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Consequences of the iron-dependent formation of ferredoxin and flavodoxin on photosynthesis and nitrogen fixation on *Anabaena* strains

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Abstract

Iron-dependent formation of ferredoxin and flavodoxin was determined in *Anabaena* ATCC 29413 and ATCC 29211 by a FPLC procedure. In the first species ferredoxin is replaced by flavodoxin at low iron levels in the vegetative cells only. In the heterocysts from *Anabaena* ATCC 29151, however, flavodoxin is constitutively formed regardless of the iron supply.

Replacement of ferredoxin by flavodoxin had no effect on photosynthetic electron transport, whereas nitrogen fixation was decreased under low iron conditions. As ferredoxin and flavodoxin exhibited the same K_m values as electron donors to nitrogenase, an iron-limited synthesis of active nitrogenase was assumed as the reason for inhibited nitrogen fixation. *Anabaena* ATCC 29211 generally lacks the potential to synthesize flavodoxin. Under iron-starvation conditions, ferredoxin synthesis is limited, with a negative effect on photosynthetic oxygen evolution.

Introduction

Flavodoxin is a low molecular weight FMN-containing flavoprotein that has been isolated from different prokaryotic as well as eukaryotic organisms (Smillie 1965, Bothe 1970, Mayhew and Ludwig 1975, Bothe 1977). They include aerobic, anaerobic and facultative heterotrophic bacteria such as *Azotobacter*, *Clostridium* and *Klebsiella*, as well as photosynthetic bacteria, cyanobacteria and algae. In some species flavodoxin has been found to be constitutive, while in other organisms it is synthesized as a consequence of the iron-limiting conditions in the culture medium. Flavodoxin can be easily purified, due to its strong acidic character and its presence in large amounts in organisms grown under low iron concentrations. Therefore it is one of the flavoproteins for which detailed structural information is available (Smith et al. 1984, Ludwig et al. 1984). Nevertheless little is known about the physiological function of this protein or the regulatory mechanism of its synthesis. It is proposed that flavodoxin replaces ferredoxin in vivo in those reactions in which this iron protein participates (Yoch and Valentine 1972). The low (very negative) oxidation-reduction potentials required for these reactions are compatible with those determined for the semiquinone/hydroquinone couple in different flavodoxins including *Anabaena* (Sykes and Rogers 1984, Fillat et al. 1990). In vitro, flavodoxin replaces ferredoxin in

the transfer of electrons between photosystem I and the enzyme ferredoxin-$NADP^+$ reductase (Smillie 1965, Trebst and Bothe 1966, Fillat et al. 1988), indicating its possible role in photosynthesis. It has also been shown to be very efficient in providing electrons to nitrogenase in the acetylene reduction assay of this enzyme (Fillat et al. 1988).

A specific ferredoxin, isolated recently by Böhme and Schrautemeier (1987) from heterocysts of *Anabaena* ATCC 29413, is reported to be different from those found in vegetative cells. The heterocystous ferredoxin has been shown to act in vivo as the immediate electron donor to nitrogenase and also to be the product of a gene which is expressed only under nitrogen fixing-conditions (Böhme and Haselkorn 1988).

In this paper three different strains of *Anabaena* are studied with regard to their ability to replace ferredoxin with flavodoxin when grown in low-iron media. In addition, the effect on photosynthesis and nitrogen fixation is compared. We also describe the presence of a flavodoxin in heterocysts of *Anabaena* ATCC 29151. Synthesis of flavodoxin, even at high concentrations of iron in the medium, indicates that in these specialized cells flavodoxin is synthesized constitutively and that the iron-dependent regulation of flavodoxin synthesis is absent, in contrast to vegetative cells of the same species.

Material and methods

Anabaena variabilis, strain ATCC 29211 (CCAP 1403.4b) and *Anabaena* strains ATCC 29151 (PCC 7119) and ATCC 29413 were grown as described by Sandmann and Böger (1980) in media containing different concentrations of iron, at 22°C under light intensities of 4000 lx. Packed cell volume (pcv) was determined by centrifugation of a cell suspension in calibrated microcentrifuge tubes.

Homogenates were prepared from 1 l of suspension of cells harvested at the end of the exponential phase of growth and disrupted during 10 min in a Vibrogen cell homogenizer using 0.45–0.5 mm glass beads. Unbroken cells and debris were removed by centrifugation at 18 000 × *g* for 20 min. Partially purified extracts for FPLC determinations were prepared on small columns containing 1 ml bed volume of Whatman DE-52 cellulose equilibrated with 0.1 M Tris-HCl pH 7.5. The crude extract was poured onto the columns and washed with 0.2 M Tris-HCl, pH 7.5, until the blue color of the phycobiliproteins disappeared. Ferredoxin and flavodoxin were eluted with 6 ml of 0.5 M Tris-HCl buffer, pH 7.5. Samples were dialyzed or diluted before FPLC separation and determination.

FPLC determinations were performed on a Pharmacia Mono Q HR 5/5 column. 0.5 ml of the eluent from the DE-52 column were diluted with an equal amount of water and injected. Salt gradients of 0–0.5 M NaCl were used and the absorbance was recorded at 465 nm. Ferredoxin and flavodoxin retention times were identified and quantified using previously purified proteins.

Antibodies against ferredoxin and flavodoxin were prepared (Lampreave and Piñeiro 1982) and immunoelectrophoresis performed using the method described by Laurell (1966) to quantify the amount of both proteins in crude or prepurified extracts. Ferredoxin and flavodoxin determined by immunoelectrophoresis were quantified in dilute extracts in the range 5–35 μg/ml. Endogenous iron content was determined by atomic absorption spectroscopy in a Varian Carbon Rod Atomic Absorption Spectrophotometer, using cells suspended in 2 N HNO_3. The kinetic parameters were measured as described previously (Fillat et al. 1988). Photosynthesis of cells was determined by measuring the oxygen evolution in a Clark type electrode. Nitrogenase activity was measured in vivo by determining the rate of acetylene reduction of illuminated cells in a Carlo Erba Fractovap gas chromatograph as described previously (Fillat et al. 1988). K_m values for flavodoxin and ferredoxin used as electron donors to nitrogenase were determined from Lineweaver-Burk plots.

Heterocysts were isolated using the method described by Fay (1980), that essentially includes several centrifugation steps at low speed and purity was checked by light microscope.

Results and discussion

The development of useful techniques for the detection and discrimination of ferredoxin and

flavodoxin is very important in order to study the distribution of these proteins in cells grown under different physiological conditions. This is particularly difficult since neither of them shows any specificity in electron transfer reactions and also because their absorbance spectra are too similar for accurate differential spectroscopy especially when both proteins are present simultaneously. Immunochemical techniques are particularly useful for determination of ferredoxin and flavodoxin since both proteins have specific antibodies. Nevertheless, immunoquantitation has the disadvantage that it shows the presence of protein irrespective of its functionality. For example, ferredoxin with its 2Fe2S cluster is determined together with its apoprotein and the immunological assay does not discriminate between FMN-containing flavodoxin and its apoprotein. This can be a problem, especially when studies are performed on the mechanisms regulating the synthesis of the functional proteins.

Therefore, a method has been developed for the rapid detection and separation of the holoproteins of both ferredoxin and flavodoxin using the FPLC technique. Figure 1 shows the separation, on a Mono Q ion exchange column, from a partially purified extract of *Anabaena* ATCC 29413 cells, grown at an iron concentration of 3 μM. Both proteins show quite different retention times: 24 and 33 min for flavodoxin and ferredoxin, respectively. It should be mentioned that under these particular FPLC conditions only one major ferredoxin was detected, while large scale purifications of ferredoxin reveals the presence of a second ferredoxin.

Flavodoxin has been isolated from *Anabaena* cultures grown in low iron media which, at the same time, contained low levels of ferredoxin (Fillat et al. 1988). It was of interest to study how the concentration of iron in the culture medium affects the level of the two proteins which can be present in the cells simultaneously. For this purpose, two different strains of *Anabaena* that have been shown to behave differently with respect to their ability to synthesize flavodoxin were grown in media containing iron in the range from 0.015 μM to 35 μM. Those strains were *Anabaena* ATCC 29413, from which flavodoxin can be isolated when grown in the absence of iron in the culture medium (Fillat et al. 1988), and *Anabaena* ATCC 29211, which we have shown (Pardo et al. 1990) to be unable to replace ferredoxin by the flavoprotein, even when grown at very low concentrations of iron. Figure 2 indicates that flavodoxin replaces ferredoxin in *Anabaena* ATCC 29413 as the con-

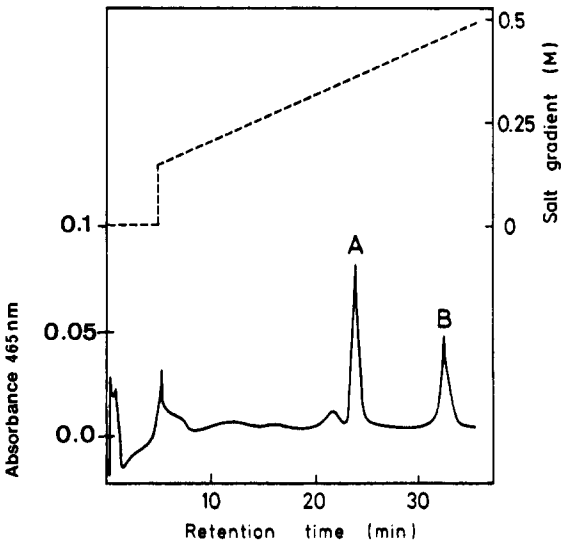

Fig. 1. FPLC determination of ferredoxin and flavodoxin from *Anabaena* ATCC 29413 grown with 3 μM iron. Peak A, with RT = 24 min, was identified as flavodoxin; peak B, with RT = 33 min, as ferredoxin. Ferredoxin and flavodoxin were identified using proteins previously purified as standars.

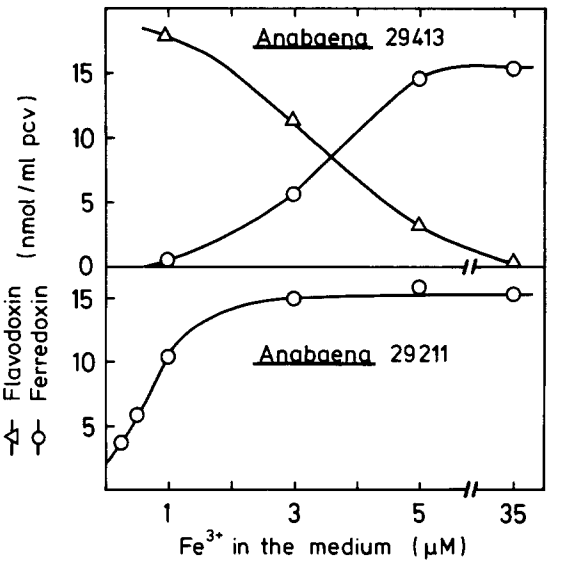

Fig. 2. Iron-dependent formation of ferredoxin and flavodoxin in two different *Anabaena* strains: *Anabaena* ATCC 29413 and *Anabaena variabilis* ATCC 29211. Cells were grown in media containing the iron concentrations indicated in the figure. Ferredoxin and flavodoxin were demonstrated by FPLC as described in Material and methods.

centration of iron decreases. The strain ATCC 29211, on the contrary, does not show any synthesis of flavodoxin even at the lowest concentration of iron in the medium studied. It is interesting to note that this strain, which is unable to synthesize flavodoxin, shows a decrease of ferredoxin formation only at iron concentrations below 1 μM. This is the concentration of iron at which ferredoxin was completely absent in the flavodoxin-synthesizing *Anabaena* ATCC 29413.

Since it has been proposed that flavodoxin replaces ferredoxin in all the reactions in which this protein participates (Ludwig and Mayhew 1975) the effect of different concentrations of iron on the levels of both proteins, as well as on corresponding physiological activities, was investigated. Cells were grown at different concentrations of iron in the medium and both the photosynthetic and nitrogen-fixing activities were determined in vivo. For this purpose, *Anabaena* ATCC 29413 was chosen because it can replace ferredoxin by flavodoxin under iron-deficient conditions (Fig. 2). Table 1 shows that flavodoxin is present only in cells which contain low levels of intracellular iron due to their growth in iron-deficient media. The level of ferredoxin is, correspondingly, low as compared to cells with high intracellular levels of iron. The photosynthetic activity, measured as the rate of oxygen evolution, shows similar values for both cultures, indicating that flavodoxin transfers electrons from photosystem I to the enzyme ferredoxin-$NADP^+$ reductase as efficiently as ferredoxin. The level of nitrogen fixation is, nevertheless, very much decreased in cells grown at low levels of iron. This could be due to the lower efficiency of flavodoxin as an electron mediator for the nitrogenase, or to a direct effect of the availability of iron on the nitrogenase itself.

The effect of iron limitation on ferredoxin content and photosynthesis was also studied in an *Anabaena* strain which was unable to synthesize flavodoxin. When *Anabaena* ATCC 29211 cells were grown in low iron media, as indicated in Table 2, a decrease in the level of intracellular iron was observed at 3 μM iron. However, this limitation did not decrease the amount of ferredoxin present or interfere with the photosynthetic activity of the cells. If the concentration of iron in the culture medium was lowered to 0.5 μM, a further depletion of endogenous iron was observed, with a concurrent decrease of the ferredoxin content. In addition photosynthetic activities of the whole cells were negatively affected.

The ferredoxin-flavodoxin exchange experiments with organisms like *Anabaena* ATCC 29413 in Fig. 2 are indicative of a mechanism that regulates the synthesis of flavodoxin when the intracellular iron concentration falls below a certain threshold. In this organism, repression of the synthesis of the iron-containing ferredoxin leaves iron available for the synthesis of other Fe-proteins. *Anabaena* ATCC 29151 behaves similarly to *Anabaena* 29413 with respect to the response to iron limitation. In the ATCC 29211 strain, in which ferredoxin cannot be replaced by

Table 1. Photosynthesis, nitrogen fixation and ferredoxin/flavodoxin content of *Anabaena* ATCC 29413 grown at high and low Fe

	Fe concentration of the medium (μM)	
	3	35
Endogenous Fe (μmol/ml pcv)	0.87 ± 0.13	2.26 ± 0.57
Content (nmol/ml pvc) of		
ferredoxin	6.1 ± 1.4	16.1 ± 2.0
flavodoxin	13.9 ± 2.9	n.d.
Photosynthetic oxygen evolution (μmol O_2/ml pcv × h)	426 ± 18	446 ± 16
Nitrogen fixation (μmol C_2H_2/ml pcv × h)	1.8 ± 0.9	5.5 ± 0.3

Values are means of 4 independent determinations.
n.d. = not detected.

Table 2. Photosynthesis and ferredoxin content in *Anabaena* ATCC 29211 grown at high and low Fe

	Fe in the medium (μM)		
	0.5	3	35
Endogenous Fe (μmol/ml pcv)	0.18 ± 0.6	0.79 ± 0.18	2.55 ± 0.42
Ferredoxin content* (nmol/ml pcv)	6.5 ± 2.9	14.7 ± 3.0	16.7 ± 2.6
Photosynthesis (μmol O_2/ml pcv × h)	214 ± 42	534 ± 89	548 ± 94

* No flavodoxin and no in vivo nitrogen fixation were detectable in any of the cultures.

flavodoxin, repression of ferredoxin synthesis does not take place and iron deprivation at levels below 0.2 nmol/μl pcv (packed cell volume) limits the synthesis of the iron-proteins. In these extreme conditions, photosynthetic activity is markedly decreased.

The kinetic parameters that indicate the efficiency of the electron transfer between ferredoxin or flavodoxin and their redox partners for the different reactions in which they participate were studied. Table 3 shows the rate constants obtained for ferredoxin and flavodoxin from *Anabaena* ATCC 29151 when interacting with photosystem I (PS I) or ferredoxin-$NADP^+$ reductase (FNR). In both cases the rate constants are of the same order for both proteins. It seems that flavodoxin is slightly better reduced by PS I or FNR than ferredoxin. Table 3 also shows that the K_m value for the nitrogenase reaction, which indicates the affinity between the two proteins and nitrogenase, are identical for ferredoxin and flavodoxin. This result shows that the negative effect of the low iron concentration on the nitrogen fixing activity of the whole cells is due to factors other than poor reactivity of flavodoxin with nitrogenase. Most likely the synthesis of nitrogenase, which contains about 30 iron atoms (Orme-Johnson 1985), is restricted.

Table 3. Kinetic parameters of ferredoxin and flavodoxin from *Anabaena* ATCC 29151 in reactions with their redox partners in photosynthetic electron transport and nitrogen fixation

Reaction	Ferredoxin	Flavodoxin*
Rate constant for reduction by PS I (M^{-1} min^{-1})	4.5×10^7	9.6×10^7
Rate constant for reduction by FNR (M^{-1} min^{-1})	4.0×10^6	6.6×10^6
K_m value (μM) in nitrogenase reaction	7.9	8.5

* Values were taken from Fillat et al. (1988) for comparison.

Evidence has been presented that flavodoxin can replace ferredoxin as the electron donor to nitrogenase. In the nitrogen-fixing heterotrophic bacterium *Klebsiella pneumoniae*, in which flavodoxin participates directly in the nitrogen fixation reaction by obtaining electrons from pyruvate through the enzyme pyruvate-flavodoxin-oxidoreductase (Shah et al. 1983), this flavodoxin is encoded by a gene that is clustered with others involved in nitrogen fixation (Deistung et al. 1985). Therefore, flavodoxin content was determined on heterocysts of the nitrogen-fixing cyanobacteria *Anabaena* and the way the level of this protein is related to the presence of iron in the culture medium was also studied. The antigens used for quantitation were monospecific, and cross-reactivity of ferredoxin with the flavodoxin antigen and vice versa was not observed. Table 4 shows that heterocysts of cells grown either at limiting or saturating levels of iron produce the same amount of flavodoxin as was detected immunochemically with antibodies raised against the main form of flavodoxin when the cells are grown under iron-deficient conditions. The presence of flavodoxin in heterocysts even at high iron concentration indicates that flavodoxin is a constitutive protein in heterocysts, synthesized irrespectively of the level of iron in the medium. It also emphasizes the importance of this protein in the transfer of electrons to nitrogenase. Flavodoxin constitutively present in heterocysts could account for the low

Table 4. Flavodoxin and ferredoxin content in isolated heterocysts from *Anabaena* ATCC 29151 grown in different media. Values are averages of 3 determinations

Grown in:					
Iron	Nitrate	% heterocysts	Chlorophyll in heterocysts	Flavodoxin	Ferredoxin
			μg/ml	μg/ml	μg/ml
35 μM	not present	5%	712	5.7 ± 0.40	47.5 ± 1.1
3 μM	not present	5%	394	4.4 ± 0.25	8.22 ± 0.53
35 μM	20 mM	2%	513	not detected	6.48 ± 0.7

amount of this protein that is found in all nitrogen-fixing cyanobacteria at high iron concentrations. According with this, in the non nitrogen-fixing species of cyanobacteria such as *Anacystis nidulans*, flavodoxin should be completely absent in high-iron media. This is supported by our observation concerning the strain 29211 of *Anabaena*, where we could not detect any appreciable nitrogen-fixing ability lacks flavodoxin and also by the report (Laudenbach et al. 1988) that in *Anacystis nidulans* flavodoxin is completely absent.

The presence of a type of ferredoxin in heterocysts which is biochemically different from the ones in vegetative cells of cyanobacteria and is more specific for the nitrogenase reaction (Schrautemeier and Böhme 1985) points to a special role of this ferredoxin in the nitrogenase reaction. Moreover, an *Azotobacter vineilandii* mutant strain described by Bennet et al. (1987) which has a deletion in the flavodoxin gene, shows diazotropic growth which is, nevertheless, below the maximal in vivo nitrogenase activity found for the wild-type cells. From these findings we could conclude that flavodoxin is a physiological, but not unique, electron carrier for nitrogenase in heterocysts of cyanobacteria and that it is synthesized in these cells independently of the level of iron in the culture medium. In vegetative cells, where the nitrogenase reaction does not occur, flavodoxin would be induced only under iron-deficient conditions to substitute for ferredoxin in photosynthetic electron transfer reactions.

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References

Bennett LT, Jacobson MR and Dean DR (1987) Isolation, sequencing and mutagenesis of the *nif* F gene encoding flavodoxin from *Azotobacter vinelandii*. J Biol Chem 263: 1364–1369

Bohme H and Schrautemeier B (1987) Comparative characterization of ferredoxins from heterocysts and vegetative cells of *Anabaena variabilis*. Biochim Biophys Acta 891: 1–7

Bohme H and Haselkorn R (1988) Molecular cloning and nucleotide sequence analysis of the gene coding for heterocysts ferredoxin from the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol Gen Genet 214: 278–285

Bothe H (1970) Photosynthetische stickstoffixierung mit einem zellfreien extrakt aus der blaualge *Anabaena cylindrica*. Ber Dtsch Bot Ges 83: 421–432

Bothe H (1977) Flavodoxin. In: Trebst A and Avron M (eds) Encyclopedia of Plant Physiology, Vol 5, pp 217–221. Springer Verlag, Berlin

Deistung J, Cannon FC, Cannon MC, Hill S and Thorneley RNF (1985) Electron transfer to nitrogenase in *Klebsiella pneumoniae*: *nif* F gene cloned and the gene product, a flavodoxin, purified. Biochem J 231: 743–753

Fay P 1980 Heterocysts isolation. Methods Enzymol 69: 801–803

Fillat MF, Sandmann G and Gómez-Moreno C (1988) Flavodoxin from the nitrogen-fixing cyanobacterium *Anabaena* PCC 7119. Arch Microbiol 150: 160–164

Fillat MF, Edmondson DE and Gómez-Moreno C (1990) Structural and chemical properties of a flavodoxin from *Anabaena* PCC 7119. Biochim Biophys Acta (in press)

Lampreave F and Piñeiro A (1982) Characterization of a new alpha-glycoprotein as the major serum component in later fetal and newborn pigs. Comp Biochem Physiol 72B: 215–219

Laudenbach DE, Reith ME and Straus NA (1988) Isolation, sequence analysis, and transcriptional studies of the flavodoxin gene from *Anacystis nidulans* R2. J Bacteriol 170: 258–265

Laurell CB (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal Biochem 15: 45–52

Ludwig ML, Pattridge KA and Tarr G (1984) FMN: protein interactions in flavodoxin from *A. nidulans*. In: Bray RC, Engel PC and Mayhew SG (eds) Flavins and Flavoproteins, pp 253–259. Walter de Gruyter, Berlin, New York

Mayhew SG and Ludwig ML (1975) In: Boyer PD (ed) The Enzymes, 3rd Ed, Vol 12, pp 57–118. Academic Press, New York

Pardo B, Gómez-Moreno C and Peleato ML (1990) Effect of iron deficiency on ferredoxin levels in *Anabaena variabilis* PCC 6309. Arch Microbiol 153: 528–530

Orme-Johnson WH (1985) Molecular basis of biological nitrogen fixation. Ann Rev Biophys Chem 14: 419–459

Sandmann G and Böger P (1980) Copper-induced exchange of plastocyanin and cytochrome *c*-553 in cultures of *Anabaena variabilis* and *Plectonema boryanum*. Plant Sci Lett 17: 417–424

Schrautemeier B and Böhme H (1985) A distinct ferredoxin for nitrogen fixation isolated from heterocysts of the cyanobacterium *Anabaena variabilis*. FEBS Lett 184: 304–308

Shah VK, Stacey G and Brill WJ (1983) Electron transport to nitrogenase. Purification and characterization of pyruvate: flavodoxin oxidoreductase, the *nif* J gene product. J Biol Chem 258: 12064–12068

Smillie RM (1965) Isolation of phytoflavin, a flavoprotein with chloroplast ferredoxin activity. Plant Physiol 40: 1124–1128

Smith WW, Pattridge KA, Ludwig ML, Petsko GA, Tsernoglou D, Tanaka M and Yasunobu KT (1983) Structure of oxidized flavodoxin from *Anacystis nidulans*. J Mol Biol 165: 737–755

Sykes GA and Rogers LJ (1984) Redox potentials of algal and cyanobacterial flavodoxins. Biochem J 217: 845–850

Trebst A and Bothe H (1966) Zur Rolle des Phytoflavins im photosynthetischen Electronentransport. Ber Dtsch Bot Ges 79: 44–47

Yoch DC and Valentine RC (1972) Ferredoxins and flavodoxins of bacteria. Ann Rev Microbiol 26: 41–51