Direct electrochemistry of two genetically distinct flavodoxins isolated from *Azotobacter chroococcum* grown under nitrogen-fixing conditions

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Two genetically distinct flavodoxins, designated AcFldA and AcFldB, were isolated from *Azotobacter chroococcum* (MCD1155) grown under nitrogen-fixing conditions. AcFldA and AcFldB differ in their midpoint potentials for the semiquinone–hydroquinone couple (E_m –305 mV and –520 mV respectively). Only AcFldB was competent to act as an electron donor to the Mo-containing nitrogenase of *A. chroococcum*. The N-terminal amino acid sequence (20 residues) of AcFldB was identical with that predicted from the *nifF* DNA sequence of *A. vinelandii* OP [Bennett, Jacobsen & Dean (1988) J. Biol. Chem. **263**, 1364–1369], suggesting that AcFldB is the *nifF* gene product of *A. chroococcum* (MCD1155). Direct fast reversible electrochemistry of these flavodoxins has been achieved at a polished edge-plane graphite electrode using the aminoglycoside neomycin as a promoter. The heterogeneous rates of electron transfer between the graphite electrode and AcFldA and AcFldB were determined to be $1.2 \times 10^{-3} \text{ cm}^2 \text{ s}^{-1}$ and $2.0 \times 10^{-3} \text{ cm}^2 \text{ s}^{-1}$ respectively. The natures of two minor species of flavodoxin designated AcFldC and AcFldD, which were resolved by f.p.l.c., are also discussed.

INTRODUCTION

Flavodoxins are small (M_r approx. 20000) FMN-containing monomeric proteins that can act as electron donors to nitrogenase in a number of organisms, including the facultative anaerobe *Klebsiella pneumoniae* (oxytoca) (Hill & Kavanagh, 1980; Nieva-Gomez, 1980; Deistung *et al.*, 1985; Thorneley & Deistung, 1988) and the obligate aerobes *Azotobacter chroococcum* (Yates, 1972), *Azotobacter vinelandii* A.T.C.C. 478 (Klugkist *et al.*, 1985, 1986) and *A. vinelandii* OP (Bennett *et al.*, 1988). Deistung & Thorneley (1986) have reviewed the role of flavodoxins in nitrogen fixation and described the purification and characterization of flavodoxin from *K. pneumoniae* (KpFld) and from *A. chroococcum* (AcFld).

The synthesis of flavodoxin in nitrogen-fixing organisms is regulated by the fixed-nitrogen status. The expression of the *nifF* gene that encodes a flavodoxin in *A. vinelandii* OP, *A. chroococcum* and *K. pneumoniae* is repressed by ammonia via a well-understood regulatory cascade mechanism [see Merrick (1988) for a review]. In these organisms flavodoxin synthesis is not significantly affected by the Fe content of the medium. However, in other organisms such as *Clostridium pasteurianum*, Fe limitation results in higher levels of flavodoxin and lower levels of ferredoxin synthesis, presumably to optimize use of the Fe in proteins such as nitrogenase (Knight & Hardy, 1966). In these organisms ferredoxin is regarded as the primary electron donor to nitrogenase under Fe-sufficient growth conditions.

It is not known whether more than one flavodoxin and/or ferredoxin are electron donors to nitrogenase in *K. pneumoniae*, *A. chroococcum* and *A. vinelandii* OP. *nifF* mutants of *K. pneumoniae* are 'leaky', with an acetylene reduction rate about 10% of that of the wild-type (Hill & Kavanagh, 1980). *NifF*

mutants of *A. vinelandii* OP reduce acetylene and grow on N_2 , indicating the presence of alternative electron donors to nitrogenase. Although a low-potential ferredoxin (AvFdl) has been characterized and shown to act as an electron donor to nitrogenase, a double mutant lacking both AvFdl and AvFdl remains capable of diazotrophic growth (Martin *et al.*, 1989). This suggests that a third electron donor to nitrogenase is present in *A. vinelandii* OP. If this is a second flavodoxin, its identification could be complicated by the possibility of post-translational modification. Boylan & Edmonson (1990) have shown that *A. vinelandii* OP synthesizes a flavodoxin with a phosphodiester linkage that is thought to stabilize the protein structure rather than regulate activity. It is not entirely clear whether the three flavodoxins isolated from *A. vinelandii* OP by Klugkist *et al.* (1985, 1986) are different gene products or modified forms of the same protein.

In the present paper we show that *A. chroococcum* synthesizes at least two genetically distinct flavodoxins, only one of which is competent in electron transfer to nitrogenase. Previously it has been assumed that only one flavodoxin was present (Yates, 1972; Deistung & Thorneley, 1986). The separation of the two flavodoxins was achieved by f.p.l.c. before attempts at direct electrochemistry at a carbon electrode (Barker *et al.*, 1988). We also present a more detailed account of this electrochemistry, which is itself important for three reasons. First, flavodoxin could be used to drive nitrogenase and a number of other enzymes used in 'biosensors' at an electrode. Secondly, it provides a logical route to the study of the nitrogenase component proteins at an electrode. Thirdly, the direct electrochemistry of flavodoxins is interesting in that X-ray structure analysis (Watenpaugh *et al.*, 1972; Burnett *et al.*, 1974; Smith *et al.*, 1977, 1983; Ludwig *et al.*, 1982) and structure predictions from DNA sequence data

Abbreviations used: HQ, hydroquinone; E_m , midpoint reduction potential; SQ, semiquinone; NHE, normal hydrogen electrode.

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(Drummond, 1986) suggest that both basic and acidic domains are present in the vicinity of the FMN. This hetero-functional binding surface may influence the interaction of the protein with the electrode surface [see Bond & Hill (1991) for a review of the factors that determine protein binding to electrode surfaces and the efficiency of electron transfer].

MATERIALS AND METHODS

Isolation of flavodoxins

Azotobacter chroococcum (strain MCD1155), which carries a deletion for *nif HDK*, the structural genes for Mo-nitrogenase, (Robson *et al.*, 1986), was grown under N_2 -fixing conditions in the presence of vanadium (Eady *et al.*, 1987). The flavodoxins were isolated during the purification of the Fe protein of V-nitrogenase. They were eluted after the Fe protein during the second gel-filtration step on Sephacryl S-200 (Eady *et al.*, 1988). Flavodoxin-containing fractions were combined, and solid $(NH_4)_2SO_4$ was added to 40% (w/v). The precipitated proteins were removed by centrifugation and the yellow supernatant loaded on to a DEAE-52 column (5 cm $\times$ 10 cm) equilibrated with 50 mM-Tris/HCl, pH 8.0, containing 40% (w/v) $(NH_4)_2SO_4$. The flavodoxins bound as a tight yellow band and were eluted with a linear gradient of $(NH_4)_2SO_4$ decreasing from 40 to 0% over a volume of 500 ml. A tight yellow-orange band comprised a mixture of flavodoxins, which were subsequently resolved by f.p.l.c. on a Mono Q HR 5/5 anion-exchange column (0.5 cm $\times$ 5.0 cm) (Pharmacia LKB Biotechnology). Protein samples (approx. 15 mg) were loaded in 20 mM-1,3-Bis-Tris Propane, pH 7.4, and eluted with a linear gradient (25 ml) of 250–500 mM-KCl. Four peaks that were eluted at KCl concentrations of 350 mM (AcFldA), 400 mM (AcFldB), 460 mM (AcFldC) and 480 mM (AcFldD) were identified as flavodoxins by their u.v.-visible spectra, which were characteristic of protein-bound oxidized FMN, and had molecular masses in the range 18–20 kDa as determined by SDS/PAGE on a 15% gel with low-molecular-mass markers (Bio-Rad). The resolution of AcFldA from AcFldB was significantly better than that reported by us in a preliminary publication (Barker *et al.*, 1988).

Preparation of protein samples for N-terminal-amino-acid determination

SDS/PAGE (15% gel) of AcFldA and AcFldB was carried out by the method of Laemmli (1970), with minor modifications. Initially the top reservoir contained running buffer to which 50 μ M-GSH had been added. The gel was pre-run at 80 V for 2 h to remove free acrylic acid. Both reservoirs were then replenished with running buffer, that in the upper reservoir containing 0.1 mM-thioglycollate. After loading, the proteins (600 μ g in 0.8 ml) were electrophoresed through the stacking gel at 60 V and subsequently through the running gel at 180 V. The protein bands were then transferred from the gel to Immobilon membrane [Millipore (U.K.) Ltd., Harrow, Middx., U.K.] using the electro-blotting procedure described by Anderson (1984). The blotted proteins were visualized by staining with Coomassie Blue (0.2% in 50% methanol/7.5% acetic acid). After destaining and drying the bands were excised by cutting the membrane into strips before N-terminal-amino-acid determination using standard procedures on an ABI pulsed liquid analyser.

Peptide mapping

A prepared mixture of AcFldA and AcFldB in 50 mM-Tris/HCl, pH 8.0, was denatured by addition of SDS (4 μ l, 10%) over a period of 20 min. Chymotrypsin A4 (1.5 μ l or

7.5 μ l; 1 mg $\cdot$ ml⁻¹) and sample buffer (Laemmli, 1970) were then added and the mixture incubated at 30 °C for times up to 23 h. Proteolysis was stopped by boiling aliquots (40 μ l) for 5 min. Samples (25 μ l) were then subjected to SDS/PAGE (15% gel), followed by staining with Coomassie Blue (Laemmli, 1970).

All biochemicals were purchased from Sigma (Poole, Dorset, U.K.), and salts were from BDH (Poole, Dorset, U.K.).

Flavodoxin assay

Reduced AcFldA and AcFldB [hydroquinone (HQ-) state] were assayed for their ability to transfer an electron to the Fe-protein of *A. chroococcum* Mo-nitrogenase (Ac2) by monitoring acetylene reduction and concomitant H_2 -evolution activities of the MoFe-protein (Ac1) in the absence of $Na_2S_2O_4$ at 23 °C. Assay mixtures (1 ml) contained 9 mM-ATP, 10 mM-MgCl₂, 130 μ g-Ac2, 100 μ g-Ac1, 20 μ M-AcFldA or AcFldB in the HQ-state or 0–20 μ M- $Na_2S_2O_4$ /25 mM-Hepes buffer, pH 7.4, equilibrated under an atmosphere comprising acetylene/ N_2 (1:3) in a glass vial (2.3 ml volume) fitted with a Suba-Seal closure. Assays were run for at least 10 min before gas sampling commenced. It was calculated that reductant exhaustion would have occurred after only a few seconds.

All procedures were carried out in a glove-box through which N_2 containing less than 1 p.p.m. of O_2 was circulated via heated catalyst columns (BASF R311). Since all solutions were saturated with N_2 , acetylene-reduction assays were done under an atmosphere of acetylene/ N_2 (1:3), rather than the customary acetylene/Ar (1:3). However, since 25% acetylene completely suppresses N_2 reduction by nitrogenase, the only reduction products that need to be determined are ethylene and H_2 . This was done as previously described (Eady *et al.*, 1972).

$Na_2S_2O_4$ (0.4 mM) was removed from a stock solution containing a mixture of Ac1 (2 mg $\cdot$ ml⁻¹) and Ac2 (2.6 mg $\cdot$ ml⁻¹) by the addition of MgATP (10 mM), which causes nitrogenase to turn over with consequent oxidation of $Na_2S_2O_4$ and partial hydrolysis of the MgATP. An aliquot (50 μ l) of this $S_2O_4^{2-}$ -free nitrogenase was used to initiate the acetylene-reduction assays. The system was calibrated by substituting a defined concentration of $Na_2S_2O_4$ (0–20 μ M) for either of the two flavodoxins in otherwise identical assays.

AcFldA and AcFldB in the HQ-state were prepared by reduction with $Na_2S_2O_4$ (2 mM). It was necessary to increase the pH to 8.0 to effect the reduction of AcFldB, which has a lower midpoint reduction potential (E_m) than AcFldA. Excess $Na_2S_2O_4$ was removed by gel filtration on a pre-calibrated column (0.7 cm $\times$ 15 cm) of P-6DG (Bio-Rad) equilibrated with 25 mM-Hepes (pH 7.4)/10 mM-MgCl₂. Complete reduction to the HQ-state was confirmed by their u.v.-visible spectra recorded on a Perkin-Elmer λ -5 spectrophotometer.

Electrochemistry

Cyclic voltammetry was carried out using an Oxford Electrodes potentiostat with all voltammograms recorded on a Bryans XY (series 60000) chart recorder. The electrochemical cell employed a three-electrode system with two electrolyte compartments. The counter-electrode was a semicylindrical piece of platinum gauze, and the reference was a saturated calomel electrode linked to the main cell compartment through a Luggin capillary tip ($\sim$ 0.1 mm diameter).

The edge-plane graphite electrode was constructed from a 5 mm disc of standard pyrolytic graphite (Le Carbone, Portslade, Sussex, U.K.) cut with the *a*-*b* plane perpendicular to the disc face and housed in a Teflon sheath. Before each measurement, the edge-plane graphite electrode was polished with a water/alumina slurry, followed by sonication and thorough rinsing with water.

RESULTS

Purification and characterization of flavodoxins

Fig. 1 shows the f.p.l.c. elution profile obtained when partially purified AcFld was run on Mono Q HR 5.5. The protein was resolved into two major components, designated AcFldA and AcFldB and two minor species, AcFldC and AcFldD. The amounts of AcFldC and AcFldD were small and varied with different preparations. In the experiment shown in Fig. 1, they comprise less than 5% of the total flavodoxin, whereas in a preliminary publication (Barker *et al.*, 1988) we showed a comparable, but less-well-resolved, elution profile in which they comprised approx. 10% of the total flavodoxin applied to the column.

AcFldA and AcFldB have different apparent molecular masses, namely 18 and 19.5 kDa respectively, as determined by SDS/PAGE (Fig. 4 below). AcFldC and AcFldD behaved similarly to AcFldB (results not shown). As reported by Klugkist *et al.* (1985), SDS/PAGE is an unreliable method of determining the molecular mass of flavodoxins, since the extent of migration relative to marker proteins varies with gel composition. We have obtained values of 21 kDa and 23 kDa for AcFldA and AcFldB with a 20%-(w/v)-polyacrylamide gel. However, the lower values quoted above are closer to those of 18.95 kDa and 19.48 kDa derived from DNA sequence data for KpFld and AvFld, which, like AcFldB, are *nifF*-gene products.

The u.v.-visible spectra of AcFldA and AcFldB (Fig. 2) are characteristic of flavodoxins and, together with an E_m of -520 mV versus normal hydrogen electrode (NHE) (see under 'Electrochemistry of purified flavodoxin components' below) establish AcFldB as being the species previously characterized by Deistung & Thorneley (1986). Although these workers did not identify the other flavodoxins reported here, they were presumably separated from AcFldB during the purification, which involved preparative gel electrophoresis (f.p.l.c. was not available).

The different *N*-terminal-amino-acid sequences of AcFldA and AcFldB establish that these two flavodoxins are genetically

Fig. 2. U.v.-visible spectra of *A. chroococcum* flavodoxins AcFldA and AcFldB

-----, Oxidized (quinone) state; ----, SQ state; -.-.-, HQ (quinol) state. The spectra were recorded with an [AcFld] of $10 \mu\text{M}$ at pH 8.0 in 25 mM-Hepes buffer in a 1 cm-pathlength anaerobic cell. Flavodoxin SQ was prepared by stoichiometric reduction of the oxidized flavodoxin with $\text{Na}_2\text{S}_2\text{O}_4$. Subsequently an excess of $\text{Na}_2\text{S}_2\text{O}_4$ was used to reduce the flavodoxin SQ to the HQ state.

Fig. 1. F.p.l.c. elution profile for separation of *A. chroococcum* flavodoxins (AcFldA, AcFldB, AcFldC and AcFldD) on a Mono-Q HR5/5 anion-exchange column

Separation of the four flavodoxins was achieved as described in the Materials and methods section. The absorbance scale was changed from 2.0 to 0.5 full scale at 0.42 M-KCl to allow the minor peaks representing AcFldC and AcFldD to be seen.

distinct (Fig. 3). A comparison with the *N*-terminal sequences of flavodoxins isolated from other organisms shows that the first 20 residues of AcFldB are identical with those of AvFld (*OP*) (Tanaka *et al.*, 1977; Bennett *et al.*, 1988). Although AcFldA has considerable sequence similarity to other flavodoxins, its *N*-terminal sequence is not identical with any one of them.

The *N*-terminal sequence of AcFldC is identical with that of AcFldB (Fig. 3). Its resolution from AcFldB by f.p.l.c. (Fig. 1) and its u.v.-visible spectrum indicates that it is either a modified (i.e. phosphorylated) or partly denatured form of the protein that retains a flavin cofactor.

The *N*-terminal sequence of AcFldD shows that it is a mixture of AcFldC or AcFldB (which have identical sequences) with another protein designated AcZ (Fig. 3). We have not been able to resolve this mixture by PAGE or f.p.l.c., and therefore are unable to say whether AcZ is a flavodoxin. The yellow coloration may be entirely due to the presence of species AcFldB or AcFldC. We have not further characterized species AcFldC and AcZ.

AcFldA and AcFldB also differ in their susceptibility to proteolysis by chymotrypsin A4. Over a 23 h incubation of the

Fig. 3. *N*-Terminal-amino-acid sequences of the four flavodoxins from *A. chroococcum*

N-Terminal-amino-acid sequence analysis was carried out as described in the Materials and methods section. Unidentified residues are represented by Xaa. The first *N*-terminal amino acids of AcFIdB are identical with those of *A. vinelandii* (OP Berkeley) flavodoxin (Tanaka *et al.*, 1977) and the *nifF* DNA sequence of *A. vinelandii* (OP) (Bennett *et al.*, 1988). AcFIdD is a mixture of AcFIdB or AcFIdC and an unknown protein (AcZ), the sequence of which is given above. There is no evidence that AcZ is a flavodoxin, since its u.v.-visible spectrum could be due to the AcFIdB or AcFIdC content of the mixture.

Fig. 4. Peptide mapping of AcFIdA and AcFIdB by limited proteolysis with chymotrypsin A4

Two samples of a mixture of AcFIdA and AcFIdB (75 µg of each in 100 µl) were denatured in 0.4% SDS over a period of 20 min. Chymotrypsin A4 [1 mg·ml⁻¹; 1.5 µl (I) or 7.5 µl (II)] and Laemmli sample buffer were added and the mixtures incubated at 30 °C for periods up to 23 h. Proteolysis was stopped by boiling samples (40 µl) for 5 min. Samples (25 µl) were then analysed on SDS/15%-PAGE. x, undigested AcFIdA + AcFIdB; y, undigested AcFIdA; z, undigested AcFIdB. Abbreviation: POP, period of proteolysis; *M*, molecular mass.

isolated proteins with chymotrypsin A4 under the conditions of Fig. 4, product analysis by SDS/PAGE showed extensive proteolysis of AcFIdA, but no detectable proteolysis of AcFIdB (results not shown). In order to eliminate the possibility that failure to detect proteolysis of AcFIdB was due to a lack of chymotrypsin activity, we repeated the digestion using a prepared mixture of AcFIdA and AcFIdB, and these data are shown in Fig. 4. As expected, AcFIdA was proteolysed into low-molecular-mass fragments, whereas AcFIdB remained intact. Whether this behaviour is due to a different number and/or distribution of aromatic amino acids or different protein conformations will not be clear until the complete sequences and X-ray structures are available for both proteins. However, the data of Fig. 4 do provide further evidence for AcFIdA and AcFIdB being different gene products.

The data presented in Fig. 5 show that AcFIdB, but not AcFIdA, can function as an electron donor to nitrogenase. The same amount of acetylene and H₂ was produced with 20 nmol of AcFIdB(HQ) as the electron donor, as was predicted from a control experiment with 10 nmol of Na₂S₂O₄ as reductant. This is consistent with AcFIdB(HQ) donating a single electron to

Fig. 5. Electron transfer from *A. chroococcum* flavodoxin to *A. chroococcum* nitrogenase

Assays to determine the ability of reduced AcFIdA and AcFIdB to transfer an electron to *A. chroococcum* nitrogenase were carried out as described in the Materials and methods section. The products H₂ (▽), ethylene (○) and ethylene + H₂ (△) are plotted as a function of [Na₂S₂O₄] in the calibration curves (a), (b) and (c). The reduction products H₂ (▼), ethylene (●) and ethylene + H₂ (▲) obtained using 20 µM-AcFIdB in the assays in the absence of Na₂S₂O₄ are shown on the graph at an equivalent concentration of Na₂S₂O₄ (20 µM electron equivalents; 10 µM-Na₂S₂O₄). Replicate determinations were within 5% of the average values shown. AcFIdA gave only background levels of ethylene + H₂ (less than 0.5 nmol in total) (points not shown on the graph). These data show that AcFIdB(HQ), but not AcFIdA(HQ), is oxidized by nitrogenase.

nitrogenase with AcFIdB(SQ) (where SQ is semiquinone) as the oxidation product (Yates, 1972). Na₂S₂O₄ donates two electrons after dissociation to yield two equivalents of the active reductant superoxide ion (SO₂⁻) (Thorneley *et al.*, 1975). It is noteworthy that, with SO₂⁻ as the electron donor, a higher percentage of the total electron flux goes into H₂ formation (20%) than does with AcFIdB(HQ) as donor (less than 5%).

Cyclic voltammetry of flavodoxin mixtures

The mixture of flavodoxins in species obtained after elution from DEAE-52, but before separation by f.p.l.c. (see the Materials and methods section), was initially studied by cyclic voltammetry.

The cyclic voltammogram of the fully oxidized flavodoxin mixture (100 µM) at a polished edge-plane graphite electrode

Fig. 6. Cyclic voltammograms at $10 \text{ mV} \cdot \text{s}^{-1}$ of purified AcFldB and FMN

AcFldB (a) at a polished edge-plane graphite electrode; (b) in the presence of neomycin (1 mM); (c) in the presence of $\text{Cr}(\text{NH}_3)_6^{3+}$ (6 mM). (d) FMN (100 μM). All determinations were carried out at pH 7.4.

shows a redox couple with an E_m of -250 mV corresponding to free FMN, which is known to be in equilibrium with flavodoxin-bound FMN. The electrochemical response of FMN (100 μM) is shown in Fig. 6(d) and yields the expected reversible two-electron couple with an E_m -240 mV at a polished edge-plane graphite electrode.

In previous electrochemical studies of *Desulfovibrio vulgaris* and *Megasphaera elsdenii* flavodoxins, fast heterogeneous reduction of the fully oxidized protein was not observed (Armstrong *et al.*, 1984). Hence the electrochemistry of semi-reduced flavodoxin was investigated. No voltammetric response corresponding to the reduction of SQ to HQ was observed at a polished edge-plane graphite electrode. The addition of either $\text{Cr}(\text{NH}_3)_6^{3+}$ or 105 kDa poly-L-lysine to act as promoters of flavodoxin electrochemistry produced, in both cases, a very unstable, ill-defined, voltammetric response. Furthermore, in the presence of poly-L-lysine, the flavodoxin was precipitated during the course of an experiment (usually lasting 2–3 h). The use of an aminoglycoside promoter, neomycin, produced a set of waves which were relatively much more stable. The two observed waves, $E_{m1} \sim -500 \text{ mV}$ and $E_{m2} \sim -300 \text{ mV}$, in addition to free FMN ($E_m \sim -250 \text{ mV}$) indicate the presence of two redox-active species in the flavodoxin sample.

Electrochemistry of purified flavodoxin components

The electrochemistry of AcFldB was investigated by using $\text{Cr}(\text{NH}_3)_6^{3+}$ or neomycin as promoter at a polished edge-plane graphite electrode. The flavodoxin was reduced to the SQ form with sodium dithionite: Fig. 6(a) depicts the voltammogram of the SQ form of AcFldB at a polished graphite electrode, and Fig. 6(b) shows the reversible couple ($E_m \sim -520 \text{ mV}$) obtained upon the addition of 1 mM-neomycin. Also shown in Fig. 6(c) is the response of AcFldB in the presence of 6 mM- $\text{Cr}(\text{NH}_3)_6^{3+}$. Dithionite, neomycin and $\text{Cr}(\text{NH}_3)_6^{3+}$ do not show any electrochemical response in the absence of AcFldB. Fig. 7(a) exhibits a set of cyclic voltammograms for the range of sweep rates 2–100 $\text{mV} \cdot \text{s}^{-1}$.

Fig. 7. Potential scan-rate-dependence of AcFldB

(a) Cyclic-voltammetric response of the SQ-HQ couple and (b) a plot of the cathodic peak current versus the square root of the potential scan rate.

From the linear portion of the cathodic peak current against (scan rate) $^{1/2}$ depicted in Fig. 7(b), a diffusion coefficient of $2.3 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ was obtained. A heterogeneous rate constant, k_s , of $2.0 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$ was also determined by plotting (scan rate) $^{1/2}$ against a kinetic parameter calculated from the voltammetric peak-to-peak separation according to the procedure outlined by Nicholson (1965). Fast reversible electrochemistry was never observed in the presence of $\text{Cr}(\text{NH}_3)_6^{3+}$ as promoter.

AcFldA was investigated under the conditions described for AcFldB. With fully oxidized or SQ-form AcFldA (120 μM) no electrical response was discernible at a polished edge-plane graphite electrode. Addition of neomycin to the SQ form of AcFldA yielded a reversible couple with $E_m = -305 \text{ mV}$. From the sweep-rate-dependence, as described above for AcFldB, the heterogeneous rate of electron transfer was determined to be $1.2 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$.

We have not undertaken detailed electrochemistry of AcFIdC and AcFIdD, owing to the limited amount of material available. However, preliminary experiments under the conditions used for AcFIdA and AcFIdB indicate that they do not undergo reversible electrochemistry. AcFIdC gave a very slight response. However, the unstable and irreproducible nature of this response made it difficult to make any conclusions, except that the potential of the observed wave was at approx. -500 mV. AcFIdD showed no electroactivity.

DISCUSSION

These data allow two important conclusions. First, *A. chroococcum* (MCD1155) contains two genes that code for flavodoxins with different amino acid sequences and midpoint potentials. Only one of these flavodoxins is competent to transfer electrons to nitrogenase. Secondly, direct electrochemistry of these flavodoxins can be achieved at a polished edge-plane graphite surface if the aminoglycoside neomycin is present as a promoter.

Although Klugkist *et al.* (1986) separated three flavodoxins from the related organism *Azotobacter vinelandii* (A.T.C.C. 478), they provided no direct evidence that these proteins were genetically distinct. They showed that the proteins, designated AvFIdI, AvFIdII and AvFIdIII, yielded different peptide fragments on partial proteolysis and were immunologically distinct, but no sequence data were presented. They also showed that only AvFIdI and AvFIdII were synthesized under the conditions of N_2 fixation employed in the present work with *A. chroococcum* (MCD1155). The *N*-terminal sequences and proteolysis data presented above unequivocally show that AcFIdA and AcFIdB are the products of different genes. However, it is likely that AcFIdA and AcFIdB are equivalent to AvFIdI and AvFIdII, since they have similar E_m values, namely -305 mV and -320 mV, and -520 mV and -500 mV, respectively with only AcFIdB and AvFIdII competent to donate electrons to nitrogenase. In addition, the first 20 *N*-terminal residues of AcFIdB are identical with those of the only flavodoxin isolated to date from *A. vinelandii* OP Berkeley) and sequenced by Tanaka *et al.* (1977). These residues also correspond to those of the *nifF* gene product of *A. vinelandii* OP identified from a DNA sequence by Bennett *et al.* (1988). These data strongly suggest that AcFIdB is the *nifF* gene product of *A. chroococcum* (MCD1155).

Although AcFIdB and AcFIdC have identical *N*-terminal sequences, they can be resolved by f.p.l.c., suggesting that AcFIdC is a modified form of AcFIdB. This may be the result of partial denaturation or modification by phosphorylation. AvFIdII from *A. vinelandii* (A.T.C.C. 478) has no protein-bound phosphate other than that of the FMN (Klugkist *et al.*, 1986), whereas the flavodoxins from *A. vinelandii* (OP 13705) and *A. vinelandii* (OP Berkeley) have an additional covalently bound phosphate (Edmonson & James, 1979; Boylan & Edmonson, 1990).

The sequence data also show that AcFIdD is a mixture of either AcFIdB or AcFIdC (identical sequences) and an unknown protein which may not be a flavodoxin. Since AcFIdC and AcFIdD were present in relatively small amounts (about 5% of the total flavodoxin content) and were not reproducibly electrochemically active, we have not attempted to characterize them further.

The reversible oxidation–reduction of AcFIdA and AcFIdB (SQ–HQ couple) at a polished edge-plane graphite electrode in the presence of neomycin is, to our knowledge, the first successful direct electrochemistry reported for flavodoxins. The aminoglycoside neomycin provides a semi-rigid array of positive charges at pH 7.4 that presumably allows the formation of a tertiary complex involving the negatively charged, partially oxidized

graphite electrode surface (carboxylate groups), the flexible cationic promoter and the flavodoxin. Within this tertiary complex, fast, reversible, electron transfer between AcFIdA or AcFIdB and the graphite electrode occurs. The potential scan rates yield rate constants (k_s) of $2 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$ and $1.2 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$ respectively for the quasi-reversible electrochemical response. These heterogeneous electron-transfer rates compare favourably with those reported for other low-molecular-mass redox proteins such as cytochrome *c* ($5 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1}$) and plastocyanin ($7 \times 10^{-2} \text{ cm} \cdot \text{s}^{-1}$) (Armstrong *et al.*, 1987).

We were surprised that the multivalent cation, $\text{Cr}(\text{NH}_3)_6^{3+}$, did not promote electron transfer between AcFIdA or AcFIdB and the polished graphite electrode. $\text{Cr}(\text{NH}_3)_6^{3+}$ has been shown to be an excellent promoter of the direct electrochemistry of most negatively charged redox proteins and has recently been used successfully with the enzyme *p*-cresol methylhydroxylase (molecular mass 115 kDa) (Guo *et al.*, 1989). The selective response of these flavodoxins with respect to neomycin at the edge-plane of graphite gives some insight into the specific binding nature of the negatively charged domain of the protein that interacts with the electrode surface. Presumably neomycin allows a sufficient degree of macromolecular recognition because of its flexibility and ability to contact the protein at several points on its surface, thereby allowing a close approach of the prosthetic FMN group to the electrode surface. We suggest that $\text{Cr}(\text{NH}_3)_6^{3+}$ is not able to induce electron transfer between the flavodoxin and the graphite electrode, presumably because, as a relatively rigid multivalent cation, it causes the positively charged protein patch surrounding the FMN, identified by Drummond (1985) by sequence comparisons, to be repelled from the electrode surface. We anticipated that this positive patch would interact directly with the negatively charged surface of the polished graphite electrode in the absence of promoters. The failure for this to occur is attributable either to this domain not being involved in binding before electron transfer or, more likely, to the inability of the rather non-specific graphite electrode surface to select for this potential binding domain.

Direct electron transfer involving the oxidized-SQ couple of these flavodoxins has not been achieved under any conditions. This is consistent with previous studies on smaller flavodoxins and is attributable to the large conformational reorganization involved in the interconversion of these redox states (Van Leeuwen *et al.*, 1983).

The ability to perform direct electrochemistry on these flavodoxins is important not only in terms of understanding the mechanism of electron transfer in biological systems, but also in terms of developing devices such as 'biosensors' that often require electrodes to be 'coupled' to enzymes that do not themselves interact directly within the electrode. The wide range of E_m values exhibited by naturally occurring flavodoxins (Sykes & Rogers, 1984), their low molecular masses and the facility with which cassette mutagenesis could be applied to the existing DNA clones encoding flavodoxins from *A. vinelandii*, *A. chroococcum* and *K. pneumoniae* make these proteins particularly interesting for future electrochemical investigation. The possibility of 'driving' nitrogenase at an electrode using a flavodoxin mediator has not escaped our attention.

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