerobic reduction of the ubiquinone and of cytochrome b_1 than in the nitrogen-free particles. The ubiquinone may, therefore, be acting either on the cytochrome b_1 pathway of electron transport, or at the point of branching. The results were further complicated by the finding that as in beef heart-muscle preparations[12,13], the rate of ubiquinone reduction was not compatible with the oxidase rates. This finding has been confirmed by direct spectrophotometric measurement of the absorbance change due to ubiquinone in *A. vinelandii* small particles[15].

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APPENDIX

Subsequent to the completion of the above work, the accidental death of Professor E. R. REDFEARN occurred. I would like to express both my deep sorrow over this tragic event and my gratitude to Prof. REDFEARN for his friendship and guidance as my research supervisor (C.J.K.).

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