ELECTRON TRANSPORT TO NITROGENASE IN *AZOTOBACTER CHROOCOCCUM*: AZOTOBACTER FLAVODOXIN HYDROQUINONE AS AN ELECTRON DONOR

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1. Introduction

The free radical flavoprotein from Azotobacter [1–3] otherwise known as the Shethna flavoprotein [4,5] azotoflavin [6,7] or azotobacter flavodoxin [8], mediates electron transfer from either spinach chloroplasts or NADPH to azotobacter nitrogenase [6–10] or soyabean bacteroid nitrogenase [11]. This protein is unusual in that the oxidised form reacts very slowly with strong reducing agents ($Na_2S_2O_4$) at neutral pH and the half-reduced, semiquinone state is exceptionally resistant to oxidation by O_2 [3,6]; the fully reduced form is readily oxidised in air. Flavoproteins with identical semiquinone spectra have been isolated from *Peptostreptococcus elsdenii* [12] and *Escherichia coli* [13]; the latter protein will also mediate the transfer of electrons from spinach chloroplasts to azotobacter nitrogenase. Van Lin and Bothe [8] showed that the azotobacter protein catalysed NADPH formation in chloroplasts and argued that in this property and others it so resembled flavodoxins that it should be called azotobacter flavodoxin rather than specifically, azotoflavin [6]. It will therefore be called azotobacter flavodoxin in this communication. Mayhew et al. [14] suggested that the biologically active redox couple of flavodoxins was between the semi-reduced and fully-reduced forms in reactions where they substituted for ferredoxins. Van Lin and Bothe [8] obtained indirect evidence in support of this suggestion when they showed that azotobacter flavodoxin catalysed NADPH formation in illuminated chloroplasts more rapidly when oxygen was absent.

Impure nitrogenase preparations from *A. Vinelandii* have been used in all previously reported investigations into the role of azotobacter flavodoxin in electron transport to nitrogenase. The present paper demonstrates that azotobacter flavodoxin hydroquinone transfers electrons directly to purified nitrogenase from *A. chroococcum* or *Klebsiella pneumoniae* and in so doing is oxidised to the semiquinone state.

2. Materials and methods

All biochemicals were purchased from Sigma Chemical Co. (London) Ltd. and all salts from British Drug Houses, Poole, Dorset. *n*-Hydroxyethylpiperazine-*N*-ethane sulfonic acid (HEPES) was obtained from Stuart, Kinney and Co. Ltd., 11 Argyll Street, London.

Azotobacter chroococcum (NCIBL 8003) was grown and harvested as described previously [15]. *Azotobacter chroococcum* flavodoxin and ferredoxin were isolated by butanol extraction [7] and purified to yield single bands on disc-gel electrophoresis. Nitrogenase from *A. Chroococcum* was purified by a method similar to that used to prepare the nitrogenase proteins from *Klebsiella pneumoniae* [16]; details will be described elsewhere [17]. Both nitrogenase proteins from *A. Chroococcum* (designated Ac1 for the iron–molybdenum protein and Ac2 for the iron protein) were pure as judged by disc-gel electrophoresis [18], sodium dodecyl sulphate electrophoresis [19] and analytical centrifugation. Ac1 had a specific activity of 1250 nmoles of acetylene reduced/mg protein/min in the presence of saturating amounts of Ac2, and Ac2 had a specific activity of 1160 nmoles of acetylene reduced/mg protein/min in the presence of saturating amounts of Ac1. Purified nitrogenase proteins of

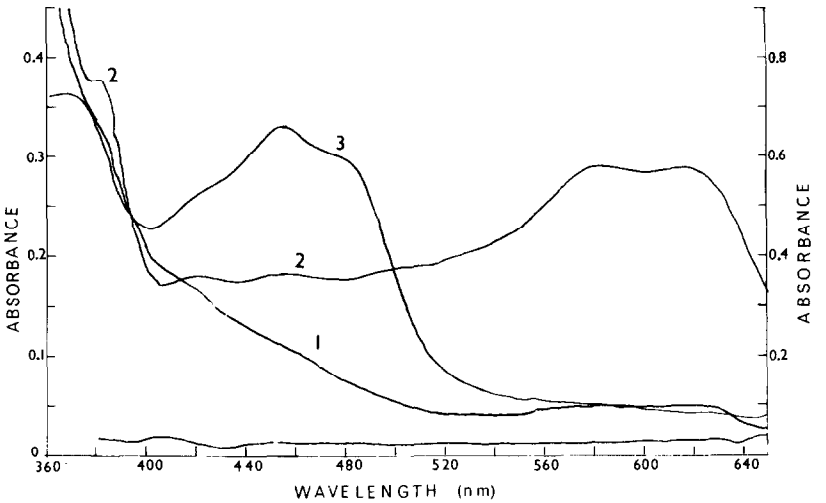

Fig. 1. The oxidation states of Azotobacter flavodoxin: (1) hydroquinone; (2) semiquinone; (3) oxidised. Each spectrum was measured with the same material during the course of an experiment run at room temperature (22°); (1) and (2) under N_2 and (3) under air. (1) was obtained in the presence of 75 μmoles of HEPES buffer, pH 7.8, an ATP-generating system [20] 350 μg of Ac1 and traces of sodium dithionite in a final volume of 2.8 ml. Spectrum (2) was measured 10 min after adding 400 μg of Ac2 (0.2 ml) to the same cell. To obtain spectrum (3) the flavodoxin, after measuring spectrum (2), was oxidised in air with solid potassium ferricyanide (< 1 mg). When necessary the material was centrifuged before measuring spectrum (3). The left ordinate is for spectrum (1) and (2) and the right ordinate for spectrum (3). The nitrogenase fractions plus the ATP-generating system had no significant absorption between 400 and 650 nm.

Klebsiella pneumoniae (Kp1 and Kp2, respectively) were kindly provided by Dr. R.R. Eady of this Unit and had similar specific activities to the Azotobacter proteins.

2.1. *Preparation of reduced azotobacter flavodoxin*

Azotobacter flavodoxin hydroquinone was obtained by dialysis overnight against 25 mM Tris buffer, pH 8.5, which was sparged with nitrogen gas and contained 0.1 mg/ml of sodium dithionite. This was readily converted to the semiquinone form when required by shaking in air for a few minutes. The semiquinone form was then made anaerobic by dialysis for 3 hr against 20 mM HEPES buffer, pH 7.2, containing 0.1 mg/ml of dithionite, and sparged with N_2. Azotobacter ferredoxin was reduced by overnight dialysis against the same HEPES buffer.

2.2. *Assay for nitrogenase*

Acetylene reduction was used as an assay for nitrogenase [20].

2.3. *Spectrophotometric assay of azotobacter flavodoxin*

Azotobacter flavodoxin hydroquinone oxidation was measured at room temperature under nitrogen at 580 nm with a Unicam SP 1800 spectrophotometer using a cell of 1 cm light path stoppered with a Subaseal. Enzymes were added with a Hamilton gas-tight syringe, if necessary against a stream of nitrogen. Flavodoxin concentration was determined by measuring the absorption at 580 nm using the extinction coefficient of 5.4×10^3 M^{-1} cm^{-1} [3] and also by oxidising aerobically with potassium ferricyanide after each experiment, centrifuging at 40,000 *g* for 10 min to remove any particulate material and measuring the absorption at 450 nm using the extinction coefficient of 10.6×10^3 M^{-1} cm^{-1} [3].

Protein concentration was determined with the Folin-Ciocalteu reagent [21].

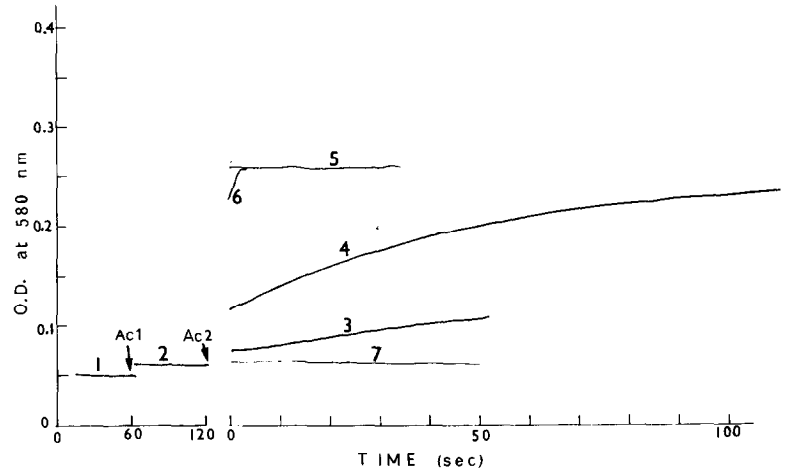

Fig. 2. The rate of oxidation of azotobacter flavodoxin hydroquinone by nitrogenase. Conditions were as described for fig. 1 under N_2; the ratio of Ac1 to Ac2 remained constant in each experiment. Line (1), hydroquinone + an ATP-generating system; line (2): (1) + Ac1 (54, 175 or 350 μg) or Kp1 (475 μg); line (3): (2) + 60 μg Ac2; line (4): (2) + 200 μg Ac2; line (5): (2) + 400 μg Ac2; line (6): (2) + 400 μg Kp2; line (7): conditions as for line (5), but without azotobacter flavodoxin. Note the change in scale on the time axis. The estimated time between adding Ac2 and measuring the absorbance was 5 sec.

3. Results

3.1. *Oxidation of azotobacter flavodoxin hydroquinone with pure azotobacter nitrogenase*

Fig. 1 shows spectra of the three oxidation states of azotobacter flavodoxin and illustrates the change from the hydroquinone to the semiquinone brought about by nitrogenase. The absorbance of the semiquinone spectrum at 580 nm was identical to that obtained within 5 seconds of adding Ac2 (fig. 2, line 5). The spectrum was unchanged 1 hr later. Since the oxidised form did not appear it follows that the reductant involved in electron transfer from azotobacter flavodoxin to nitrogenase is the hydroquinone and not the semiquinone. Because the nitrogenase was pure the experiment also provides evidence that the flavodoxin transfers electrons directly to nitrogenase and does not require an intermediate electron carrier.

Addition of Ac2 caused a rapid increase in absorbance at 580 nm; the increase appears to be biphasic (fig. 2, line 4) with a very rapid initial oxidation. The estimated time elapsed between adding Ac2 and recording the spectrum was 5 sec. The presence of, and possible competition by sodium dithionite may account for the slowing of the rate of hydroquinone oxidation as the concentration of hydroquinone diminished; this point needs further investigation. If Ac1 or ATP was added last instead of Ac2 a similar increase in absorbance occurred.

3.2. *Acceleration of acetylene reduction by azotobacter flavodoxin hydroquinone.*

The presence of sodium dithionite offers great advantages in handling oxygen-sensitive proteins but has the disadvantage that a percentage of electron transfer in these experiments may occur through dithionite. Removal of dithionite made the handling of the hydroquinone, in particular, very difficult: some oxidation occurred however stringent the precautions taken to exclude oxygen. Table 1 shows the amounts of acetylene reduced in the presence and absence of azotobacter flavodoxin. In four experiments the ethylene produced in the control, attributable to the residual dithionite in the hydroquinone preparation ranged from 0–30% of the total produced in the presence of azotobacter flavodoxin hydroquinone. The rate of acetylene reduction with the hydroquinone was as rapid as that with sodium dithionite. The specific activity of Ac2 at 22° with saturating Ac1 was 495 nmoles of acetylene reduced/mg Ac2/min with flavodoxin hydroquinone as the electron donor and 440 nmoles of acetylene reduced/mg Ac2/min with

Table 1

Acetylene reduction by purified nitrogenase fractions (Ac1 and Ac2) from *A. chroococcum* with various electron donors.

	Electron donor (nmoles)	nmoles C_2H_2 produced per min		
		Expt. 1	Expt. 2	Expt. 3
Control (0.9 ml)		11.5	0	
Control (0.6 ml)		5		6
Azotoflavin hydroquinone	186	31.5	26.0	26
Azotoflavin hydroquinone	124	18		
Azotoflavin semiquinone	124		0	
Sodium dithionite	20 μmoles			17

The experimental procedure has been described previously [20]. Assays contained 85 μg of Ac1, 45 μg of Ac2 and electron carriers as indicated. The reaction was started by adding Ac2. The control is the same volume of dialysing buffer without the electron donor. Experiments 1 and 2 were at 30° and were stopped after 5 min; experiment 3 was at 22° and was stopped after 1 min.

sodium dithionite. A specific activity can also be estimated from the initial rate of flavodoxin hydroquinone oxidation assuming that all the electrons are transferred to nitrogenase for enzymic reduction of substrate. From the absorption at 450 nm in spectrum 3 (fig. 1) the amount of flavodoxin per assay was 142 nmoles; this was converted to the semiquinone in 5 sec (fig. 2, line 5). From the absorption at 580 nm in spectrum 2 (fig. 1) the amount of semiquinone was 135 nmoles. On these bases the specific activity of Ac2 was the equivalent of 2140 or 2030 nmoles H_2 evolved/mg Ac2/min, respectively. These figures may be quite different from the true specific activity with azotobacter flavodoxin since the reaction characteristics have not yet been defined (apparent K_m for the flavodoxin, pH optimum, correct ratio of the nitrogenase proteins etc.).

Azotobacter flavodoxin semiquinone, obtained by dialysis against 25 mM HEPES buffer, pH 7.2, but still containing traces of sodium dithionite gave identical rates of acetylene reduction to the control without flavodoxin (table 1). This observation confirms the evidence in fig. 1 that the flavodoxin semiquinone cannot donate electrons to nitrogenase.

3.3. *Oxidation of azotobacter flavodoxin hydroquinone with pure Klebsiella nitrogenase*

Fig. 2, line 6 shows that Klebsiella nitrogenase will also oxidise the flavodoxin hydroquinone rapidly. This indicates that the reductant is non-specific as is clostridial ferredoxin when it donates electrons to azotobacter nitrogenase [22]. The presence of a flavodoxin in *K. pneumoniae* has not yet been reported.

3.4. *Effect of azotobacter ferredoxin*

Attempts to reduce azotobacter ferredoxin with a similar technique to that used with azotoflavin were only partially successful. While some reduction occurred, reoxidation with oxygen did not restore the fully oxidised spectrum as observed by Yoch et al. [7]. This reduced ferredoxin preparation did not support acetylene reduction with Ac1 and Ac2, nor would it reduce azotoflavin semiquinone to hydroquinone.

3.5. *Effect of sodium dithionite*

10 mM sodium dithionite completely inhibited the oxidation of azotobacter flavodoxin hydroquinone by nitrogenase.

4. Discussion

Two important observations are reported in this paper: firstly that the hydroquinone-semiquinone couple of azotobacter flavodoxin is the electron-donating system to nitrogenase and that the hydroquinone donates electrons as rapidly as any other donor; secondly, that it does so without an intermediate electron carrier. The rapid rates observed with the hydroquinone as the electron donor with purified

nitrogenase contrast sharply with the slower rates observed with crude nitrogenase preparations using chloroplasts or an NADH-generating system to reduce the flavodoxin [6,8,9]. There are several possible explanations for this, including: the slow rate of reduction by chloroplasts of the oxidised flavodoxin to the semiquinone [6]; competition from other possible electron acceptors in crude extracts or even slow penetration of the hydroquinone into the nitrogenase-containing particle. The negative result with azotobacter ferredoxin may mean that this carrier will not transfer electrons directly to nitrogenase [23] or it may be because the carrier was damaged by sodium dithionite [8]. This aspect needs further study.

The presence of such a highly oxygen-sensitive electron carrier in azotobacter must have significance in the oxygen relations of this nitrogen-fixing aerobe. Auto-oxidation of azotobacter flavodoxin hydroquinone could protect nitrogenase against oxygen damage in the cell. Oxidation of the hydroquinone could also account for the non-competitive inhibition of acetylene reduction by oxygen in the presence of illuminated spinach chloroplasts and azotobacter flavodoxin [10].

What biological system can reduce azotobacter flavodoxin semiquinone *in vivo* remains to be discovered. Presumably illuminated chloroplasts are capable of this reduction although Benemann et al. did not observe reduction beyond the semiquinone state in their experiments [6]. This failure may have been due to incomplete inactivation of the oxygen-producing system of chloroplasts; traces of oxygen would be sufficient to reoxidise the hydroquinone. Vetter and Knappe [13] claimed that illuminated spinach chloroplasts partly reduced the flavodoxin from *E. coli* to the hydroquinone, whereas the flavodoxin from *P. elsdenii* was not reduced beyond the semiquinone state by NADPH and spinach NADPH-ferredoxin reductase, but was reduced by H_2 and hydrogenase [14]. If the redox potential of the azotobacter flavodoxin hydroquinone-semiquinone couple is similar to that of the flavodoxin from *P. elsdenii* (−0.373 mV), [14]) then it is feasible that the NADH-benzyl viologen reductase from azotobacter [20] could catalyse hydroquinone formation. Attempts to show such a reaction have not yet proved successful.

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