Electron transfer to nitrogenase

Characterization of flavodoxin from *Azotobacter chroococcum* and comparison of its redox potentials with those of flavodoxins from *Azotobacter vinelandii* and *Klebsiella pneumoniae* (*nifF*-gene product)

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Flavodoxin in the hydroquinone state acts as an electron donor to nitrogenase in several nitrogen-fixing organisms. The mid-point potentials for the oxidized–semiquinone and semiquinone–hydroquinone couples of flavodoxins isolated from facultative anaerobe *Klebsiella pneumoniae* (*nifF*-gene product, KpFld) and the obligate aerobe *Azotobacter chroococcum* (AcFld) were determined as a function of pH. The mid-point potentials of the semiquinone–hydroquinone couples of KpFld and AcFld are essentially independent of pH over the range pH 7–9, being -422 mV and -522 mV (normal hydrogen electrode) at pH 7.5 respectively. The mid-point potentials of the quinone–semiquinone couples at pH 7.5 are -200 mV (KpFld) and -133 mV (AcFld) with $\Delta E_m/\text{pH}$ of -65 ± 4 mV (KpFld) and -55 ± 2 mV (AcFld) over the range pH 7.0–9.5. This indicates that reduction of the quinone is coupled to protonation to yield a neutral semiquinone. The significance of these values with respect to electron transport to nitrogenase is discussed. The amino acid compositions, the *N*- and *C*-terminal amino acid sequences and the u.v.–visible spectra of KpFld and AcFld were determined and are compared with published data for flavodoxins isolated from *Azotobacter vinelandii*.

INTRODUCTION

A flavodoxin, the product of *nifF* gene in the facultative anaerobe *Klebsiella pneumoniae*, mediates electron transfer from the *nifJ*-gene product, a pyruvate : flavodoxin oxidoreductase, to the Fe protein of nitrogenase (Kp2) (Scheme 1) (Hill & Kavanagh, 1980; Nieva-Gomez *et al.*, 1980; Shah *et al.*, 1983). The *nifF* gene has recently been cloned and a strain of *K. pneumoniae* constructed that synthesizes and retains an elevated concentration of flavodoxin (Deistung *et al.*, 1985). This flavodoxin was unambiguously shown to be the product of the *nifF* gene by a comparison of its amino acid composition and *N*- and *C*-terminal amino acid sequences with those predicted from the DNA sequence determined by Drummond (1985) (Deistung *et al.*, 1985).

The electron-transport chain to nitrogenase in the obligate aerobe *Azotobacter chroococcum* is less well understood. A gene analogous to *nifJ* in *K. pneumoniae* has not been identified in *A. chroococcum* (Evans *et al.*, 1985). This is consistent with the extremely low pyruvate-supported acetylene-reduction activity of *A. chroococcum* crude extracts prepared from de-repressed cultures (Yates & Daniel, 1969). However, evidence for

a *nifF* gene analogue in *A. chroococcum* has been obtained by using DNA hybridization to a *K. pneumoniae* *nifF*-gene probe (Evans *et al.*, 1985). The synthesis of *A. chroococcum* flavodoxin is repressed by NH_4^+ ions, suggesting that it is a *nif*-specific gene product (R. Robson & R. Jones, personal communication).

Yates (1972) partially purified this flavodoxin and showed that in the hydroquinone state it could effect a single electron transfer to the oxidized Fe protein of *A. chroococcum* nitrogenase, flavodoxin in the semiquinone state being a product of this reaction.

Benemann *et al.* (1969, 1971) and Van Lin & Bothe (1972) showed that a flavodoxin was necessary for electron transport from NADPH to nitrogenase in *A. vinelandii* (strain OP Berkeley). Scherings *et al.* (1977), Hageman & Burris (1978) and Klugkist *et al.* (1985) observed that, under certain conditions, flavodoxin stimulates dithionite-supported acetylene reduction by purified *A. vinelandii* nitrogenase. Mechanisms to explain this observation have been discussed by Hageman & Burris (1978) and Thorneley & Lowe (1984b).

The situation in *A. vinelandii* (strain A.T.C.C. 478) is further complicated by the presence of three different flavodoxins (Klugkist *et al.*, 1985, 1986). Flavodoxin I

Scheme 1. Electron-transport chain in *K. pneumoniae*

Abbreviations used: NHE, normal hydrogen electrode; Ac, *Azotobacter chroococcum*; Av, *Azotobacter vinelandii*; Kp, *Klebsiella pneumoniae*; Cp, *Clostridium pasteurianum*; Fld, flavodoxin; the nitrogenase components from various organisms are denoted by the numbers 1 (MoFe-containing protein, e.g. Kp1) and 2 (Fe-containing protein, e.g. Kp2).

has a mid-point potential for the semiquinone–hydroquinone couple of -320 mV (NHE), which is too positive for this protein to be an effective electron donor to nitrogenase. Flavodoxins II and III have $E_m = -500$ mV, which is similar to the values reported by Yoch (1972) and Watt (1979) for a flavodoxin isolated from *A. vinelandii* (strain OP). All three flavodoxins are present in cells grown on ammonium acetate, but under N_2 -fixing conditions only flavodoxins I and II are present. Klugkist *et al.* (1986) suggest that the semiquinone form of flavodoxin II is reduced by a cytoplasmic membrane-bound NADPH dehydrogenase complex and that it is this flavodoxin, in its hydroquinone state, that reduces the oxidized form of the Fe protein of *A. vinelandii* nitrogenase.

In the present paper we present the amino acid composition and the C- and N-terminal amino acid sequences of a flavodoxin from *A. chroococcum*. These data are potentially useful for identifying the gene that codes for this flavodoxin by either direct comparison with DNA sequence data or by the synthesis of oligonucleotide DNA probes. A comparison of the structure and function of the *nif* genes and their products in *A. chroococcum* (Jones *et al.*, 1984; Kennedy *et al.*, 1985) with those of *K. pneumoniae* (Dixon, 1984) is important for understanding how the apparent incompatibility of O_2 and N_2 fixation is resolved in obligate aerobes. In addition, an alternative N_2 -fixation system that functions under Mo-deficient conditions has been identified in *Azotobacter vinelandii* (Bishop & Eady, 1985; Chisnell & Bishop, 1985; Hales *et al.*, 1985) and *A. chroococcum* (Kennedy *et al.*, 1985). In *A. chroococcum* the alternative system utilizes vanadium instead of molybdenum and an Fe-containing protein that is similar to, but genetically distinct from, Ac2 (Robson *et al.*, 1986). The electron-transport systems in these organisms are therefore of particular interest. We also present u.v.-visible spectroscopic data and mid-point potentials as a function of pH for flavodoxins from *K. pneumoniae* and *A. chroococcum*. These data are not only relevant to understanding structure–function relationships in flavodoxins in general (Ludwig *et al.*, 1984; Sykes & Rogers, 1984; Drummond, 1985, 1986), but are prerequisites for detailed kinetic investigations *in vitro* of the mechanism of electron transfer to *K. pneumoniae* and *A. chroococcum* nitrogenases from their natural electron donors. We have therefore obtained these data under the same conditions (23°C , pH 7.5) previously used in this laboratory to study the kinetics and mechanisms of nitrogenase action (Thorneley & Lowe, 1983, 1984a,b, 1985; Lowe & Thorneley, 1984a,b).

MATERIALS AND METHODS

Flavodoxin preparation and characterization

A flavodoxin (*nifF*-gene product) from *K. pneumoniae* (strain U.N.F. 5112) was purified as previously described (Deistung *et al.*, 1985). The procedure of Yates (1972) with an additional preparative gel-electrophoresis step (Deistung *et al.*, 1985) was used to purify a flavodoxin from *A. chroococcum* (strain N.C.I.M.B. 8003).

The amino acid composition and the N-terminal and C-terminal amino acid sequence analyses of the Ac flavodoxin were determined as previously described for the Kp flavodoxin (Deistung *et al.*, 1985). FMN was

shown to be the prosthetic group in both flavodoxins by paper chromatography by the method of Kilgour *et al.* (1957). This method allows a clear distinction to be made between FMN (R_F 0.76) and FAD (R_F 0.58).

U.v.-visible absorption spectra were recorded with a Perkin-Elmer Lambda-5 spectrophotometer, thermostatically maintained at 23°C in quartz cuvettes (1 cm path length). Strict anoxia was maintained by preparing solutions and filling the cuvette inside a glove-box through which N_2 gas was circulated via catalyst columns (BASF R311) to maintain the O_2 concentration at less than 1 p.p.m. as monitored by a coulometer (Chandos Intercontinental, Stockport, Cheshire, U.K.).

All biochemicals were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K., and salts from BDH Chemicals, Poole, Dorset, U.K.

Measurement of oxidation–reduction potentials

Oxidized–semiquinone couple. The oxidation–reduction potential for the oxidized flavodoxin–flavodoxin semiquinone couple at pH 7.0 was determined by the method of Mayhew (1971). Experiments were performed at 23°C on mixtures of flavodoxin ($10\ \mu\text{M}$) and indigo-5,5'-disulphonate [$E_m = -125$ mV (NHE)] in 25 mM-Hepes buffer, pH 7.0, in the presence of 30 mM-EDTA. This mixture was exposed to visible light for short intervals of time (approx. 5 min). After each period of illumination, the system was allowed to equilibrate in darkness and the percentage reduction of flavodoxin and dye was determined spectrophotometrically at 460 nm (an isobestic point between the oxidized and reduced forms of indigo-5,5'-disulphonate). The extent of dye reduction was calculated from the decrease in absorption at 610 nm after correction for the absorption of flavodoxin semiquinone at this wavelength (Mayhew *et al.*, 1969).

Oxidation–reduction potentials for the oxidized flavodoxin–semiquinone couple at pH 8.3 and above were determined spectrophotometrically essentially as described by Vetter & Knappe (1971) and Yoch (1972) by equilibration of oxidized benzyl viologen [$E_m = -359$ mV (NHE)] ($5\text{--}30\ \mu\text{M}$) with flavodoxin semiquinone ($20\ \mu\text{M}$). Flavodoxin semiquinone was prepared by stoichiometric reduction of the oxidized flavodoxin with $\text{Na}_2\text{S}_2\text{O}_4$ that had been standardized by titration with $\text{K}_3\text{Fe}(\text{CN})_6$. The reduction was monitored spectrophotometrically at 580 nm to ensure complete formation of flavodoxin semiquinone. SO_3^{2-} ions then removed from the flavodoxin semiquinone by anaerobic gel filtration on Sephadex G-25 equilibrated in 25 mM-Hepes buffer, pH 8.3 or pH 8.5, or 25 mM-Tris buffer, pH 9.5.

Semiquinone–hydroquinone couple. Oxidized flavodoxin solutions (1 ml; approx. $10\ \mu\text{M}$) were standardized spectrophotometrically by using the molar absorption coefficients given in Table 3. An excess of $\text{Na}_2\text{S}_2\text{O}_4$ (0.45–9 mM) was used to reduce the flavodoxin to its hydroquinone state. Portions of Na_2SO_3 (1 μl ; 2 M) were added to the cuvette anaerobically, and the degree of oxidation to the semiquinone state was monitored at 580 nm. Na_2SO_3 concentrations in the range 0–100 mM were used. The effect of ionic strength on the redox equilibrium was determined by the addition of Na_2SO_4 . The redox potential, set principally by the dithionite–bisulphite couple, was determined in parallel experiments in which methyl viologen ($50\ \mu\text{M}$) substituted for flavodoxin. A molar absorption coefficient for the methyl

Table 1. Amino acid compositions of flavodoxins from N₂-fixing organisms

Amino acid	<i>K. pneumoniae</i>		<i>A. chroococcum</i>	<i>A. vinelandii</i>
	Composition from purified flavodoxin (Deistung <i>et al.</i> , 1985)	Composition from gene sequence (Drummond, 1985)	Composition from purified flavodoxin (present work)	Composition from purified flavodoxin sequence (Tanaka <i>et al.</i> , 1977)
Asx	20.4*	20	21.3	20
Thr	8.6	10	9.9	8
Ser	11.2	12	13.0	15
Glx	18.4†	18	21.7	21
Pro	7.6	7	5.3	5
Gly	16.7	20	19.9	20
Ala	15.6	15	14.7	14
Cys + Val	8.8	9	10.5	11
Met	3.1	4	2.2	1
Ile	10.2	8	5.3	6
Leu	20.1	20	18.4	20
Tyr	3.0	3	2.8	5
Phe	9.1	10	13.6	13
His	N.D.‡	1	1.2	—
Lys	7.3	7	10.8	12
Arg	8.0	8	5.3	5
Trp	3	3	N.D.‡	3
		Total 175		Total 175

* In Deistung *et al.* (1985) it was not made clear that this refers to the sum of aspartic acid and asparagine residues.

† In Deistung *et al.* (1985) it was not made clear that this refers to the sum of glutamic and glutamine residues.

‡ Not determined.

Table 2. N- and C-Terminal amino acid sequences of flavodoxins from N₂-fixing organisms

Source	N-Terminal amino acid residue	C-Terminal amino acid residues	References
<i>K. pneumoniae</i> (<i>nifF</i> -gene product)	Ala	Leu-Val-Thr-Arg	Deistung <i>et al.</i> (1985), Drummond (1985)
<i>A. chroococcum</i>	Ala	Leu-Ser-Leu-Gly-Phe	Present work
<i>A. vinelandii</i>	Ala	Leu-Ser-Leu-Gly-Phe	Tanaka <i>et al.</i> (1977)

viologen monocation $\epsilon = 1.3 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 604 nm (Thorneley, 1974) and a pH-independent value for the mid-point potential for the methyl viologen dication-monocation couple of -446 mV (NHE) (Mayhew, 1978) were used to calculate the potentials. A PDP1134 computer and a program using NAG subroutine E04 JBF was used to obtain least-squares fits of the data to the Nernst equation.

RESULTS AND DISCUSSION

The amino acid composition for Ac flavodoxin is given in Table 1 together with previously published data for Kp and Av flavodoxins. The C- and N-terminal amino acid sequences are given in Table 2. These data should help in the identification of the genes that code for these proteins. Although the characterization of *nif* genes in *K. pneumoniae* is at a relatively advanced stage (Dixon, 1984), the analysis in *A. chroococcum* and *A. vinelandii* has only recently commenced (Kennedy *et al.*, 1985). The identical N- and C-terminal amino acid sequences and similar amino acid compositions and mid-point potentials (see below) for Ac and Av flavodoxins indicate a

high degree of structural and functional homology. Drummond (1985) used the graphics program DIAGON (Staden, 1982) to compare the amino acid sequences of five flavodoxins. A high degree of sequence homology between Kp and Av flavodoxins was demonstrated (sequence data for Ac flavodoxin are not available). This result is perhaps surprising in that the mid-point potentials for the semiquinone-hydroquinone couple of these flavodoxins differ by approx. 100 mV (see below). The 'tuning' of the redox potential of the FMN cofactor in these proteins is therefore achieved by relatively small changes in the amino acid sequence that have yet to be identified.

The u.v.-visible spectral characteristics of Ac and Kp flavodoxins are given in Table 3, and the spectra of the oxidized, semiquinone and hydroquinone forms of Ac flavodoxin in Fig. 1. The spectra of the corresponding forms of Kp flavodoxin are very similar to those for Ac flavodoxin, with only the minor differences indicated in Table 3. The isosbestic points at 410 nm (Kp flavodoxin) and 417 nm (Ac flavodoxin) between the semiquinone and hydroquinone forms are potentially useful for monitoring absorption changes in the Fe protein of

Table 3. U.v.-visible-absorption-spectra characteristics

Source	Wavelength (nm)	Molar absorption coefficient ($\text{mm}^{-1} \cdot \text{cm}^{-1}$)			Reference
		Oxidized	Semiquinone	Hydroquinone	
<i>K. pneumoniae</i> (<i>nifF</i> -gene product)	Maxima 340	7.1	8.0	4.9	Present work
	370	9.6	5.9	4.3	
	454	10.1	2.9	1.3	
	580	—	4.1	—	
	Isosbestic points 352	8.8		—	
<i>A. chroococcum</i>	410	—	—	2.4	Present work
	502	2.7	—	—	
	Maxima 350	7.7	8.0	5.4	
	370	9.0	5.2	5.5	
	452	10.0	2.6	1.6	
<i>A. vinelandii</i> flavodoxin II	580	—	5.2	—	Klugkist <i>et al.</i> (1986)
	Isosbestic points 358	8.5		—	
	417	—	—	1.8	
	506	3.0	—	—	
	Maxima 452	11.3	3.0	1.5	
	581	—	5.7	—

Fig. 1. U.v.-visible spectra of *A. chroococcum* flavodoxin

—, Oxidized (quinone) state; — · —, semiquinone state; —, hydroquinone (quinol) state. The spectra were recorded with $[\text{AcFld}] = 33 \mu\text{M}$ at pH 8.0 in 25 mM-Hepes buffer at 23 °C in a 1 cm-path-length cell. Molar absorption coefficients are given in Table 3. The corresponding spectra of Kp flavodoxin are very similar, with the small differences in peak position and molar absorption coefficients given in Table 3.

nitrogenases on electron transfer from the hydroquinone form of flavodoxins.

Fig. 2 shows redox titration curves for Kp and Ac flavodoxins that are typical of those used to determine the mid-point potentials (E_m) for the semiquinone-hydroquinone couple given in Table 4 and summarized in Fig. 3. There was excellent agreement between the limiting absorbance at 580 nm corresponding to complete formation of flavodoxin semiquinone (calculated from

Fig. 2. Potentiometric titrations of Kp and Ac flavodoxins at pH 7.5 at 23 °C

The potential was varied by increasing $[\text{NaHSO}_3]$ from 0 to 100 mM with $[\text{Na}_2\text{S}_2\text{O}_4] = 0.72 \text{ mM}$ for KpFld and 0.9 mM for AcFld. The potential at each NaHSO_3 concentration was independently determined with methyl viologen as described in the Materials and methods section. $[\text{AcFld}] = 11 \mu\text{M}$; $[\text{KpFld}] = 11 \mu\text{M}$. The curves are computed best fits to the data points for KpFld (●) and AcFld (■) with mid-point potentials for the semiquinone-hydroquinone couple $E_m = -422 \text{ mV}$ and -522 mV respectively. The broken lines show the limiting absorbance calculated from the ϵ_{580} (Table 3) and flavodoxin concentration assuming complete oxidation to the semiquinone state. Data and E_m values at other pH values are given in Table 4 and summarized in Fig. 3.

the spectral data in Table 3) with that computed from the data points at lower potentials by using the Nernst equation. Allowing the n value in the Nernst equation to float caused E_m to shift by at most 4 mV with a deviation of 0.1 in the value of n . A disadvantage of

Table 4. Redox potentials for the flavodoxins from *K. pneumoniae* and *A. chroococcum* at 23 °C

pH	E_m [mV (NHE)]	
	<i>K. pneumoniae</i>	<i>A. chroococcum</i>
Oxidized-semiquinone couple*		
7.0	-158 ± 2	-115 ± 2
8.0	-228 ± 3	—
8.3	-247 ± 3	—
8.5	—	-198 ± 3
9.5	-322 ± 2	-253 ± 3
Semiquinone-hydroquinone couple†		
7.0	-412	—
7.5	-422	-522
8.0	-425	-524
8.5	—	-518
9.0	—	-512

* Values are averages for three independent determinations. Least-squares analysis gives $\Delta E_m/\Delta \text{pH} = -65 \pm 4$ for Kp flavodoxin and -55 ± 2 mV for Ac flavodoxin. The data in Table 4 are summarized in Fig. 3.

† Computed best fit to Nernst equation with $n = 1.0$.

the dithionite-bisulphite couple is that with $[\text{NaHSO}_3] < 100$ mM the maximum potential that can be obtained is approx. -400 mV (NHE). This results in partial titration curves owing to incomplete oxidation of the hydroquinone form, particularly at the higher pH values with Kp flavodoxin. However, we consider this to be preferable to the use of dye mediators and a platinum electrode because of the uncertainties associated with dye binding to the protein and absorption of both dyes and protein on the surface of the platinum electrode. It is also possible to study the kinetics of oxidation-reduction of flavodoxins and the Fe protein of nitrogenases by using $\text{S}_2\text{O}_4^{2-}$ and SO_3^{2-} ions. These kinetic data can be used to validate the mid-point potentials presented in the present paper. However, the procedure used involves an increase in ionic strength with increasing potential. Therefore the effect of high ionic strength was investigated at one potential close to E_m on each titration curve. Na_2SO_4 , added such that $[\text{HSO}_3^-/\text{SO}_3^{2-} + \text{SO}_4^{2-}] = 100$ mM with $[\text{HSO}_3^- + \text{SO}_3^{2-}] = 4\text{--}30$ mM, depending on pH, did not change the absorbance at 580 nm, indicating no detectable ionic-strength effect on the redox equilibrium. This is consistent with the excellent fit of the data points to the Nernst equation.

The pH-dependences of the mid-point potentials for the oxidized-semiquinone couple of Kp and Ac flavodoxins are given in Table 4 and summarized in Fig. 3. The values of $\Delta E_m/\Delta \text{pH}$ of -65 ± 4 mV (Kp) and -55 ± 2 mV (Ac) are close to the theoretical value of -59 mV if reduction of the oxidized form is coupled to a protonation to yield a neutral semiquinone form (Mayhew & Ludwig, 1975). The mid-point potentials of the oxidized-semiquinone couples are too positive for the semiquinone to be a likely donor to nitrogenase (see below). The only plausible way in which oxidized flavodoxin might be involved in N_2 fixation would be if the hydroquinone form donated two electrons to nitrogenase, an unlikely event since the consensus view is that the Fe protein of nitrogenase undergoes one-electron oxidation-reduction in its catalytic cycle (Stephens, 1985). Although the oxidized-semiquinone mid-point potentials for Ac flavodoxin are about 50 mV more positive than those for Kp flavodoxin, the

semiquinone-hydroquinone couple of Ac flavodoxin is about 100 mV more negative than that of Kp flavodoxin (Fig. 3). This suggests that the modulation of mid-point potential is achieved primarily by modification of interactions between the polypeptide and FMN in the semiquinone form. Stabilization of this state will lower the E_m for the semiquinone-hydroquinone couple and increase the E_m for the oxidized-semiquinone couple. Ludwig *et al.* (1984) have discussed how this might occur in *Clostridium pasteurianum* MP and *Anacystis nidulans* flavodoxins by a comparison of X-ray crystal-structure data.

The pH-independent behaviour of the semiquinone-hydroquinone couple for Ac and Kp flavodoxins over the range pH 7–9 is consistent with the formation of the hydroquinone anion. Protonation of this form occurs with pK_a of 5.8 (*Megasphaera elsdenii*) and 6.7 (*C. pasteurianum*) and is associated with an increase in mid-point potential as the pH is decreased from 7 to 5 (Mayhew & Ludwig, 1975). No attempt was made to study this phenomenon with Kp and Ac flavodoxin because of the instability of the dithionite-bisulphite couple in this pH range. Studies with nitrogenase are usually at above pH 7.0.

The mid-point potential for the semiquinone-hydroquinone couple of the Ac flavodoxin (-522 mV, pH 7.5) is close to the values of -500 ± 10 mV (pH 7.0) for flavodoxin II from *A. vinelandii* (strain A.T.C.C. 578) (Klugkist *et al.*, 1986) and -515 mV (pH 8) for a flavodoxin from *A. vinelandii* (strain OP) (Watt, 1979). These potentials are about 100 mV more negative than that of Kp flavodoxin (-422 mV, pH 7.5). Since flavodoxin acts as an electron carrier, its mid-point potential is presumably optimized between those of the electron donor and the Fe protein of nitrogenase. Lowe & Thorneley (1984a) showed that with $\text{Na}_2\text{S}_2\text{O}_4$ as the electron donor it is $\text{Kp}_{2\text{ox}}(\text{MgADP})_2$ that is reduced in the catalytic cycle. The low mid-point potentials of the Av and Ac flavodoxins suggest that this is also the case *in vivo*, where flavodoxin is the electron donor. Watt (1985) reported mid-point potentials of -310 mV for Av2, -425 mV for Av2(MgATP)₂ and -480 mV for Av2(MgADP)₂. Thus a low-potential flavodoxin

Fig. 3. Mid-point potentials for the oxidized-semiquinone and semiquinone-hydroquinone couples of flavodoxins from *K. pneumoniae* (*nifF*-gene product) and *A. chroococcum* at 23 °C

▲, *A. chroococcum* flavodoxin; ●, *K. pneumoniae* flavodoxin. The data are given in Table 4 and the procedures used described in the Materials and methods section.

($E_m < -500$ mV) is only required if $\text{Av}_{2\text{ox}}(\text{MgADP})_2$ is the electron acceptor. E_m values for $\text{Ac}_{2\text{ox}}$ have not been reported. Equilibration of Kp flavodoxin hydroquinone with $\text{Kp}_{2\text{ox}}(\text{MgADP})_2$, followed by e.p.r. monitoring of the $g = 1.95$ feature, gave a mid-point potential of -398 mV (pH 7.5) for $\text{Kp}_{2\text{ox}}(\text{MgADP})_2$. Thus a flavodoxin with a mid-point potential of -422 mV (pH 7.5) would be an effective electron donor in *K. pneumoniae*. The *K. pneumoniae* potentials are very similar to those of the corresponding proteins in *C. pasteurianum*, which has either a ferredoxin ($E_m = -420$ mV; Benemann & Valentine, 1972) or flavodoxin ($E_m = -419$ mV; Mayhew, 1971) as an electron donor to $\text{Cp}_{2\text{ox}}(\text{MgADP})_2$ ($E_m = -380$ mV; Zumft *et al.*, 1974). The detailed interpretation of E_m values for nitrogenase Fe proteins is complicated because (i) two interconvertible Fe-S cluster conformations with $S = \frac{1}{2}$ and $S = \frac{3}{2}$ spin systems exist (Lindahl *et al.*, 1985; Hagen *et al.*, 1985; Watt & McDonald, 1985) and (ii), E_m values obtained at low temperatures by using e.p.r. spectroscopy may differ from room-temperature values. However, it seems likely that the Kp2 and Cp2 and their electron donors operate at a potential close to -400 mV, whereas the *A. vinelandii* and *A. chroococcum* systems operate close to -500 mV. The origin and functional significance of this difference are intriguing,

since a high degree of sequence homology (60–70%) has been demonstrated between the nitrogenase structural genes from *A. vinelandii*, *A. chroococcum* and *K. pneumoniae* (Lowe *et al.*, 1985; Hennecke *et al.*, 1985).

On the basis of the mid-point potentials reported in the present paper, the *nifJ*-gene product, a flavodoxin: pyruvate oxidoreductase, should have a mid-point potential significantly lower than -420 mV for the efficient reduction of flavodoxin to the hydroquinone state in *K. pneumoniae*. Similarly the electron donor(s) to flavodoxin in *A. chroococcum* and *A. vinelandii* should have mid-point potentials below -515 mV.

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