Flavodoxin 1 of *Azotobacter vinelandii*: characterization and role in electron donation to purified assimilatory nitrate reductase

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Flavodoxins synthesized by *Azotobacter vinelandii* strain UW 136 during growth on nitrate as nitrogen source were separated by FPLC on a Mono Q column into two species, flavodoxin 1 (AvFld 1) and flavodoxin 2 (AvFld 2). Both proteins migrated as single bands on SDS/PAGE. AvFld 1 was approx. 5-fold more abundant than AvFld 2 in the unresolved flavodoxin mixture. N-terminal amino acid analysis showed the sequence of AvFld 2 to correspond to the *nif F* gene product, an electron donor to nitrogenase. The sequences also show that these species corresponded to the flavodoxins Fld A and Fld B isolated from N_2-grown cultures of the closely related organism *Azotobacter chroococcum* [Bagby, Barker, Hill, Eady and Thorneley (1991) Biochem. J. **277**, 313–319]. Electrospray mass spectrometry gave M_r values for the polypeptides of 19430 ± 3 and 19533 ± 5 respectively. ^{31}P-NMR measurements showed that in addition to the phosphate associated with the FMN ($\delta = -136.3$ p.p.m. and -135.48 p.p.m.), AvFld 1 had a signal at $\delta = -142.1$ p.p.m. and AvFld 2 at $\delta = -138.59$ p.p.m. present in substoichiometric amounts with FMN. These appeared to arise from unstable species since they were readily lost on further manipulation of the proteins. The mid-point potentials of the semiquinone–hydroquinone redox couples were -330 mV and -493 mV for AvFld 1 and AvFld 2 respectively, but only AvFld 1 was competent in donating electrons to the purified assimilatory nitrate reductase of *A. vinelandii* to catalyse the reduction of nitrate to nitrite. Flavodoxin isolated from NH_4^+-grown cells (Fld 3) also functioned as electron donor at half the rate of AvFld 1, but ferredoxin 1 from *A. chroococcum* did not.

INTRODUCTION

Flavodoxins are low-M_r, flavin mononucleotide (FMN)-containing proteins which can act as low potential electron donors to a number of redox enzymes. Of those which have been investigated, the physiologically important redox couple has been shown to be the semiquinone–hydroquinone oxidation couple (SQ/HQ with mid-point potentials in the range -100 to -520 mV NHE). In most organisms the expression of flavodoxin genes is controlled by the availability of iron, apparently at the level of transcription, and flavodoxin levels are increased during growth under iron limitation. Under these conditions, flavodoxin can replace ferredoxin in the pyruvate phosphoroclastic system, and also in the photosynthetic reduction of pyridine nucleotides. In some organisms, including *Azotobacter*, the level of flavodoxin does not respond to the availability of iron, and high levels are synthesized irrespective of the iron status, see [1] for review.

In *Klebsiella pneumoniae* a flavodoxin, the *nif F* gene product, has been clearly established to be the physiological electron donor to nitrogenase, where it links a pyruvate-flavodoxin oxidoreductase (EC 1.2.7.1) to the Fe protein of nitrogenase [2,3]. A homologous *nif F* gene is located in the cluster of genes encoding the Mo-nitrogenase of *Azotobacter*, but mutations in this gene do not result in the loss of the ability to grow on N_2, indicating that *Azotobacter* has more than one electron donor capable of supporting nitrogenase activity [4].

For many years it was not recognized that *Azotobacter vinelandii* was capable of synthesizing more than a single type of flavodoxin. However, the use of conventional purification methods was shown to result in flavodoxins co-purifying as a mixture [5], although the preparations appeared homogeneous by several analytical methods. These were resolved by FPLC separation on 'Q'-based column materials to yield two flavodoxins designated AvFld 1 and AvFld 2. Organisms grown on dinitrogen as nitrogen source had AvFld 2, the product of the *nif F* gene, as the major species, and minor amounts of AvFld 1. *A. vinelandii* grown on ammonium as nitrogen source contained a different flavodoxin, AvFld 3, which if present in a mixture can also be resolved from the other flavodoxins by FPLC [5].

It was subsequently shown that the closely related organism *Azotobacter chroococcum*, synthesized two flavodoxins during growth on N_2. These were resolved by FPLC [6], and were shown to be homologous to the species Fld 1 and Fld 2 isolated previously from *A. vinelandii* [5]. The N-terminal sequences of these flavodoxins from *A. chroococcum* were shown to be different, indicating that they were the products of distinct genes [6]. The sequence of AvFld 2 identified it as the product of the *nif F* gene which is located within the major *Nif* gene cluster which encodes the Mo-nitrogenases of *A. vinelandii* [4] and *A. chroococcum* (R. L. Robson, personal communication). The level of expression of *nif F* is decreased in the presence of ammonium and it is generally considered that although not essential, under normal conditions the physiological role of this flavodoxin is that of an electron donor to nitrogenase. The function of the other flavodoxins is unknown, but in *Azotobacter* species it is apparent that the nitrogen status of the organism, rather than the availability of iron, controls the level and type of flavodoxin synthesized.

We have recently purified the assimilatory nitrate reductase of *A. vinelandii* and shown that it has Fe/S and a molybdenum

Abbreviations used: AvFld 1 and AvFld 2, flavodoxins 1 and 2 from *A. vinelandii*.

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cofactor as redox centres [7]. During purification the enzyme was assayed using reduced Methyl Viologen, and no activity was observed when NADH or NADPH were tested as electron donors to the purified enzyme. Here we report that when *A. vinelandii* is grown on nitrate as nitrogen source, the predominant flavodoxin is AvFld 1, and that the semiquinone–hydroquinone redox couple, and to a lesser extent the semiquinone–quinone redox couple of this flavodoxin, are effective electron donors to purified nitrate reductase.

AvFld 2, the predominant flavodoxin of N_2-grown cells, was not active, but, in contrast, AvFld 3 isolated from ammonium-grown *A. vinelandii* was. The activity with AvFld 3 was lower than with AvFld 1, indicating that factors other than redox potential are important in determining the reactivity of flavodoxins with nitrate reductase. These findings, obtained with purified proteins, substantiate the suggestion that in *Azotobacter* reduced flavodoxin functions as the source of electrons for nitrate assimilation [8]. Our results provide a putative biological role for one of the additional flavodoxins we [6], and others [5], have isolated from *Azotobacter* species, in addition to that for Fld 2 which is implicated in nitrogen fixation.

MATERIALS AND METHODS

Growth of the organism and preparation of crude extracts

The strain used throughout this work was *A. vinelandii* UW 136, a rifampicin-resistant mutant derived from *A. vinelandii* strain OP [9]. Cultures were grown aerobically at a dissolved oxygen concentration of 10% of air saturation in a 200 l pilot plant fermenter (New Brunswick Scientific) in modified Burk's medium [10], supplemented with 10 mM $NaNO_3$ as nitrogen source. The cells were harvested during logarithmic growth, resuspended in 50 mM Hepes buffer, pH 8.0, containing 0.1 g/l dithiothreitol and disrupted using a Manton Gaulin homogenizer at 27 MPa pressure. The crude extract was obtained by centrifugation at 27000 ***g*** for 30 min at 10 °C and the clear brown supernatant frozen in liquid nitrogen until required.

Preparation of an unresolved mixture of AvFld 1 and AvFld 2

Crude flavodoxin was isolated during the purification of the assimilatory nitrate reductase of *A. vinelandii* [7]. Crude extract (approx. 1 l) was loaded in three separate batches on to separate columns of DEAE-cellulose (15 cm × 5 cm) equilibrated with 50 mM Tris/HCl buffer, pH 8.0. The columns were washed with 200 ml of the same buffer flowing at 200 ml/h. Absorbed proteins were then eluted with a linear gradient of NaCl (0 to 0.4 M). Nitrate reductase activity eluted as a single peak between 0.18 and 0.22 M NaCl and a mixture of flavodoxins eluted as a single yellow band at NaCl concentrations above 0.3 M.

Combined fractions from several columns (850 ml, 6.6 mg/ml of protein) were subjected to ammonium sulphate fractionation. Solid ammonium sulphate was added to 60% satn. and the resulting precipitate removed by centrifuging at 27000 ***g*** for 10 min and discarded. The supernatant was then made 95% satd. with ammonium sulphate and centrifuged as before. The pellet, containing the flavodoxins, was redissolved in 65 ml of 50 mM Tris/HCl buffer, pH 8.0, and dialysed against the same buffer.

Separation of AvFld 1 and AvFld 2

The procedure used is essentially as described for resolving the flavodoxins of *A. chroococcum* [6]. The fraction containing flavodoxins was loaded on a Pharmacia-LKB Biotechnology Mono 'Q' HR 5/5 anion-exchange FPLC column (0.5 cm × 5 cm) equilibrated with 20 mM Bistris buffer, pH 6.4, and eluted with a linear gradient of 0.25 to 0.55 M KCl in a total volume of 30 ml. This resolved two yellow bands (AvFld 1 and AvFld 2) with spectral characteristics typical of oxidized flavodoxin with two broad absorption bands in the regions 372–379 nm and 443–465 nm. The NaCl concentrations at which these proteins eluted (0.37 M KCl and 0.42 M KCl) correlated with the flavodoxin species isolated from extracts of N_2-grown *A. vinelandii* (A.T.C.C. 478) [5], except that the relative abundance of the two was reversed, i.e. in our case there was approx. 3-fold more AvFld 1 than AvFld 2 (see Figure 1). These species were assigned as different gene products, rather than post-translationally modified species which have also been resolved using this technique [11], since they differed in their sensitivity to proteolytic digestion and showed different antigenic cross-reactivity. This assignment was subsequently confirmed for the homologous proteins of *A. chroococcum* by N-terminal amino acid sequence determination [6].

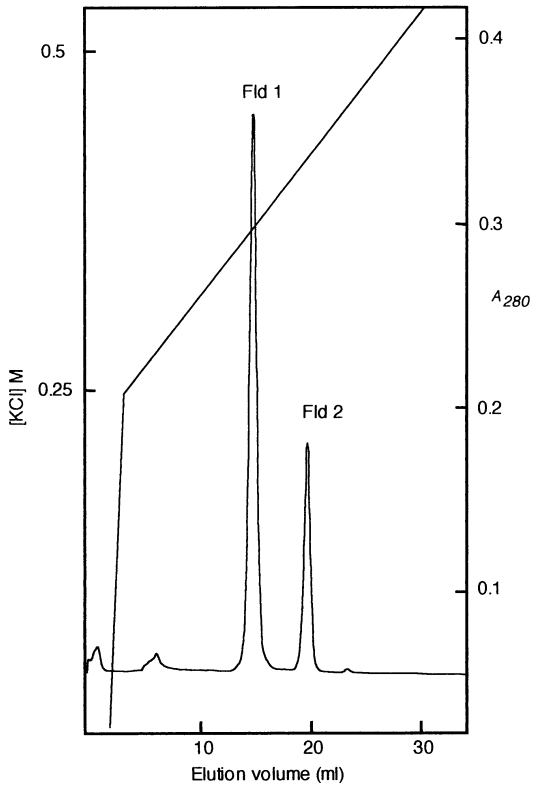

Figure 1 FPLC elution profile of the separation of AvFld 1 and AvFld 2 from a crude flavodoxin fraction on a Mono Q HR 5/5 anion-exchange column

The KCl gradient and running conditions are described in the Materials and methods section. Two yellow bands, AvFld 1 and AvFld 2, were separated in the fractions corresponding to the peaks in the A_{280} profile as indicated.

^{31}P-NMR spectroscopy

Flavodoxins were concentrated to ~ 250 μM in 25 mM Hepes, pH 8.0, containing 20% ^1H_2O. NMR spectra were recorded at room temperature on a JEOL 270 GSX spectrometer, operating at 109.25 MHz in Fourier transform mode as described previously [11]. All chemical shifts are referenced to phosphoric acid at 0 p.p.m. (downfield shifts positive).

Preparation of protein samples for N-terminal amino acid determination

The samples were run on SDS/PAGE before analysis was carried out using standard procedures on an ABI pulsed liquid analyser [6].

Determination of redox potentials

Redox measurements of the mid-point potential of the semiquinone/hydroquinone redox couple of both flavodoxins were made spectrophotometrically under anaerobic conditions at 25 °C in 50 mM phosphate buffer. AvFld 1 was titrated to equilibrium with NADPH in the presence of ferredoxin-NADP oxidoreductase [12]. The concentration of the semiquinone form at equilibrium was calculated using a value of ϵ_{mM} of 4.8 at 596 nm [5]. AvFld 2 was titrated with bisulphite/dithionite using solutions previously standardized by determination of the mid-point potential of Methyl Viologen [13]. The extent of formation of the hydroquinone species was determined from the absorbance at 580 nm.

Flavodoxin and ferredoxin-nitrate reductase activity measurements

All manipulations up to the point when the reduced flavodoxin or ferredoxin samples were transferred to the spectrophotometer cuvette were carried out in a glove box under N_2 containing less than 1 p.p.m. of O_2. Proteins were reduced by the addition of $Na_2S_2O_4$ (2 mM). After several minutes of incubation excess reductant was removed by gel filtration on a P6DG (Bio-Rad) desalting column (1.5 cm × 15 cm) equilibrated with 50 mM phosphate buffer, pH 7.0. The reduced proteins were then transferred to a cuvette closed with a rubber closure.

After the spectrophotometric measurements had been made, flavodoxin and nitrate reductase were removed by centrifuging the reaction mixture in a mini-centricon tube and the nitrate concentration of the clear supernatant determined colorimetrically as described in [7].

RESULTS AND DISCUSSION

N-terminal amino acid sequences of flavodoxins isolated from NO_3^--grown *A. vinelandii*

The N-terminal amino acid sequences of AvFld 1 and AvFld 2 were determined, and although over the first 20 residues they show some similarities, they are clearly different (Table 1). The sequence of AvFld 1, the major component in our preparations, corresponds to the minor species of the flavodoxins isolated from N_2-grown *A. chroococcum* [6], and designated as AcFld A by these authors. The sequence of AvFld 2, the minor component in our preparations, corresponds to the major species isolated from N_2-grown *A. chroococcum* (designated as AcFld B) [6]. AvFld 2 and Fld B are identical in this N-terminal region and have the sequences predicted for the *nif F* gene products of *A. vinelandii* [4] and *A. chroococcum* (R. L. Robson, personal communication).

These sequences also show that the species AvFld 1 and Fld A are homologous flavodoxins of *A. vinelandii* and *A. chroococcum* which are not encoded by *nif F*, since the sequences of these proteins do not correspond to the N-terminal sequence, and are not contained within the *nif F* sequence. These data also show that the flavodoxins from *A. vinelandii* first described in [5] are, as suggested by these workers, the products of different genes.

This work also shows that *A. vinelandii* strain UW 136, like *A. vinelandii* (A.T.C.C. 478) and *A. chroococcum* MCD 1155, is capable of synthesizing multiple flavodoxin species. This property is clearly more widespread in *Azotobacter* species than has been proposed on the basis of studies with *A. vinelandii* strain OP which appears from DNA hybridization studies to have only a single flavodoxin gene [4]. It is not apparent why multiple flavodoxins can be isolated from *A. vinelandii* UW 136, a rifampicin derivative of *A. vinelandii* OP, but not from the nominally isogenic strain *A. vinelandii* (OP, Berkeley).

Gene fusion and Northern blot analysis of *nif F* mRNA in *A. vinelandii* strain OP have shown that significant levels of transcription and translation of the *nif F* gene occurs under non-nitrogen-fixing conditions [4]. As a consequence of the absence of tight regulatory control, in those *Azotobacter* species which have multiple flavodoxin genes, at least two types of flavodoxin are synthesized during growth with N_2 or NO_3^- as N-source.

Relative molecular mass

Both flavodoxins described above migrated as single bands on SDS/PAGE with approximate M_r values of 20000. Electrospray mass spectrometry gave values of 19430 ± 3 for AvFld 1 and 19533 ± 5 for AvFld 2. These values indicate that both AvFld 1 and AvFld 2 belong to the class of long-chain flavodoxins which have M_r values in the range 19500 to 23000. The experimental value we obtained of M_r 19533 for AvFld 2 compares with that of 19663 determined from the DNA sequence of *nif F* of *A. vinelandii* OP [4]. This small difference may arise from limited mutational events leading to differences in the amino acid composition of this flavodoxin between these strains on prolonged subculture in different laboratories. The genes encoding Fld 1 and Fld 3 in *Azotobacter* have yet to be identified and sequenced.

^{31}P-NMR measurements of purified AvFld 1 and AvFld 2

The ^{31}P-NMR spectra of purified AvFld 1 and AvFld 2 (Figure 2) showed signals at −136.3 p.p.m. (Fld 1), and −135.48 p.p.m. (Fld 2), characteristic of the dianionic form of phosphate, arising from the FMN group which these proteins contain. These small differences in chemical shifts presumably arise from the slight

Table 1 N-terminal amino-acid sequence of flavodoxins from NO_3^--grown *A. vinelandii*

Protein samples were electroblotted before N-terminal amino-acid sequence analysis as described in the Materials and methods section. AvFld 1 has the same sequence as the minor species Fld A isolated from N_2-grown *A. chroococcum* [6], and AvFld 2 the same sequence as the *nif F* gene product of *A. vinelandii* [4].

Flavodoxin	Sequence
Flavodoxin 1 (Fld1)	Ser-Arg-Ile-Gly-Ile-Phe-Tyr-Gly-Ser-Ser-Ser-Gly-Val-Thr-Gly-Lys-Val-Ala-Glu-Lys…
Flavodoxin 2 (Fld 2)	Ala-Lys-Ile-Gly-Leu-Phe-Phe-Gly-Ser-Asn-Thr-Gly-Lys-Thr-Arg-Lys-Val-Ala-Lys-Ser…

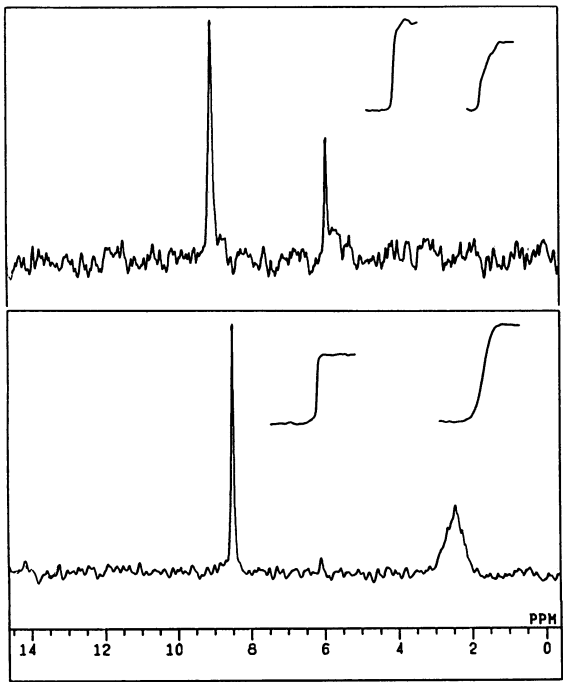

Figure 2 ^{31}P-NMR spectra of AvFld 1 and AvFld 2 isolated from nitrate-grown *A. vinelandii*

The proteins were in 25 mM Hepes, pH 8.0, containing 20% ^2H_2O. Top: AvFld 1; bottom: AvFld 2. Spectra were collected as described in the Materials and methods section, the protein concentrations were: AvFld 1, 260 μM; AvFld 2, 250 μM.

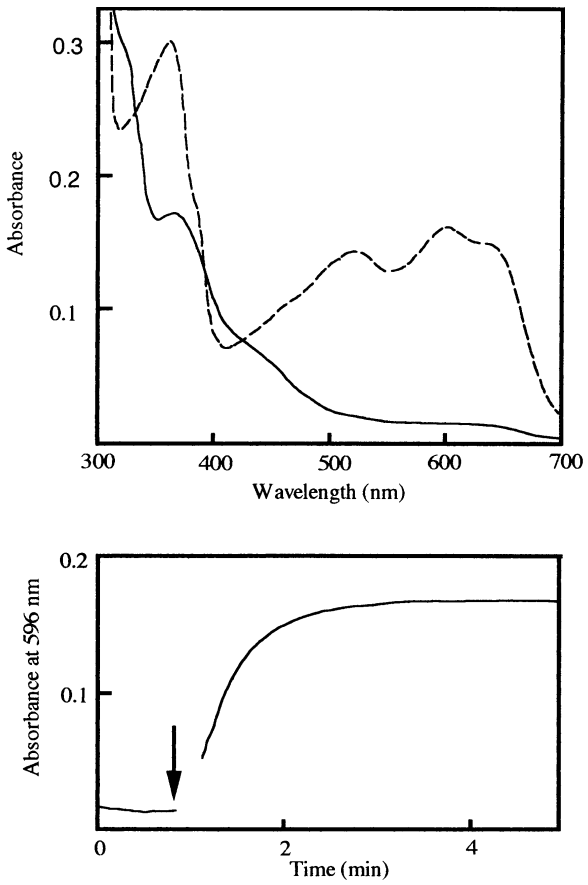

Figure 3 Electron donation to purified nitrate reductase by reduced AvFld 1$_{HQ}$

Top: AvFld 1 was reduced by the addition of excess dithionite which was then removed by gel filtration on a Bio-Gel P6DG desalting column (1.5 cm × 15 cm) equilibrated with 50 mM phosphate buffer, pH 7.0. The reduced AvFld 1 (42 μM) was transferred to a cuvette which was then capped with a rubber closure and removed from the anaerobic enclosure. Spectrum 1 (solid line) was recorded and then nitrite (5 mM) and purified nitrate reductase (38 nM) were added by syringe to the cuvette. Spectrum 2 (dashed line) was recorded 8 min after the addition of enzyme. Bottom: time course of the oxidation of AvFld 1$_{HQ}$ by nitrite reductase measured from the increase in A_{596}. Nitrate reductase was added as indicated by the arrow, the conditions were as described for the top panel.

perturbations in the environment of the FMN groups in the two proteins. Similar values were reported for the equivalent flavodoxin species of *A. vinelandii* A.T.C.C. 478 [5].

The ^{31}P-NMR spectra of both AvFld 1 and AvFld 2 also exhibited an additional signal to that associated with FMN, (Figure 2) with chemical shifts of −142.1 p.p.m. and −138.59 p.p.m. respectively. Comparison of the intensities of the signals relative to the intensity of that of FMN gave P/FMN ratios of 1.54:1 and 0.64:1 for AvFld 1 and AvFld 2 respectively. The non-FMN phosphate of AvFld 1 was unstable and was lost on further manipulation of the protein. Similar lability of bound phosphate in preparations of AvFld 3 was noted previously [5].

The presence of additional phosphate groups in flavodoxins was first detected by NMR spectroscopy of flavodoxin isolated from N_2-grown *A. vinelandii* (strain OP Berkeley), which was shown to contain three phosphate groups [14]. Subsequently, ^{1}H–^{31}P multiquantum two-dimensional NMR techniques were used to show one of these ($\delta = -140.2$ p.p.m.) to be a bi-substituted phosphate residue involving serine and threonine [15]. Phosphate was incorporated into newly synthesized flavodoxin *in vivo* under both N_2-fixing and NH_4^+-sufficient conditions and this modification has been suggested to have a structural role [16]. The other phosphate residue ($\delta = -140.8$ p.p.m.) was labile and lost on storage of the protein [14].

No systematic study of the properties of the phosphate groups of AvFld 1 and AvFld 2 was made, but the non-stoichiometric amounts compared with the FMN and the observed lability of this group on AvFld 1 would suggest they correspond with the non-covalently bound species (i.e. the phosphate residue which is released on precipitation of the protein with trichloroacetic acid) [14]. Our findings provide additional evidence for the existence of additional phosphate binding sites on *Azotobacter* flavodoxins, a proposal which has not been universally accepted [17,18].

Redox potential of the semiquinone/hydroquinone couple

The mid-point potential of the semiquinone/hydroquinone redox couple (SQ/HQ) of AvFld 1 was determined from equilibrium measurements of the ratios of NADP/NADPH and AvFld 1$_{SQ}$/AvFld.$_{HQ}$ in the presence of excess NADPH and catalytic amounts of NADP reductase [12]. This method, which is applicable to flavodoxins of relatively high redox potential, gave a value of −330 mV, in good agreement with that of −320 mV determined by others [5].

The mid-point potential of the semiquinone/hydroquinone redox couple of AvFld 2 was determined to be −493 mV by spectrophotometric titration using bisulphite/dithionite to poise the redox potential [13]. This value is very close to that of −500 mV determined from equilibration measurements under H_2 in the presence of catalytic amounts of hydrogenase [5].

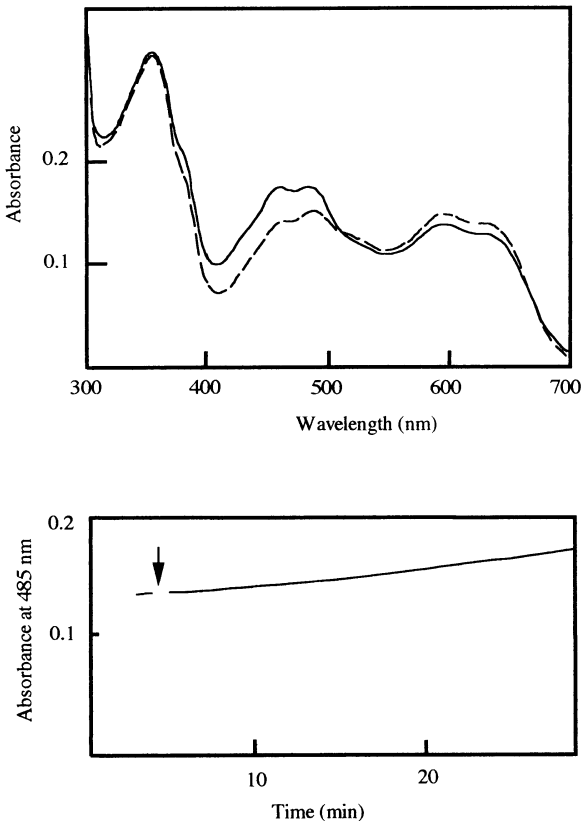

Figure 4 Electron donation to purified nitrate reductase by reduced AvFld $1._{SQ}$

Top: AvFld $1._{SQ}$ was generated by enzymic oxidation with nitrate reductase as described in the legend of Figure 3. Spectrum 1 (dashed line) was recorded 8 min after the addition of enzyme and nitrate when AvFld $1._{HQ}$ present initially had been oxidized to the semiquinone level. Spectrum 2 (solid line) was recorded after 10 min when AvFld $1._{SQ}$ had been partially converted into the quinone form. Bottom: time course of the oxidation of AvFld $1._{SQ}$ by nitrite reductase measured from the increase in A_{485} as in the top panel.

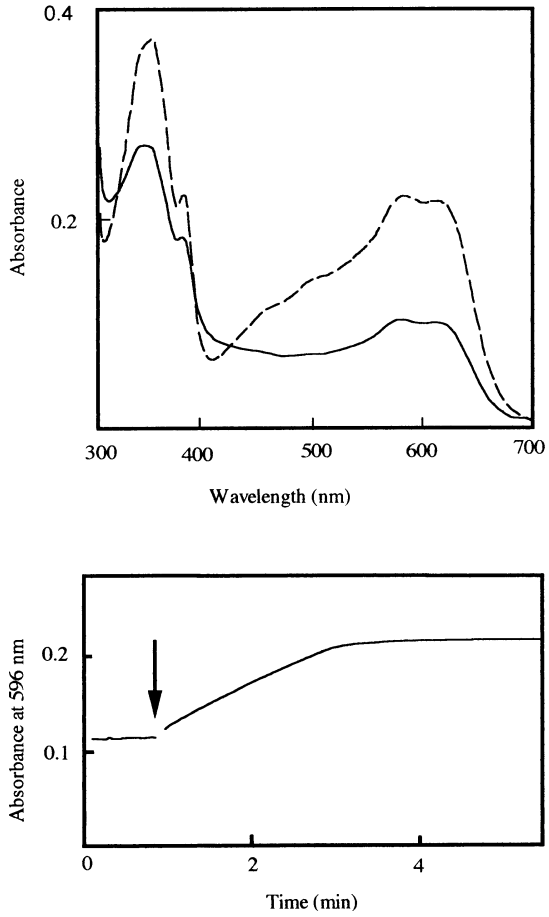

Figure 5 Electron donation to purified nitrate reductase by reduced AvFld $3._{HQ}$

Top: AvFld 3 was reduced by the addition of excess dithionite which was then removed by gel filtration on a Bio-Gel P6DG desalting column (1.5 cm × 15 cm) equilibrated with 50 mM phosphate buffer, pH 7.0. The reduced AvFld 3 (40 μM) was transferred to a cuvette which was then capped with a rubber closure and removed from the anaerobic enclosure. Spectrum 1 (dashed line) was recorded and then nitrite (5 mM) and purified nitrate reductase (38 nM) were added by syringe to the cuvette. Spectrum 2 (solid line) was recorded 8 min after the addition of enzyme. Bottom: time course of the oxidation of AvFld $3._{HQ}$ by nitrite reductase measured from the increase in A_{580}. Nitrate reductase was added as indicated by the arrow, the conditions were as described for Figure 3 (top).

Flavodoxins and ferredoxin 1 as putative electron donors to assimilatory nitrate reductase

Previous work has shown that flavodoxin or ferredoxin purified from N_2-grown cultures could function as electron donors to crude preparations of solubilized assimilatory nitrate reductase from *A. vinelandii* [8]. We have recently characterized a soluble form of this enzyme and shown it to contain acid-labile iron and sulphide and molybdenum pterin cofactor (Moco) as redox centres. The purified enzyme was not active when tested with NADH or NADPH as electron donors [7].

Since the work described above shows that *A. vinelandii* is capable of synthesizing a mixture of flavodoxins during growth on NO_3^-, as had previously been shown for N_2-grown organisms [5], we investigated the ability of these purified flavodoxin species to donate electrons to purified nitrate reductase.

The fully reduced hydroquinone oxidation level of flavodoxins and reduced ferredoxin 1 were prepared by reduction by excess dithionite, and excess reductant was subsequently removed by gel filtration in an anaerobic chamber (< 1 p.p.m. O_2), as described in the Materials and methods section. The extent of reduction of flavodoxins to the hydroquinone level was determined spectrophotometrically, since the low potential of the semiquinone/hydroquinone couple, particularly in the case of AvFld 2 and AvFld 3, prevents complete conversion into the hydroquinone level with dithionite as reductant at pH 7.

The rate and extent of reaction of nitrate reductase with AvFld $1._{HQ}$ was followed spectrophotometrically, and also by the amount of nitrite formed during the reaction. Figure 3 (top) shows that following the addition of nitrate reductase to an anaerobic solution of nitrate and AvFld $1._{HQ}$ a rapid oxidation occurred as indicated by the increase in absorbance at 595 nm. The spectral features of the reaction mixture after 10 min reaction was characteristic of AvFld $1._{SQ}$ (Figure 3, top). The initial rate of oxidation of AvFld $1._{HQ}$ determined from Figure 3 (bottom) was 42 nmol/min corresponding to a specific activity of 7 μmol/min per mg of nitrate reductase, compared with 10.5 μmol of NO_3^- formed/min per mg measured for this preparation in the standard assay system with reduced Methyl Viologen as electron donor. The initial rate of oxidation was proportional to the nitrate reductase concentration up to at least 200 nM at an AvFld 1 concentration of 29 μM. No change in

absorbance was observed when nitrate or nitrate reductase was omitted. In this experiment, 50 nmol of AvFld $1._{HQ}$ were oxidized to AvFld $1._{SQ}$ before a sample was removed for estimation of the amount of nitrite formed as described in the Materials and methods section. Unexpectedly, analysis gave 32 nmol of nitrite instead of the expected 25 nmol for the two-electron reduction reaction of nitrate to nitrite. Subsequently, we observed a slow reaction of AvFld $1._{SQ}$ with nitrate reductase (see Figure 4) and we attribute the increased yield of nitrite as arising from the continued reaction of this oxygen-stable redox level of AvFld 1 with the enzyme during the manipulation of the samples before analysis for nitrite. The rate of reaction with AvFld $2._{SQ}$ was proportional to the amount of nitrate reductase added to the assay, but at an equivalent enzyme concentration was some 30-fold slower than the rate observed with AvFld $2._{HQ}$ as electron donor.

The rapid oxidation of all the AvFld $1._{HQ}$ in an assay to AvFld $1._{SQ}$ indicates that the proportion of this species which NMR indicates is modified by phosphorylation (64 %), does not affect the ability to transfer electrons to nitrate reductase. Either the two species show the same reactivity, or they are in redox equilibrium.

In contrast, AvFld $2._{HQ}$, despite having an E_m some 170 mV more negative than AvFld $1._{HQ}$, was not oxidized in the presence of nitrate reductase and NO_3^-, indicating that it did not function as an electron donor to nitrite reductase. This lack of activity is not due to phosphorylation since only 60 % of the protein is phosphorylated. Studies on this protein lacking the phosphate residue have shown that the redox couple AvFld $2._{HQ}$ to AvFld $2._{SQ}$ is 50–60 mV more positive than the phosphorylated protein [19].

Dithionite-reduced ferredoxin 1 from *A. chroococcum* (E_m -300 mV) was also not oxidized when tested in this system as an electron donor to nitrate reductase.

Although AvFld 3 (E_m of -500 mV, the predominant flavodoxin in NH_4^+-grown cells) is not present in NO_3^--grown organisms, it was tested as an electron donor to probe the specificity of the electron transfer partner to nitrate reductase. In experiments similar to those described above, AvFld $3._{HQ}$ was oxidized at a rate of 24.6 nmol/min per mg of nitrate reductase, approximately half the rate observed with AvFld $2._{HQ}$ (Figure 5). No oxidation of AvFld $3._{SQ}$ was observed. These data show that factors other than the difference in redox potential are important in these protein–protein redox coupling reactions.

As shown above the predominant flavodoxin species present in nitrate-grown *A. vinelandii*, AvFld 1, is capable of providing reducing equivalents for nitrate reductase, making it a likely candidate for the physiological electron donor *in vivo*. The ability of flavodoxin to couple to nitrate reductase reported previously [8], using a crude solubilized preparation of nitrate reductase and an unresolved flavodoxin mixture isolated from N_2-grown cells, was presumably due to the presence of some AvFld 1 in a preparation containing predominantly AvFld 2.

Our finding that the semiquinone/hydroquinone redox couple of flavodoxin is the more effective in coupling to nitrate reductase suggests that, like nitrogenase, membrane energization may be involved in generating reduced flavodoxin [20]. If this is the case, the assimilation of nitrate may well be more effective at low dissolved oxygen concentrations, and like nitrogenase function may require respiratory protection to allow this process to occur effectively in aerobic organisms.

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