Posttranslational Modification of *Klebsiella pneumoniae* Flavodoxin by Covalent Attachment of Coenzyme A, Shown by ^{31}P NMR and Electrospray Mass Spectrometry, Prevents Electron Transfer from the *nifJ* Protein to Nitrogenase. A Possible New Regulatory Mechanism for Biological Nitrogen Fixation

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Received August 1, 1991; Revised Manuscript Received October 10, 1991

ABSTRACT: A strain of *Escherichia coli* (71-18) that produces ca. 15% of its soluble cytoplasmic protein as a flavodoxin, the *Klebsiella pneumoniae nifF* gene product, has been constructed. The flavodoxin was purified using FPLC and resolved into two forms, designated KpFldI and KpFldII, which were shown to have identical N-terminal amino acid sequences (30 residues) in agreement with that predicted by the *K. pneumoniae nifF* DNA sequence. ^{31}P NMR, electrospray mass spectrometry, UV–visible spectra, and thiol group estimations showed that the single cysteine residue (position 68) of KpFldI is posttranslationally modified in KpFldII by the covalent, mixed disulfide, attachment of coenzyme A. KpFldII was inactive as an electron carrier between the *K. pneumoniae nifJ* product (a pyruvate–flavodoxin oxidoreductase) and *K. pneumoniae nifH* product (the Fe-protein of nitrogenase). This novel posttranslational modification of a flavodoxin is discussed in terms of the regulation of nitrogenase activity in vivo in response to the level of dissolved O_2 and the carbon status of diazotrophic cultures.

Klebsiella pneumoniae (oxytoca) flavodoxin [KpFld, M_r = 18 981 + 454 (FMN)] is a small low-potential [E_m = −422 mV (NHE) for the semiquinone–hydroquinone couple] flavoprotein (*nifF* gene product) that mediates electron transfer between a pyruvate–flavodoxin oxidoreductase (*nifJ* gene product) and the Fe protein of nitrogenase (*nifH* gene product) (Hill & Kavanagh, 1980; Nieva-Gomez et al., 1980; Shah et al., 1983; Drummond, 1986; Wahl & Orme-Johnson, 1987; Thorneley & Deistung, 1988). KpFld contains a single FMN prosthetic group (Deistung & Thorneley, 1986) and, of importance in the context of the present paper, a single cysteine residue, located from the DNA sequence of Drummond (1985) at position 68.

KpFld is present at very low concentrations in *K. pneumoniae*, even when growth is under N_2-fixing conditions (Hill & Kavanagh, 1980; Wahl & Orme-Johnson, 1987). Hence, for biochemical studies, it has been overexpressed previously in *Escherichia coli* (Deistung et al., 1985). However, the yields of KpFld from this construct were, by present standards, relatively poor (ca. 3% of the total soluble protein in crude extracts). We report in the present paper the construction of a new strain of *E. coli* that produces up to 15% of its soluble protein as KpFld. During the final stages of purification of this protein, originally destined for crystallization trials and kinetic studies, we separated two forms of KpFld by FPLC. We report below the characterization of these two forms (designated KpFldI and KpFldII) by several techniques, including ^{31}P NMR and electrospray mass spectrometry. We also show that KpFldI but not KpFldII is competent to couple the oxidation of pyruvate by the *nifJ* protein to the reduction of nitrogenase Fe protein. The posttranslational attachment of coenzyme A, by a mixed disulfide bond to Cys68, distinguishes KpFldII from KpFldI. This modification is discussed in terms of the possible regulation of biological nitrogen fixation in response to the dissolved oxygen concentration of *K. pneumoniae* cultures. Edmonson and James (1979) and Live and Edmonson (1988) used ^{31}P NMR to assign a posttranslational modification of *Azotobacter vinelandii* flavodoxin (AvFld) to a phosphodiester link between a serine and a threonine. Subsequent ^{32}P-labeling studies showed that the extent of phosphate incorporation did not depend on the growth conditions, hence a "structural" rather than a "regulatory" role was assigned to the modification (Boylan & Edmonson, 1990). The ^{32}P NMR data for AvFld obtained by Edmonson and co-workers are compared in the present paper with our data for KpFldII since it is clearly important to establish that they are different and are the result of distinct modifications.

Materials and Methods

Construction of E. coli Strain 71-18. The *nifF* coding sequence was cloned from pRD545 (Drummond, 1985) to the high copy number expression vector pDK6 (Kleiner et al., 1988) using standard techniques (Sambrook et al., 1989) to generate pSH219.

Growth of E. coli Strain 71-18. E. coli strain 71-18 carrying pSH219 was grown in a 200-L pilot plant fermenter (New Brunswick Scientific U.K., Hatfield, U.K.) at 37 °C in an L-broth medium containing kanamycin (30 $\mu g\ mL^{-1}$) to an A_{600} of 0.25 (1-cm path length) before addition of IPTG to a final concentration of 30 μM. Growth was continued for 16 h during which period flavodoxin appeared to accumulate in the cells during stationary phase, as judged by blue pigmentation of the culture. Cells were harvested using a Westfalia continuous centrifuge and stored frozen in liquid nitrogen.

Purification of KpFldI and KpFldII. A crude extract of *E. coli* strain 71-18 was prepared by homogenizing cell paste (2 kg) suspended in 2 L of Tris-HCl (25 mM, pH 7.5) using

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0006-2960/92/0431-1216$03.00/0

a Manton–Gaulin homogenizer at 4 °C followed by centrifugation of the resulting suspension in a Hi-Spin 21 centrifuge at 4 °C, for 3 h, at 9000 rpm.

A portion of the blue-green crude extract (750 mL, 50 mg of protein mL^{-1}) was loaded onto a DEAE-52 column (7 cm × 10 cm) equilibrated with Tris-HCl buffer (25 mM, pH 7.4, 4 °C) under aerobic conditions. Flavodoxins were eluted as a broad dark green band following a stepped gradient of NaCl (100, 150, 200, and 350 mM, 500 mL of each concentration). Freezing of green flavodoxin fractions caused their oxidation to give a yellow solution. Each fraction (500 mL) was concentrated on DEAE-52, eluted with Tris-HCl buffer (25 mM, pH 7.4, 4 °C) containing NaCl (350 mM), and then subjected to gel filtration on Sephacryl S-200 equilibrated with Tris-HCl buffer (25 mM, pH 7.4, 4 °C, 100 mM NaCl). Yellow flavodoxin fractions (ca. 100 mL) were concentrated on DEAE-52 at 4 °C and subjected to FPLC using a Hi-Load 26/10 Q-Sepharose high-performance anion exchange column (Pharmacia) at room temperature. Samples [10 mL, 40 mg mL^{-1}, diluted with Bis-Tris propane buffer (20 mM, pH 7.4) to 10 mg mL^{-1} to decrease the NaCl concentration from 350 to 90 mM in order to facilitate binding to the FPLC column] were loaded and then eluted with a three-stage KCl gradient (0–250 mM, 150 mL; 250–375 mM, 350 mL; 375–500 mM, 150 mL). KpFldI was eluted at 300 mM KCl, KpFldII at 340 mM KCl, and a third FMN-containing protein at 460 mM KCl. Purified samples from a series of runs were obtained, concentrated on DEAE [eluted in Tris-HCl buffer (25 mM, pH 7.4) containing NaCl (350 mM)], and, after characterization by UV–visible spectroscopy, stored frozen at −18 °C.

N-terminal Amino Acid Sequence Determination. The N-terminal amino acid sequences of KpFldI and KpFldII (30 residues) were determined as described previously in Bagby et al. (1991).

Thiol Group Estimation. The presence of one free thiol group in KpFldI (Cys68) and its absence in KpFldII was shown by a spectrophotometric determination based on the method of Riddles et al. (1983). A solution (0.9 mL) of guanidinium chloride (6.2 M), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 32 μM), and HEPES/NaOH (25 mM, pH 7.4) was added to either 0.1 mL of KpFldI and KpFldII (100 μM) or 0.1 mL of HEPES/NaOH buffer, and the absorbance at 412 nm determined. The free thiol group concentration was calculated using a $\Delta\epsilon_{412} = 13.7\ mM^{-1}\ cm^{-1}$.

In Vitro Interconversion of KpFldI and KpFldII by Addition and Removal of Coenzyme A. KpFldI (1 mM) was incubated at room temperature for 5 days with coenzyme A (2 mM) in Tris-HCl buffer (10 mL, 25 mM, pH 7.4) containing NaCl (350 mM) and NaN_3 (5 mM). The extent of conversion of KpFldI to KpFldII was determined by comparison of peak areas following spectrophotometric monitoring at 280 nm of the eluent after FPLC using a small-volume column (Hi-Load 16/10 Q-Sepharose) (see above for running conditions).

KpFldII (68 μM) isolated from *E. coli* and purified as described above was converted to KpFldI by incubation with dithiothreitol (6.5 mM, 1 mg mL^{-1}) in Bis-Tris propane/HCl buffer (1 mL, 20 mM, pH 8.0) at 30 °C for 24 h in a shaking water bath. The extent of conversion to KpFldI was estimated as described in the preceding paragraphs. In experiments designed to quantify the amount of coenzyme A formed, a 60-min incubation at pH 9.0 was used in order to minimize the decomposition and maximize the rate of release of coenzyme A from FldII. The amount of coenzyme A released was determined enzymatically using acetyl-CoA:orthophosphate acetyltransferase [EC 2.3.1.8.] (Michal & Bergmeyer, 1985) and also by integration of the FPLC peak after calibration of the system with coenzyme A standards.

"Synthetic" samples, designated KpFldI* and KpFldII*, were also characterized and compared with the two "natural" forms of KpFld isolated from *E. coli*.

UV–Visible Spectroscopy. Spectra were recorded using a Lambda-5 spectrophotometer (Perkin-Elmer, Beaconfield, U.K.) in quartz cuvettes, 1-cm path length, at 23 °C. Spectra of the semiquinone and hydroquinone forms of KpFldI and KpFldII were obtained by the addition of sodium dithionite under strictly anaerobic conditions, as previously described (Deistung & Thorneley, 1986).

^{31}P NMR. NMR spectra were recorded on a Jeol 270 GSX spectrometer, operating at 109.25 MHz in Fourier transform mode. Field/frequency lock was provided by the ^2H_2O signal. Relaxation time measurements obtained by using the inversion–recovery sequence allowed optimum pulse repetition times and pulse angles to be determined. Generally 2 s and 60° were used. This also allowed integrations to be quantitatively related. Exponential broadening factors were applied to $^{31}P\{^1H\}$ FID's, and proton-coupled spectra were also investigated using Gaussian resolution-enhancement functions. Spectra were recorded at room temperature in 10-mm o.d. tubes; sample heating was minimized using Waugh decoupling techniques. All chemical shifts are referenced to trimethyl phosphite at 0 ppm (downfield shifts positive).

Each flavodoxin sample was concentrated to between 1 and 8 mM using Centriprep-10 concentrators (Amicon Ltd., Stonehouse, U.K.). Each concentrated sample (1 mL) was then loaded onto a column of Bio-Gel P-6DG (Bio-Rad) (1 cm × 15 cm) equilibrated with HEPES/NaOH buffer (25 mM, pH 7.4), $MgCl_2$ (10 mM), or NaCl (100 mM) made up in ^2H_2O. Flavodoxin (3 mL), eluted from the column, was made 5 mM in NaN_3 to prevent degradation by microbial growth and stored frozen at −18 °C until required for NMR experiments. Further details are given in the legends to the appropriate figures and in the accompanying text.

Electrospray Mass Spectrometry. Samples of KpFldI and KpFldII (30 μM) prepared in HEPES/NaOH (25 mM, pH 7.5) were dialyzed against Tris-HCl (5 mM, pH 7.0) and diluted with an equal volume of water. Sample solutions (10 μL, 150 pmol) were injected into the carrier solvent stream (50:50 mixture of water/methanol containing 1% acetic acid, at a flow rate of 4 μL/min) of a VG BIO Q quadrupole mass spectrometer. The spectrometer scanned over a range of 700–1500 *m/e* and was calibrated with myoglobin. The results were analyzed and data printed using the software provided by the instrument manufacturer (VG Analytical, Manchester, U.K.).

Pyruvate–Flavodoxin–Nitrogenase Coupled Assay. Pyruvate–flavodoxin oxidoreductase from *K. pneumoniae* strain M5a1 (*nifJ* gene product) was partially purified from a crude extract obtained from 100 g of cell suspension disrupted in a French Press at 4 °C under strictly anaerobic conditions in Tris-HCl buffer (25 mM, pH 8.7) followed by centrifugation at 4 °C, for 1.5 h, at 13000 rpm. The crude extract (200 mL) was loaded onto DEAE-Sephacel (5 cm × 9 cm) equilibrated with Tris-HCl buffer (25 mM, pH 7.5) containing dithiothreitol (0.1 g L^{-1}), $Na_2S_2O_4$ (50 μM), and thiamine pyrophosphate (1 mM) and eluted with a stepped gradient of NaCl (150 mM, 300 mL; 200 mM, 200 mL; 250 mM, 200 mL). Yellow-brown fractions were combined and concentrated on DEAE-Sephacel followed by anaerobic gel filtration using Bio-Gel P-6DG (1 cm × 20 cm) equilibrated with Tris-HCl

a)

	EcoRI	XhoI	PstI	MluI	SstII	NotI	NcoI	NruI	NheI	ApaI	SstI	HindIII
pMD220	GAATTCCTCGAGCTGCAGACGCGTCCGCGGCGGCCGCCATGGTCGCGAGCTAGCGGGCCCGAGCTCAAGCTT											

b)

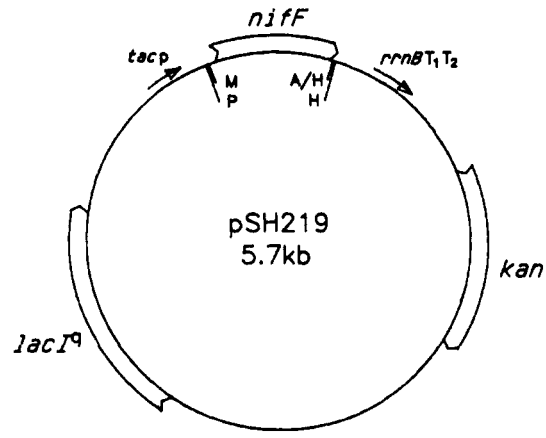

FIGURE 1: Construction of the *nifF* overproducing plasmid. (a) Polylinker of pMD220 into which a *Mlu*I–*Hpa*I fragment carrying *nifF* was cloned. (b) Structure of pSH219, *rrnB*T_1T_2 is a strong terminator from the *E. coli rrnB* operon which prevents potentially deleterious read-through from the *tac* promoter (*tac*p) into the rest of the replicon. A/H is the hybrid *Apa*I/*Hpa*I site, H is the *Hin*dIII site, M is the *Mlu*I site, and P is the *Pst*I site.

buffer (25 mM, pH 7.4) to remove $Na_2S_2O_4$ and dithiothreitol. Samples were stored frozen in bead form in liquid N_2. *K. pneumoniae* nitrogenase component proteins were purified as described previously (Eady et al., 1972).

Assays were performed in glass vials (7.8-mL volume) fitted with rubber "suba seal" closures and flushed with Ar. The inclusion of a glucose/glucose oxidase O_2-scavenging system (as described below) increased the reproducibility of these dithionite-free nitrogenase assays.

Each Ar-flushed assay vial contained, in a total volume of 1 mL, ATP (5 μmol), $MgCl_2$ (12 μmol), HEPES/NaOH buffer (25 μmol), creatine phosphate (10 μmol), creatine kinase (12 units), sodium pyruvate (5 μmol), thiamine pyrophosphate (24 nmol), coenzyme A (0.2 μmol), glucose (2.4 μmol), glucose oxidase (5.0 units) and was equilibrated in a shaking water bath at 23 °C before the addition of partially purified *nifJ* protein (50 μL, 16.8 mg mL^{-1}), acetylene (1 mL), and various volumes (1–15 μL) of KpFldI (780 μM) or KpFldII (440 μM). Assays were started by the addition of 20 μL of nitrogenase (mixture of Kp1 and Kp2 at 11.4 mg mL^{-1} and 5.8 mg mL^{-1}, respectively). Assays were stopped after 4- and 8-min reactions by the addition of trichloroacetic acid (0.1 mL, 30% w/v). Ethylene production was determined by taking a 0.5-mL gas sample for injection into a gas chromatograph as previously described (Eady et al., 1972).

Reagents. All biochemicals and enzymes were purchased from Sigma Chemical Co. (Poole, U.K.) or Boehringer Mannheim (Lewes, U.K.) unless otherwise specified. Salts were purchased from BDH Chemicals (Poole, U.K.), FSA Laboratory Supplies (Loughborough, U.K.), and Rhône Poulenc (Manchester, U.K.).

Results and Discussion

Overproduction of nifF Flavodoxin. The *nifF* gene of *K. pneumoniae* was cloned into the expression vector pDK6 (Kleiner et al., 1988), where it is transcribed from the *tac* promoter (Amann et al., 1983). The coding sequence was first excised on a 572 bp *Mlu*I–*Hpa*I fragment from the plasmid pRD545 (Drummond, 1985). The *Hpa*I site lies just 5 bp downstream from the *nifF* stop codon, while *Mlu*I cuts 38 bp upstream from the start codon, 6 bp downstream from the transcription start. The *Mlu*I–*Hpa*I fragment thus does not carry the *nifF* promoter but does include the native Shine–Dalgarno sequence. It was first cloned between the *Mlu*I and *Apa*I sites of pMD220 (Drummond, unpublished), an *αlacZ* complementing vector carrying the polylinker shown in Figure 1a. The 3′ extension was removed from the *Apa*I site using T4 DNA polymerase to allow ligation to the *Hpa*I site. A *Pst*I–*Hin*dIII fragment carrying *nifF* was then cloned from this construct to pDK6, yielding pSH219 (Figure 1b).

pDK6 carries *lacI*^{q}, which is necessary to repress transcription from the *tac* promoter, since the high copy number of this plasmid results in titration of the chromosomally encoded *lac* repressor. In *E. coli* 71-18 (Messing et al., 1977), IPTG added to 30 μM was sufficient to give complete derepression of *nifF* transcription. To maximize yields, cells were grown at 37 °C in L-broth with kanamycin at 30 μg/mL, IPTG was added to 30 μM when the cell density was about one-tenth of the final density, and incubation was continued overnight as KpFld continued to accumulate in stationary phase. Under this regime, flavodoxin comprised 10–15% of the total protein, as judged by densitometry of Coomassie blue stained SDS gels. Harvested cells were visibly pigmented blue-green, presumably reflecting a predominance of the semiquinone form of the flavodoxin.

Preparation and Interconversion of KpFldI and KpFldII. KpFldI was resolved from KpFldII using FPLC as described under Materials and Methods. Figure 2 shows a representative elution profile. The ratio of peak areas for KpFldI and KpFldII was 11:3, respectively, in the FPLC experiment shown. The salt concentration at which each flavodoxin eluted was subsequently used as a diagnostic for the interconversion of KpFldI and KpFldII.

Figure 2 also shows a third FMN-containing protein (designated X) that eluted from the FPLC at a high KCl concentration (460 mM). It has only been partially characterized since PAGE and electrospray mass spectroscopy showed it to have a $M_r \sim 28K$. ^{31}P NMR showed it to contain another phosphorus resonance in addition to that assigned to FMN. Since this protein is clearly not the *nifF* gene product

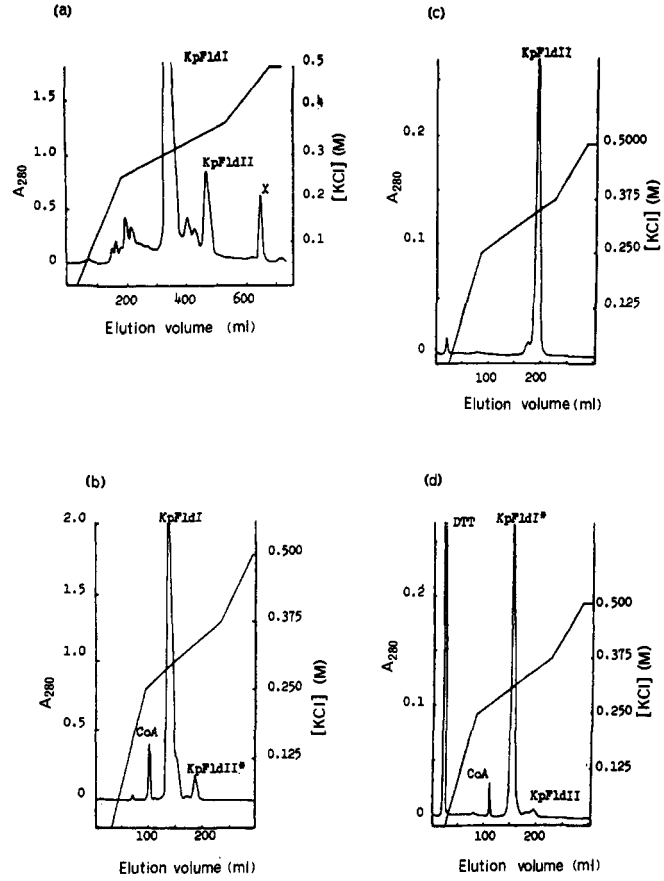

FIGURE 2: FPLC elution profiles for KpFldI, KpFldII, KpFldI*, and KpFldII*. FPLC trace a was obtained as described under Materials and Methods. Traces b, c, and d, with decreased elution volumes relative to trace a, were obtained with a smaller FPLC column (Hi-Load 16/10 Q-Sepharose). (a) Separation of KpFldI and KpFldII. A third FMN-containing protein designated "X" elutes at 460 mM KCl. (b) Conversion of KpFldI to ca. 5% KpFldII* by incubation with coenzyme A (2 mM) for 5 days, as described under Materials and Methods. (c) Purified KpFldII before treatment with dithiothreitol. (d) Essentially complete conversion of KpFldII to KpFldI* by incubation with dithiothreitol. Note the appearance of the free coenzyme A peak eluting at the same KCl concentration as the excess coenzyme A added in trace b.

and because it was isolated in variable amounts comprising between 1 and 6% of the total flavodoxin content, we do not wish to comment further on its structure or function.

After it had been established that the difference between KpFldI and KpFldII was the posttranslational modification of Cys68 by mixed disulfide formation with coenzyme A (see below), attempts were made to convert KpFldI into KpFldII by incubation with coenzyme A and KpFldII to KpFldI by incubation with dithiothreitol. These reactions were performed as described under Materials and Methods, and confirmation that the interconversion had been achieved was initially obtained by FPLC. Figure 2b shows the formation of KpFldII* (where the asterisk indicates the synthetically modified flavodoxin) after incubation of KpFldI with coenzyme A. The elution profile of KpFldII* (peak at 340 mM KCl) was identical with that of the KpFldII isolated directly from *E. coli* (Figure 2a). Integration of the peak areas showed that 5% of the KpFldI initially present was converted to KpFldII* over the 5-day period of incubation. The extreme slowness of this reaction makes it very unlikely that the attachment of coenzyme A to KpFldI is an artifact occurring spontaneously during the isolation and purification of flavodoxin from the crude extract of *E. coli* strain 71-18. Samples of KpFldII* were combined and concentrated and subjected to ^{31}P NMR,

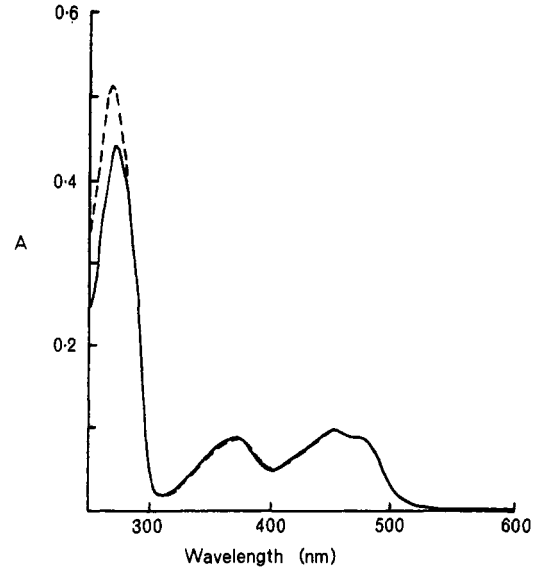

FIGURE 3: UV–visible spectra for oxidized KpFldI and KpFldII. Spectra were obtained as described under Materials and Methods. The spectra are identical in the visible region, indicating that covalent attachment of the coenzyme A does not perturb the FMN. The shift in the UV maximum from 272 nm (KpFldI) (—) to 268 nm (KpFldII) (---) with enhanced absorption is attributed to the adenosine group of coenzyme A. Conditions: [KpFldI] = [KpFldII] = 10 μM, HEPES/NaOH (25 mM, pH 7.4), 23 °C. Extinction coefficients are given in Table I.

Table I: UV–Visible Absorption Spectra Characteristics of KpFldI and KpFldII

wavelength (nm)	molar absorption coefficient (mM^{-1} cm^{-1})[a] oxidized	semi-quinone	hydro-quinone
maxima			
268	– (54.0)	– –	– –
272	46.5 –	– –	– –
340	7.0 (6.7)	8.4 (9.1)	– –
372	9.8 (9.7)	5.7 (6.4)	5.5 (5.5)
453	10.0 (10.0)	3.0 (4.0)	2.2 (2.4)
588	– –	3.3 (4.1)	– –
isosbestic points			
353	8.3 (8.7)		
420	– –	2.7 (2.6)	
505	2.5 (3.4)		

[a] Figures in brackets relate to KpFldII.

electrospray mass spectroscopy, UV–visible spectroscopy, and thiol group analysis and in all cases gave identical results to those obtained with KpFldII (see below).

Figure 2c shows the elution profile for purified KpFldII. A sample of this protein was incubated with dithiothreitol as described under Materials and Methods and subsequently rerun on FPLC (Figure 2d). These data show essentially complete conversion after 24 h of KpFldII to KpFldI* and the appearance of free coenzyme A. At shorter incubation times, only partial conversion occurred. Samples of KpFldI* prepared and purified in this way were shown to be identical to KpFldI using the other techniques described below.

UV–Visible Spectra. Figure 3 shows the UV–visible absorption spectra for KpFldI and KpFldII with their FMN cofactors in the oxidized state. At wavelengths greater than 300 nm, the spectra are essentially identical. However, the 272-nm peak of KpFldI shifts to 268 nm for KpFldII with an increase in ϵ_{max} from 46 to 54 mM^{-1} cm^{-1} (Table I). This is consistent with the posttranslational modification of KpFldI by addition of coenzyme A to yield KpFldII since coenzyme

Table II: Thiol Group Estimations of KpFldI and KpFldII[a]

isolated from *E. coli* strain 71-18	% SH
KpFldI	86 ± 6
KpFldI*[b]	91
KpFldII	10 ± 4
KpFldII*[c]	7 ± 4

[a] Percentage of Cys68 detected spectrophotometrically as a free thiol by reaction with DTNB, as described under Materials and Methods. The results are expressed as a percentage of the total flavodoxin concentration calculated using $\epsilon_{max\,453}$ = 10 mM cm^{-1} (see Table I). [b] Synthesized by removal of coenzyme A from KpFldII. [c] Synthesized by addition of coenzyme A to KpFldI.

A has $\epsilon_{max\,259nm}$ = 15 mM^{-1} cm^{-1} (see below), the shift and increased absorbance being due to the adenosine moiety of coenzyme A. Table I also gives the spectral characteristics for the semiquinone and hydroquinone states of KpFldI and KpFldII. The spectra are not shown since they are essentially the same as those published previously by Deistung and Thorneley (1986) with no significant differences at wavelengths greater than 300 nm. The small differences in extinction coefficient for the semiquinone state of KpFldI and KpFldII in Table I are attributed to the difficulty in obtaining precise reduction to this state with $Na_2S_2O_4$ without the generation of any hydroquinone. These spectra are consistent with both forms of flavodoxin containing a single FMN cofactor. This was confirmed by paper chromatography using the method of Kilgour et al. (1957), which readily distinguishes FMN from FAD and riboflavin.

N-Terminal Amino Sequence. The N-terminal amino acid sequence (30 residues) for KpFldI and KpFldII were identical and also in agreement with the *nifF* DNA sequence data of Drummond (1985). Thus both forms of KpFld are the product of the *nifF* gene with no posttranslational modification of any one of the first 30 residues detected by the amino acid analysis procedure used.

Thiol Group Estimation. The DNA sequence data of Drummond (1985) show that the *nifF* gene product has a single cysteine residue at position 68. Table II shows the result of thiol group estimations on KpFldI and KpFldII. KpFldI has the predicted single free thiol (86 ± 6%), but KpFldII has only 10% ± 4% of the thiol accessible for reaction with DTNB. Similar results were obtained with KpFldII* synthesized from KpFldI by incubation with coenzyme A and with KpFldI* synthesized from KpFldII by removal of coenzyme A by incubation with dithiothreitol (see Materials and Methods). We conclude that Cys68 is posttranslationally modified and that it is this modification that distinguishes KpFldI from KpFldII. Since the modifying group was removed by reaction with dithiothreitol, we conclude that this group, shown below to be coenzyme A, is attached to Cys68 by a mixed disulfide bond.

Electrospray Mass Spectroscopy. Rapid and remarkably accurate molecular weight determinations of proteins (2 mass units in 20K) can be made by electrospray ionization using a quadrupole mass spectrometer (Fenn et al., 1989). Proteins are desorbed from liquid droplets as a series of multiply charged gas-phase ions, $(M + nH)^{n+}$, which have m/e ratios generally in the range 700–1500. Each peak in the mass spectrum arises from many ions of the same m/e ratio. Because the protein is present in a number of charged states, a series of peaks is observed in the mass spectrum. The charge state corresponding to each peak in the spectrum is determined from a simple calculation which relies on the fact that adjacent peaks in the series correspond to species which differ by one charge. The molecular mass of the protein is calculated for each peak in the series from the product of the charge and the

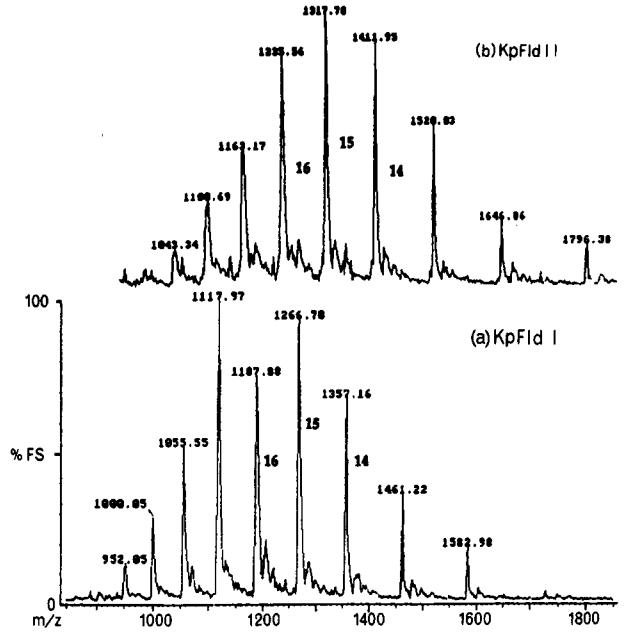

FIGURE 4: Electrospray mass spectra of KpFldI and KpFldII. (a) KpFldI and (b) KpFldII. The number at the top of each peak is the m/e ratio; the number beside the peak corresponds to n in the formula $(M + nH)^{n+}$ for a particular charged ion. The difference in mass (767.5, Table III) is consistent with KpFldII being identical to KpFldI except for the covalent mixed disulfide attachment of coenzyme A to Cys68 of the flavodoxin.

Table III: Electrospray Mass Spectrometry Data

	molecular weight		
	calcd[a]	obsd[b]	obsd av difference
KpFldI	18981.4	18984.0 ± 3.8 18984.2 ± 3.5	765.6 ± 3.5
KpFldII	19747.9	19748.7 ± 1.7 19750.7 ± 4.9	
KpFldII*	19747.9	19754.0 ± 7.1	
CoA-SH	767.5		

[a] Molecular weights calculated using the average atomic weight weighted for isotopic abundance: H = 1.008; C = 12.011; O = 15.999; N = 14.007; S = 32.066. [b] Standard deviation calculated automatically in fitting a family of peaks.

m/e ratio. From these multiple determinations, a mean molecular weight and standard deviation are derived. The physical state of the highly charged protein species in the spectrometer is uncertain, although it is likely to be partially or fully unfolded (Chowdhury et al., 1990) and dissociated from noncovalently bound ligands. Very recent reports describe the use of electrospray mass spectroscopy to detect an enzyme–metal complex in isopenicillin N synthase (Aplin et al., 1990a) and enzyme–substrate complexes of β-lactamase (Aplin et al., 1990b) and a nitrilase (Stevenson et al., 1990).

We have used this technique to determine the difference in molecular weight between KpFldI and KpFldII and use this information to identify the nature of the posttranslational modification that distinguishes these two forms of the *nifF* gene product.

Figure 4a shows the electrospray mass spectrum of KpFldI and Figure 4b that of KpFldII. The main feature of each spectrum is a series of peaks corresponding to a family of multiply charged ions $(M + nH)^{n+}$, where n ranges from 12 to 20 and 11 to 19, respectively. These arise from proteins of molecular weight 18984.1 (KpFldI) and 19749.7 (KpFldII) (average of two determinations, Table III). The value of

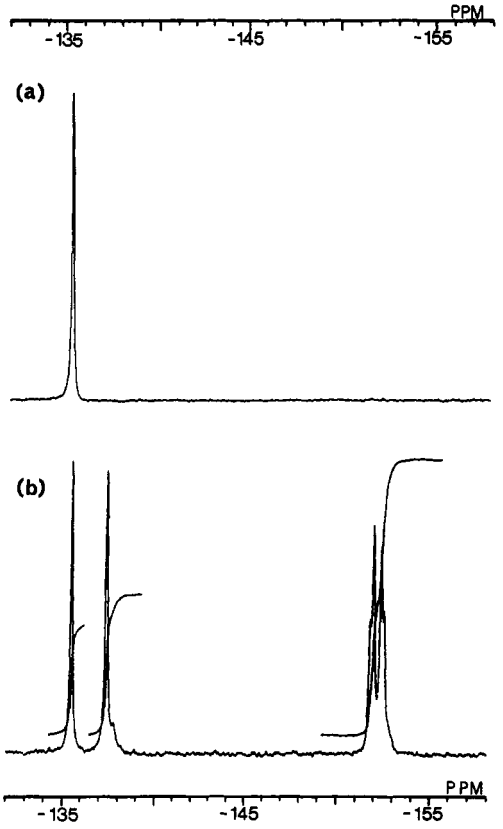

FIGURE 5: ^{31}P NMR spectra of KpFldI and KpFldII. (a) $^{31}P\{^1H\}$ NMR spectrum of KpFldI showing the single peak due to FMN. (b) Spectrum of KpFldII with the integrals of the peaks overlaid. The spectra were the result of 5000 acquisitions with the samples prepared and run as described under Materials and Methods.

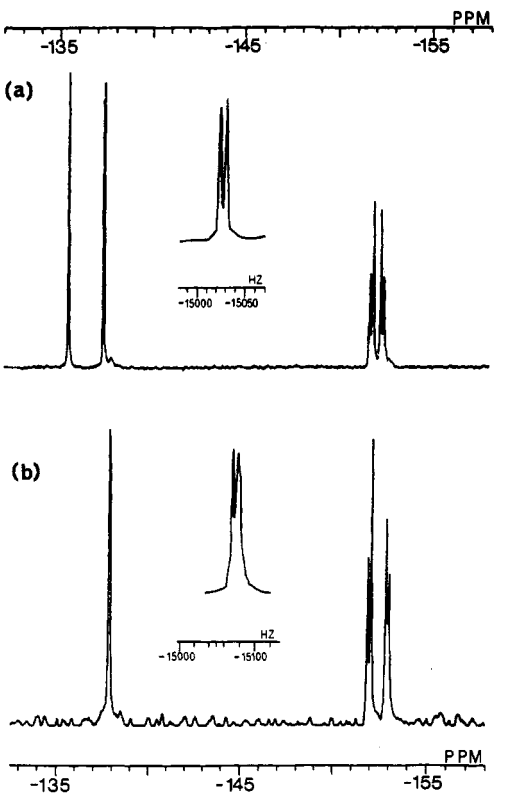

FIGURE 6: Proton-coupled ^{31}P NMR spectra of KpFldII and free coenzyme A. (a) KpFldII. (b) Free coenzyme A. The samples were prepared as described under Materials and Methods and recorded overnight (16 h). Resolution enhancement methods were applied to these data.

18 984.1 for KpFldI is very close to that of 18 981.4 calculated from the DNA-derived amino acid sequence (Drummond, 1985) assuming the loss of the N-terminal methionine and the dissociation of noncovalently bound FMN cofactor from the peptide in the electrospray apparatus. The difference in molecular weights of the two species allowed us to assign coenzyme A as the most likely modifying group. The measured increase in molecular weight, 765.6, is remarkably close to the molecular mass of coenzyme A 767.5 (CoA–SH) and in exact agreement if coenzyme A is attached to KpFldI by a mixed disulfide bond formed with the loss of two protons. The phosphate groups of coenzyme A will be protonated because of the acid medium in which samples are introduced into the mass spectrometer (see Materials and Methods). KpFldII* prepared by incubation of KpFldI with coenzyme A as described above gave essentially the same mass spectrum as that shown in Figure 4b, giving a molecular weight of 19 754 (Table III).

Identification of Coenzyme A as the Modifying Group. The conversion of KpFldII to KpFldI by treatment with dithiothreitol (see above) was associated with the appearance of a new peak in the FPLC elution profile (Figure 2d). The elution characteristics of this compound were identical to those of authentic coenzyme A. Unequivocal identification was shown by its activity in a coenzyme A specific assay using acetyl-CoA:orthophosphate acetyltransferase. After a 60-min incubation with dithiothreitol at pH 9.0, 30 °C, 61% of the KpFldII had been converted to KpFldI (70 nmol) and coenzyme A (74 nmol) as calculated from FPLC peak areas. Coenzyme A (65 nmol) was determined in this sample using the acetyl-CoA:orthophosphate acetyltransferase assay. A short incubation time (60 min) and high pH (9.0) were used in this experiment in order to minimize any degradation of the coenzyme A released and to allow a quantitative estimation.

We conclude that KpFldII is a posttranslationally modified form of KpFldI formed by single covalent mixed disulfide attachment of coenzyme A to Cys68 of the protein. ^{31}P NMR provided additional data to support this conclusion.

^{31}P NMR of KpFldI and KpFldII. The ^{31}P NMR spectra of these two proteins are shown in Figure 5a,b. The signal at −135.4 ppm is characteristic of the dianionic form of phosphate, arising from the FMN group common to KpFldI and KpFldII. The spectrum of KpFldII clearly shows two additional signals at $\delta_1 = -137.4$ ppm and δ_2 centered at −152.2 ppm. The multiplet (AB quartet) at −152.2 ppm is typical of the pyrophosphate group ($^2J_{P-O-P} = 18.2$ Hz). The signal at −137.4 ppm forms a doublet in the proton-coupled spectrum $J_{PH} = 6.9$ Hz (Figure 6a) and shows a chemical shift/pH dependence comparable with that observed for free coenzyme A of 1.6 ppm/pH unit (data not shown). This signal is therefore clearly not of the phosphodiester type which shows no chemical shift/pH dependence, as observed by Edmonson and James (1979) for AvFld. In addition, they did not report any resonances in the pyrophosphate region. The ^{31}P NMR spectrum of the modifying group associated with KpFldII is therefore unique and unlike any previously reported for other flavoproteins. Figure 5b shows the integration of the three phosphorus signals of KpFldII. After correction for relaxation effects, these give a FMN to monophosphate to pyrophosphate ratio of 1:1.1:1.1.

The ^{31}P NMR spectrum of free coenzyme A (Figure 6b) shows similar, but slightly shifted, signals ($\delta_1 = -137.8$ ppm, $\delta_2 = -152.4$ ppm, $^2J_{P-O-P} = 18.5$ Hz) to those given above for KpFldII. In addition, the proton-coupled spectrum of free

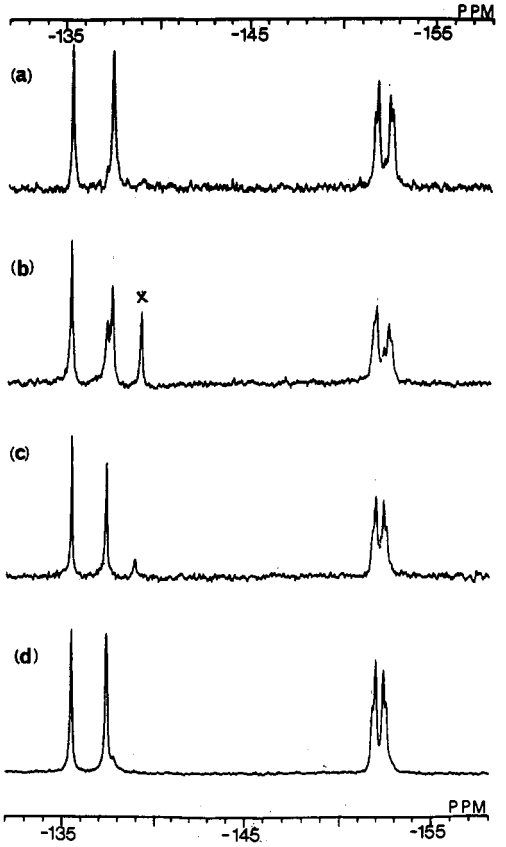

FIGURE 7: Effect of incubation of KpFldI with coenzyme A and a comparison of the reaction product ^{31}P NMR spectrum with that of KpFldII. (a) Initial spectrum obtained after mixing KpFldI with coenzyme A. The spectrum is that predicted from the spectra of KpFldI (Figure 5a) and free coenzyme A (Figure 6b). (b) Reaction product spectra recorded after 4 days incubation at 23 °C. (c) Reaction product purified by FPLC (KpFldII*) whose spectrum should be compared with that of KpFldII shown in panel d with which it is identical in terms of chemical shifts and coupling constants. The samples were prepared and spectra recorded as described under Materials and Methods.

coenzyme A exhibits a J_{PH} value of 6.2 Hz, again comparable to that observed for KpFldII. The small differences in chemical shift for free coenzyme A and KpFldII are most likely due to the effect of the protein environment. The effect of incubating KpFldI with coenzyme A in an NMR tube for 4 days at room temperature is shown in Figure 7a,b. The growth of the peak at −137.4 ppm is clearly seen to correspond to the resonance found in the original KpFldII sample. The spectrum of KpFldII* purified from the reaction mixture by FPLC is shown in Figure 7c. This spectrum is identical to that of KpFldII (Figure 7d).

An additional peak (labeled X in Figure 7b) essentially disappears from the sample after purification by FPLC (Figure 7c). We have not been able to assign this resonance but note that its chemical shift is close to that of the additional phosphorus resonance associated with the 27K flavoprotein designated X in Figure 2 and briefly discussed above.

We conclude that these ^{31}P NMR data are entirely consistent with the covalent attachment of coenzyme A to Cys68 by a mixed disulfide bond. The integrations that show that the ratio of FMN to coenzyme A is 1:1 in both KpFldII and KpFldII* is in complete agreement with the electrospray mass spectrometry and thiol group data reported above.

Posttranslational Covalent Attachment of Coenzyme A to Kp Flavodoxin Prevents Pyruvate-Supported Nitrogenase Activity. Pyruvate is oxidized by the *nifJ* gene product of *K. pneumoniae* to CO_2 with concomitant formation of acetyl-CoA and two electrons which are transferred to the *nifF* gene product, KpFld (Hill & Kavanagh, 1980; Nieva-Gomez et al., 1980; Wahl & Orme-Johnson, 1987). The enzyme uses thiamine pyrophosphate as a cofactor and coenzyme A as a substrate. The two electrons are used sequentially to effect the one-electron reductions of 2 equiv of KpFld semiquinone to the hydroquinone state. KpFld hydroquinone is an electron donor to nitrogenase. This sequence of reactions is demonstrated by the data for pyruvate-supported, nitrogenase-catalyzed, acetylene reduction activity using KpFldI as the electron-mediating protein shown in Figure 8. Under the conditions used, a relatively low concentration of KpFldI is required (ca. 6 μM) to obtain a maximum rate of acetylene reduction. KpFldII, in which Cys68 has been posttranslationally modified by covalent attachment of coenzyme A via a mixed disulfide, was unable to mediate electron transfer between *nifJ* protein and nitrogenase (Figure 8).

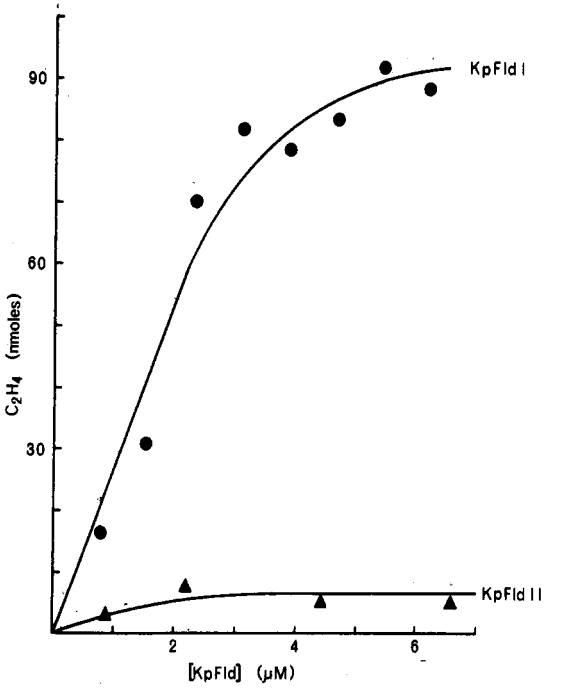

FIGURE 8: Pyruvate flavodoxin oxidoreductase supported acetylene reduction, catalyzed by nitrogenase, is mediated by KpFldI but not by KpFldII. The assay procedure is described under Materials and Methods. The data clearly show that KpFldI (●) but not KpFldII (▲) mediates electron transfer from *nifJ* protein to nitrogenase. The attachment of coenzyme A to KpFldI to yield KpFldII may have a regulatory function in vivo.

KpFldI and KpFldII both undergo facile reduction to the semiquinone and hydroquinone states using dithionite ion as reductant. The UV–visible spectra of two forms show no significant differences in any of the three oxidation levels (except for the 272–268-nm region) (Figure 3 and Table I). These data indicate that the environment of the FMN is not significantly altered by attachment of coenzyme A at Cys68. Although modeling studies suggest that Cys68 is located on the same face of protein as the FMN (Drummond, 1986), its precise position is difficult to ascertain, since it lies on a loop of the protein which is not present in the flavodoxins whose X-ray structure has been determined. The most likely explanation for the inactivity of KpFldII (Figure 8) is that the presence of coenzyme A prevents the effective "docking" of the flavodoxin with either the *nifJ* product or the Fe-protein of nitrogenase, thereby preventing electron transfer to and from the FMN (i.e., steric hindrance). An attractive alternative explanation is that the presence of coenzyme A on the flavo-

doxin stabilizes the protein complex formed with *nifJ*. The coenzyme A attached to Cys68 could occupy the site on the *nifJ* protein that is normally involved in the conversion of coenzyme A to acetyl coenzyme A during the oxidation of pyruvate, i.e., KpFldII is a competitive inhibitor of coenzyme A binding (as a substrate) to *nifJ* protein. It should be possible to distinguish these alternatives by studying the partial reactions of the *nifJ* product with KpFldII semiquinone and of KpFldII hydroquinone with the oxidized Fe-protein of nitrogenase.

A Possible New Regulatory Mechanism for Nitrogenase Activity in Vivo. Nitrogen fixation is an energetically costly process since nitrogenase can comprise 15% of the soluble cell protein and has a very high ATP requirement for activity. In most organisms, nitrogenase synthesis is repressed in the presence of a source of fixed nitrogen (when nitrogenase activity is not required for growth) or in the presence of excess oxygen (when nitrogenase is irreversibly inactivated). Although there is a well-described model for the regulation of *nif* gene expression in *K. pneumoniae* in response to ammonia and oxygen concentration [see Dixon (1988) and Hill (1988) for reviews], little is known about the regulation of nitrogenase activity in vivo. There is no evidence with *K. pneumoniae* for an ADP-ribosylation system such as that which operates in *Rhodospirillium rubrum* to render the nitrogenase Fe-protein inactive when cultures are exposed to elevated ammonia concentrations (Ludden & Roberts, 1989). However, chemostat studies have shown that nitrogenase activity of *K. pneumoniae* can be rapidly "switched on and off" in response to the concentration of dissolved O_2, in the absence of protein synthesis (Hill, 1988). The modification of KpFldI by coenzyme A at Cys68 may be part of this regulatory mechanism. In support of this hypothesis, sequence comparisons show that this cysteine is conserved in the *nif* specific flavodoxins of *Azotobacter vinelandii* and *Azotobacter chroococcum*, species which also regulate nitrogenase activity in response to dissolved oxygen. A chromosomal *nifF* (Cys68) mutant would provide a means of investigating the consequences of removing this level of regulation on nitrogen fixation in vivo under conditions of O_2 stress or carbon limitation.

We think it unlikely that the attachment of coenzyme A to KpFld is a consequence of overexpression of the *nifF* gene in *E. coli* and an attempt by the organism to inactivate the flavodoxin. The organism continues to grow well even though the levels of unmodified flavodoxin (ca. 10% of soluble protein) are many times greater than those normally present in *E. coli* or *K. pneumoniae* (Hill & Kavanagh, 1980; Wahl & Orme-Johnson, 1987). We are also surprised that, if the modification is a consequence of overproduction, there are no other reports, of which we are aware, of coenzyme A attachment to any one of the many proteins overproduced in *E. coli*. The extremely low levels of flavodoxin present in *K. pneumoniae* (Hill & Kavanagh, 1980; Wahl & Orme-Johnson, 1987), even under diazotrophic growth conditions, will make it necessary to use either [^{14}C]coenzyme A labeling or the site-directed mutagenesis approach outlined above to confirm the significance of our findings in terms of the regulation of nitrogen fixation.

The posttranslational modification of proteins by coenzyme A has very recently been reported for the rat liver mitochondrial matrix enzymes acetyl-CoA acetyltransferase and 3-oxoacetyl-CoA thiolase (Huth et al., 1991). However, the ^{14}C-labeling techniques employed were unable to determine precisely the mode of binding of coenzyme A to the proteins, although the authors do suggest a covalent mixed disulfide linkage as the most likely method. Since the radiolabeling with [^{14}C]coenzyme A of these proteins in vivo was transient, and the fully active enzymes remained unlabeled, it was suggested that coenzyme A modification is a "signal" involved in the assembly or degradation of distinct mitochondrial matrix proteins.

To our knowledge, the results of the present paper are the first to show coenzyme A modification of a prokaryotic protein. The extremely slow rate of the spontaneous reaction of coenzyme A with KpFldI and the slow rate of reduction of the resulting mixed disulfide by dithiothreitol suggest that these reactions are enzyme-catalyzed in vivo. The existence of such a system in *E. coli* implies that it may regulate processes common to enteric bacteria as well as nitrogenase activity in diazotrophs.

Acknowledgments

We thank Dr. R. A. Dixon for the gift of pRD545, Mr. N. Henderson for assistance in the bulk fermentation of bacteria, and Dr. B. Dunbar of the Aberdeen Amino Acid Sequencing Facility for N-terminal sequence determinations. The VG BIO Q quadrupole mass spectrometer facility is part of the SERC Cambridge Centre for Molecular Recognition. A.S. is grateful to SERC for graduate student funding. We also thank Mrs. R. Foote for preparation of the manuscript.

Registry No. CoA, 85-61-0; Nitrogenase, 71822-26-9.

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Inhibition of Tyrosine Z Photooxidation after Formation of the S_3 State in Ca^{2+}-Depleted and Cl^--Depleted Photosystem II

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Received July 22, 1991; Revised Manuscript Received October 18, 1991

ABSTRACT: Ca^{2+} and Cl^- are obligatory cofactors in photosystem II (PS-II), the oxygen-evolving enzyme of plants. The sites of inhibition in both Ca^{2+}- and Cl^--depleted PS-II were compared using EPR and flash absorption spectroscopies to follow the extent of the photooxidation of the redox-active tyrosine (TyrZ) and of the primary electron donor chlorophyll (P_{680}) and their subsequent reduction in the dark. The inhibition occurred after formation of the S_3 state in Ca^{2+}-depleted PS-II. In Cl^--depleted photosystem II, the inhibition occurred after formation of the S_3 state in about half of the centers and probably after S_2TyrZ^+ formation in the remaining centers. After the S_3 state was formed in Ca^{2+}- and Cl^--depleted photosystem II, electron transfer from TyrZ to P_{680} was inhibited. This inhibition is discussed in terms of electrostatic constraints resulting from S_3 formation in the absence of Ca^{2+} and Cl^-.

Photosystem II (PS-II)[1] catalyzes light-driven water oxidation resulting in oxygen evolution. The reaction center of PS-II is made up of two membrane-spanning polypeptides (D1 and D2) analogous to the L and M subunits of the purple photosynthetic bacteria [see Michel and Deisenhofer (1988) for a review]. Absorption of a photon leads to a charge separation between a chlorophyll molecule, designated P_{680}, and a pheophytin molecule. The pheophytin anion transfers the electron to a quinone, Q_A, and P_{680}^+ is reduced by a tyrosine residue, TyrZ, tyrosine-161 of the D1 polypeptide (Barry & Babcock, 1987; Debus et al., 1988a,b; Vermaas et al., 1988; Metz et al., 1989). A cluster of four Mn located in the reaction center of PS-II probably acts both as the active site and as a charge-accumulating device of the water-splitting enzyme [see Rutherford (1989) for a review]. During the enzyme cycle, the oxidizing side of PS-II goes through five different redox states that are denoted S_n, n varying from 0 to 4 (Kok et al., 1970). In oxygen-evolving PS-II, reduction of P_{680}^+ by TyrZ, after a flash, occurs in the tens to hundreds of nanoseconds time range depending on the S state (Brettel et al., 1984).

Three extrinsic polypeptides, with molecular masses of 17, 23, and 33 kDa, are bound to the PS-II reaction center on the inside of the thylakoid membrane. Removal of these three polypeptides by Tris washing results in the depletion of the Mn cluster and in inhibition of oxygen evolution [reviewed in Murata and Miyao (1985)]. In Tris-washed PS-II, reduction of P_{680}^+ by TyrZ occurs in about 5 μs at high pH and in 40-50 μs at low pH (Conjeaud & Mathis, 1980). This pH effect was interpreted as being due to protonation of a group near TyrZ (Conjeaud & Mathis, 1986). When TyrZ is already oxidized by a preflash, the charge recombination between P_{680}^+ and Q_A^- takes 200 μs (Conjeaud & Mathis, 1980). When the donation by TyrZ to P_{680}^+ is inhibited in the presence of acetate, the charge recombination between P_{680}^+ and Q_A^- takes 500 μs (Saygin et al., 1986). In a mutant of Synechocystis 6803 in which tyrosine-161 of the D1 polypeptide was replaced by a phenylalanine residue, the charge recombination between P_{680}^+ and Q_A^- occurs with a $t_{1/2}$ of 1 ms (Metz et al., 1989). Recently, Hogansson et al. (1991) reported that, in Tris-washed PS-II, the binding of Mn^{2+}, in a site probably corresponding to a Mn site in the functional enzyme, slowed down P_{680}^+ reduction by TyrZ from $\approx$5 to $\approx$40 μs. The variations in P_{680}^+ reduction kinetics described above were generally interpreted as being due to electrostatic restraints (Brettel et al., 1984; Hogansson et al., 1991; Metz et al., 1989).

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‡ Supported by the CNRS.

[1] Abbreviations: P_{680}, reaction center chlorophyll (Chl) of photosystem II (PS-II); TyrZ, tyrosine acting as the electron donor to P_{680}; TyrD, tyrosine acting as a side-path electron donor of PS-II; Q_A, primary quinone electron acceptor of PS-II; Q_B, secondary quinone electron acceptor of PS-II; EPR, electron paramagnetic resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PPBQ, phenyl-*p*-benzoquinone.

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