Azotobacter vinelandii Flavodoxin: Purification and Properties of the Recombinant, Dephospho Form Expressed in *Escherichia coli*†

Matthew F. Taylor,‡ Maire H. Boylan,§ and Dale E. Edmondson*

Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322

Received January 24, 1990; Revised Manuscript Received March 27, 1990

ABSTRACT: The *nifF* gene coding for the flavodoxin from the nitrogen-fixing bacterium *Azotobacter vinelandii* (strain OP) was cloned into the plasmid vector pUC7 [Bennett, L. T., Jacobsen, M. R., & Dean, D. R. (1988) *J. Biol. Chem. 263* 1364–1369] and the resulting plasmid transformed and expressed in *Escherichia coli* strain DH5. Recombinant *Azotobacter* flavodoxin is expressed at levels 5–6-fold higher in *E. coli* than in comparable yields of *Azotobacter* cultures grown under nitrogen-fixing conditions. Even higher levels were observed with flavodoxin expressed in *E. coli* under control of a *tac* promoter. Electron spin resonance spectroscopy on whole cells and in cell-free extracts showed the flavodoxin to be largely in the semiquinone form. The flavodoxin purified from *E. coli* exhibited the same molecular weight, isoelectric point, flavin mononucleotide (FMN) content, N-terminal sequence, and carboxyl-terminal amino acids as for the wild-type *Azotobacter* protein. The recombinant flavodoxin differed from native flavodoxin in that it exhibited an increased antigenicity to flavodoxin antibody and did not contain a covalently bound phosphate. Small differences are also observed in circular dichroism spectral properties in the visible and ultraviolet spectral regions. The recombinant, dephospho flavodoxin exhibits an oxidized/semiquinone potential (pH 8.0) of −224 mV and a semiquinone/hydroquinone couple (pH 8.0) of −458 mV. This latter couple is 50–60 mV higher than that exhibited by the native flavodoxin. Resolution of recombinant dephospho flavodoxin resulted in an apoflavodoxin that was much less stable than that prepared from the native protein. Recombinant apoflavodoxin rapidly lost its FMN binding ability on dilution or on storage and was not able to bind riboflavin. These results are consistent with the view that the phosphodiester cross-link in *Azotobacter* (strain OP) flavodoxin is incorporated via a posttranslational process and that its function in the protein is primarily a structural role.

The first direct demonstration of an intramolecular phosphodiester bond linking two amino acid residues in a protein was published 10 years ago (Edmondson & James, 1979), in which ^{31}P NMR and chemical approaches on the flavodoxin isolated from *Azotobacter vinelandii* (strain OP, Berkeley) were used. Subsequent β-elimination experiments (Edmondson & James, 1982) identified the amino acids involved in this unusual linkage to be serine and threonine. Evidence supporting this assignment from ^{1}H–^{31}P multiquantum two-dimensional NMR techniques on the intact protein has been recently published (Live & Edmondson, 1988). Further studies on a model dipeptide in which serine and threonine are linked via a phosphodiester bond between their respective hydroxyl groups (Dreef-Tromp et al., 1989) also provide support for the assignment of this unusual cross-link in *Azotobacter* flavodoxin. Additional evidence for the presence of a covalent phosphorus moiety has been shown by in vivo ^{32}P labeling studies in intact cells in which it has been demonstrated that the flavodoxin is phosphorylated in vivo and the incorporation of the phosphodiester cross-link into the protein is a posttranslational process (Boylan & Edmondson, 1990). Taken together, this combined body of data listed above provides unequivocal proof for the existence of a phosphodiester cross-link in *Azotobacter* flavodoxin, which should satisfy doubts expressed in the literature (Vogel, 1984; Johnson et al., 1989) that are based on little or no experimental data. The flavodoxin isolated from *A. vinelandii* (strain OP, Berkeley) is unique among the flavodoxins tested, to date, from various microorganisms in containing this covalent phosphate. We have examined approximately ten flavodoxins from other organisms by chemical analysis and found the only phosphorus residue present to be that of the FMN coenzyme. These results demonstrate other flavodoxins tested do not contain covalent phosphorus but do not provide any information on whether other nonphosphorus cross-links might be present.

The structural information available on this phosphodiester linkage would seem to suggest that it plays a structural role in stabilization of the three-dimensional structure of *Azotobacter* flavodoxin. This flavodoxin is among the largest of the known flavodoxins (Mayhew & Ludwig, 1975) and also has the most negative semiquinone/hydroquinone oxidation–reduction potential of the known proteins in this class (Watt, 1979). Previous ^{31}P NMR studies have demonstrated this diester to be exposed to solvent and to be remote (>10 Å) from the flavin mononucleotide (FMN) ring (Edmondson & James, 1982), as well as not to be shielded from the solvent on complexation of the flavodoxin with cytochrome *c* (Tollin et al., 1987) or with ferredoxin-$NADP^+$ oxidoreductase (Gomez-Moreno et al., 1987). Although these model system studies suggest that this phosphodiester linkage does *not* play a direct role in protein–protein interactions involving flavodoxin, further work is required to determine if this situation is still valid in

† This work was supported in part by grants from the National Science Foundation (DMB-8616952) and from the National Institutes of Health (GM-29433). Funds for the purchase of the circular dichroism spectrometer used in this work were provided by an NIH equipment grant. Funds for the purchase of the ESR spectrometer used in this work were provided by Emory University.

‡ Present address: Department of Biochemistry, King's College, University of London, United Kingdom.

§ Present address: Monsanto Co., 700 Chesterfield Village Parkway, St. Louis, MO 63198.

0006-2960/90/0429-6911$02.50/0

the interaction of this flavodoxin with its physiological donor/acceptor proteins.

A structural role for this phosphodiester could be envisaged as functioning as an intramolecular cross-link such as is a well-known function for disulfide bonds or for ionic interactions between charged amino acid residues (such as that occurring in chymotrypsin). Since this flavodoxin functions in low-potential electron-transfer reactions to the nitrogenase complex of *Azotobacter*, the substitution of a disulfide with a phosphodiester could be a reasonable alternative to ensure that the cross-link could not be reductively cleaved during normal biological function. The testing of concepts such as those listed above requires comparison of structural properties of flavodoxin preparations that differ only by the presence or absence of this phosphodiester linkage. Efforts in the past in this laboratory to prepare dephospho flavodoxin by incubation of the protein with various phosphatases or phosphodiesterases have been unsuccessful. As an alternative approach, we initiated a program of characterizing the recombinant flavodoxin obtained on expressing the cloned *A. vinelandii* flavodoxin (strain OP) gene in *E. coli*. This approach was possible with the availability of the cloned *nifF* gene (Bennett et al., 1988), in which the deduced amino acid sequence from the DNA sequence is essentially identical with that determined by classical methods for the flavodoxin from *A. vinelandii* (strain OP, Berkeley) (Tanaka et al., 1977) (the DNA sequence shows a Gln at position 157 rather than a Glu as determined by protein sequencing). The idea behind this approach was that if the phosphodiester were incorporated into the protein by a posttranslational process (Boylan & Edmondson, 1989), expression of the flavodoxin in *E. coli* might possibly result in the formation of a dephosphoprotein. Previous unpublished work in this laboratory has shown that *E. coli* K-12 flavodoxin (Vetter & Knappe, 1971) does not contain a covalently bound phosphate, which suggests that the enzymes responsible for the incorporation of this phosphate into *Azotobacter* flavodoxin probably are not present in *E. coli* or, if they do exist, may not recognize this flavodoxin as a viable substrate. As demonstrated in this paper, the expression of the *Azotobacter* flavodoxin gene in *E. coli* results in a protein that has the same molecular weight, N-terminal sequence, and carboxyl-terminal amino acids as the native *Azotobacter* protein with the exception that it no longer contains a covalently bound phosphodiester linkage. Comparison of the spectral, redox, and FMN binding properties of this recombinant dephosphoprotein with those of the native phosphoprotein provides direct evidence for the concept of a structural role for the phosphodiester linkage in *Azotobacter* flavodoxin.

Materials and Methods

Bacterial Strains and Plasmids. Plasmids pDB93 and pDB230 were provided by Dr. Dennis Dean, Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA. Plasmid pDB93 was constructed by cloning a 2.0-kb *Xho*I restriction fragment (which encodes *nifF*) of *A. vinelandii* (strain OP) DNA into the *Sal*I restriction site of the plasmid vector pUC7 (Bennett et al., 1988). Plasmid pDB230 is comprised of the vector pKK223-3 (Brosius & Holy, 1984), containing the same *nifF* DNA fragment. *E. coli* strain DH5 (F^- *rec*A1 *hsd*R17 ($r_k^-m_k^-$) *sup*E44-*thi*-1 *gyr*A *rel*A1) was obtained from Dr. June Scott, Department of Microbiology & Immunology, Emory University School of Medicine. This strain was used as the host organism for plasmid pDB93. Transformation of pDB93 into this host strain of *E. coli* was performed by using the method published by Dagert and Ehrlich (1979). This bacterial system provided the recombinant flavodoxin used in all studies reported here using purified protein. For ESR experiments on whole cells and on cell-free extracts, plasmid pDB230 was transformed into *E. coli* strain RB791 (W3110 *lac8I*^{q}) (Frost et al., 1984) (this strain of *E. coli* was obtained from Dr. J. Knowles, Department of Chemistry, Harvard University). Construction of this *E. coli* transformant allowed induction and overproduction of recombinant flavodoxin by the addition of IPTG[1].

Bennett et al. (1988) have described the construction of a mutant of *Azotobacter vinelandii* (strain DJ130) in which the *nifF* gene has been replaced with an in-phase translational gene fusion of *nifF-lacZ*. Colonies of strain DJ130 do not express normal flavodoxin and appear blue on plates in which the β-galactosidase substrate 4-chloro-3-indolyl-β-galactoside has been added to a final concentration of 0.005% (w/v). A culture of this organism was generously provided by Dr. Dennis Dean, Virginia Polytechnic Institute and State University, and transformed with purified pDB93 DNA according to the method described by Page and von Tigerstrom (1979). Transformants that had lost their β-galactosidase activity were obtained at a frequency of 10^{-2}–10^{-3}. These transformants represent cells in which the *nif-lacZ* gene fusion has been replaced with the gene for OP flavodoxin. These cells were grown on a large scale (10 L) in nitrogen-free Burk's salts containing 2% (w/v) sucrose, the flavodoxin was isolated by published procedures (see below), and the purified protein was analyzed for its covalent phosphorus content.

Bacterial Cell Growth and Purification of Flavodoxin. E. coli DH5 containing plasmid pDB93 was grown at 37 °C in 10 L of LB broth containing 100 μg/mL ampicillin in a New Brunswick Fermenter with a stirring rate of 300 rpm and an aeration pressure of 15 psi for a period of 16 h. Cells were harvested by centrifugation at 8000*g* for 20 min at 4 °C and the resulting pellet was washed once by resuspension in 2 volumes of distilled water and recentrifuged. On the average, 50 g wet weight of cells was obtained from 10 L of culture. Flavodoxin purification was then performed by using the procedure described by Hinkson and Bulen (1967) for its isolation from *Azotobacter* with the modifications used by Edmondson and James (1979). The flavodoxin preparation, after the final Sephadex G-100 column, still contained a 20% protein impurity, which was identified as *E. coli* acyl carrier protein, which exhibits similar hydrodynamic properties as flavodoxin in spite of its smaller size (Rock & Cronan, 1981). Further purification was achieved by gradient chromatography on DE-52 in 50 mM Tris–acetate and 1 mM DTT, pH 8.0, with a NaCl gradient between 0.25 and 0.6 M. Flavodoxin eluted off the column at 0.45 M NaCl while the acyl carrier protein elutes at 0.33 M. We were also successful in resolving the two proteins by complexation of the flavodoxin with cytochrome *c* (Dickerson et al., 1985) under conditions of low ionic strength (5 mM Tris-acetate, pH 8.0) and chromatography on Sephadex G-50 in the same buffer. Cytochrome *c* is then simply removed from the flavodoxin by Chelex column chromatography, which strongly binds cytochrome *c* but not flavodoxin. The flavodoxin preparation was judged to be 99+% pure by the citeria of SDS–PAGE and visualization by silver staining.

Native *Azotobacter vinelandii* (strain OP, Berkeley) was purified from cells grown under nitrogen-fixing conditions described earlier (Edmondson & James, 1979).

IPTG Induction of Flavodoxin Expression. Two cultures of *E. coli* cells containing plasmid pDB230 (see above) were grown for 2 h in LB media at 37 °C in the presence of 100

μg/mL ampicillin. IPTG (1 mM final concentration) was added to one culture while an equal volume of sterile H_2O was added to the other. Both cultures were incubated for 15 h at 37 °C. Cells from both cultures were harvested by centrifugation and the resulting pellets washed once with distilled water before further manipulations.

Methods. SDS–polyacrylamide gel electrophoresis was run by using 15% gels according to the procedure of Laemmli (1970). Isoelectric focusing was carried out by using SERVALYT precote strips in the pH range 3–6 in accord with manufacturer's instructions. Amino-terminal sequence analysis was done by the Emory University microchemical facility using Applied Biosystem equipment. Carboxyl-terminal amino acids were determined after digestion with carboxypeptidase A (Sigma Chemical Co.) by using the procedure described by Allen (1981) as used by Deistung et al. (1985). Phosphorus analysis was performed by using the Bartlett (1959) procedure. Rocket immunoelectrophoresis was performed (Owen, 1985) by using rabbit polyclonal antisera raised against *Azotobacter* (OP Berkeley) flavodoxin by Pel Freeze Biologicals, Rogers, AR. The antibody was further purified by using a flavodoxin affinity column prepared from *Azotobacter* flavodoxin by the procedure described by Mayhew and Strating (1975) for *Megasphera elsdenii* flavodoxin. Circular dichroism spectra were recorded on an Aviv 60DS instrument. Quartz cells with a 10-mm path length were used in the visible and near-UV spectral region, and cells with a 1-mm path length were used in the far-UV spectral region. Fluorescence spectra, used in flavin binding studies, were recorded with a Perkin-Elmer MPF-44 instrument. Oxidation–reduction potentials were determined by using a spectrocoulometric apparatus similar in design to that described by Stankovich (1980) with equipment described previously (Williamson & Edmondson, 1985). ESR spectral data were recorded in 3-mm quartz tubes at 80 K using an IBM/Bruker ER 220 spectrometer equipped with an Oxford cryostat. Protein concentrations were determined by using the biuret procedure (Gornall et al., 1949). Flavodoxin concentrations (both native and recombinant) were estimated by using E_{452} =10600 M^{-1} cm^{-1} (Hinkson & Bulen, 1967); apoflavodoxin concentrations were estimated by their absorption at 280 nm ($E_M = 3.2 \times 10^4$) (D'Anna & Tollin, 1971).

Results

Properties of Recombinant Flavodoxin. The amount of recombinant flavodoxin purified from *E. coli* was always consistently larger than that isolated from *A. vinelandii* grown under nitrogen-fixing conditions. Our best preparation yielded 4.5 μmol of recombinant flavodoxin from 80 g wet wt of *E. coli* cell paste. In contrast, a similar amount of *A. vinelandii* cell paste would yield approximately 0.8 μmol of purified flavodoxin. These relative values are in agreement with estimates of flavodoxin content by rocket immunoelectrophoresis of cell extracts of *Azotobacter* and of *E. coli*, which showed a 4–5-fold higher level in transformed *E. coli* cells than in *Azotobacter* grown under nitrogen-fixing conditions. The reason for the high levels of expression in *E. coli* is probably the relatively high number of copies of the plasmid. The isolated recombinant flavodoxin contained 1 mol of FMN per mole of protein and exhibited an identical molecular weight and isoelectric point as the wild-type flavodoxin (Figure 1). The data in Figure 1 also demonstrate that the preparation of recombinant flavodoxin has the requisite purity for in-depth comparative chemical and physical studies with the native flavodoxin. The recombinant flavodoxin exhibits identical absorption spectral properties in the visible and near-UV region

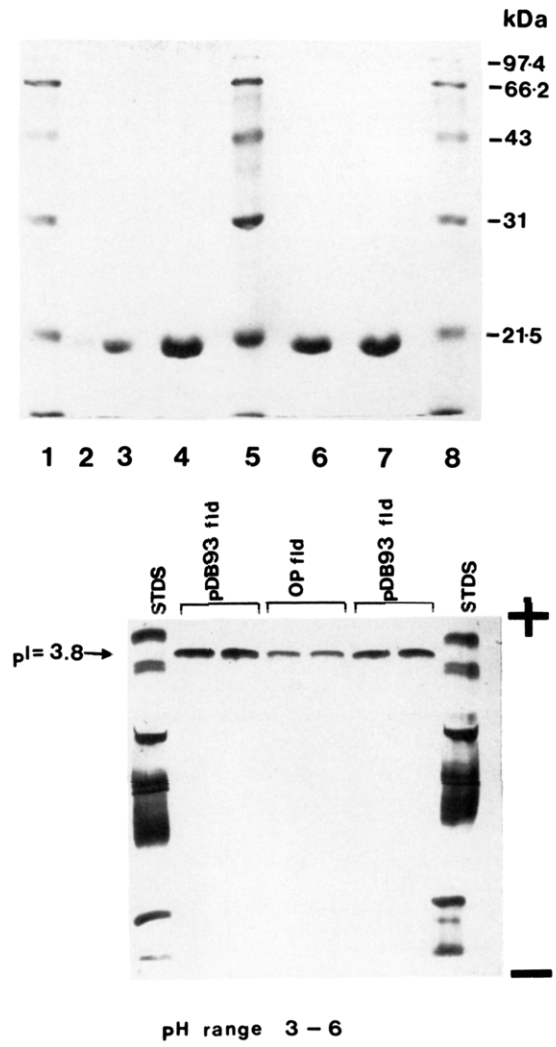

FIGURE 1: Electrophoretic comparison of native and recombinant flavodoxins. (Top) SDS–polyacrylamide gel electrophoresis of purified native and recombinant flavodoxins. A 15% gel matrix was used and the protein bands were detected by silver staining. Lanes 1, 5, and 8: molecular weight standards. Recombinant flavodoxin was loaded in lanes 2–4 with 2.2, 10.8, and 21.5 μg, respectively. Lane 7 contained 17 μg of flavodoxin isolated from *Azotobacter* (strain OP, Berkeley) and lane 6 contained 21.5 μg of flavodoxin isolated from *Azotobacter* (strain 478). (Bottom) Isoelectric focusing gel of preparations of purified recombinant and native flavodoxins. Lanes 1 and 8 contained standards; lanes 2 and 3 contained 7.5 μg and lanes 6 and 7 contained 5 μg each of recombinant flavodoxin; and lanes 4 and 5 contained 5 μg each of *Azotobacter* (strain OP, Berkeley) flavodoxin. Protein bands were detected by staining with Coomassie Blue.

as the native form. Amino-terminal sequencing studies resulted in the following sequence for the first 12 residues: Ala-Lys-Ile-Gly-Leu-Phe-Phe-Gly-Ser-Asn-Thr-Gly. This is identical with the sequence determined for a sample of wild-type flavodoxin and with the published sequences for this flavodoxin by either classical methods (Tanaka et al., 1977) or DNA sequencing (Bennett et al., 1988). Treatment of the apoprotein prepared from either recombinant or native flavodoxin with carboxypeptidase A results in the liberation of 2 mol of Leu and 1 mol each of Ser, Gly, Phe, and Glu. These experiments demonstrate the expected amino acids at the carboxyl terminal of the recombinant flavodoxin. Taken together, the above data provide convincing evidence that the recombinant flavodoxin expressed in *E. coli* has the same polypeptide chain as that isolated from *Azotobacter*.

When comparative rocket immunoelectrophoresis on recombinant and native flavodoxins was done by using antibody

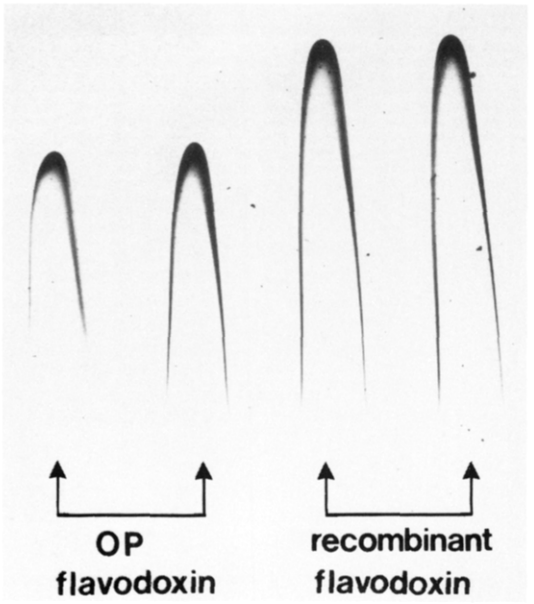

FIGURE 2: Rocket immunoelectrophoresis of native and recombinant preparations of *Azotobacter* flavodoxin. The rabbit antisera used was raised against the native flavodoxin. Identical quantities of each flavodoxin (0.3 μg) were loaded into each well. Visualization of the rockets was achieved by staining with Coomassie Blue.

Table I: Phosphorus Composition of Recombinant *Azotobacter* Flavodoxin

sample	phosphate (nmol)	phosphorus/protein (mol/mol)
22.1 nmol of flavodoxin[a] expressed in *E. coli*	21.9	0.99
17.8 nmol of flavodoxin[b] expressed in *E. coli* after FMN removal by trichloroacetic acid precipitation	0	0

[a] Average of four determinations with phosphate analysis ranging from 21.6 to 22.1 nmol. [b] Three determinations.

raised against the flavodoxin isolated from *Azotobacter*, the recombinant flavodoxin exhibited an increased antigenicity as compared to the native form. This is documented in Figure 2 where it is observed that the height of the rocket for the recombinant protein is 30% greater than that for the native protein when identical amounts of each protein were used in the experiment. These data suggest that the two proteins are not identical, which was verified when each sample was analyzed for phosphorus content. The native flavodoxin preparation contained 2 mol of phosphate; one is due to the FMN residue, which was dissociable from the protein on acid treatment, while the other was covalently bound and is due to the phosphodiester linkage between a seryl residue and a threonyl residue (Edmondson & James, 1979, 1982; Live & Edmondson, 1988) [the labile phosphate observed previously (Edmondson & James, 1979, 1982) was not present as it dissociates on storage and is removed by either dialysis or gel filtration]. Analysis of the recombinant flavodoxin for phosphorus content resulted in the finding of 1 mol of acid-dissociable phosphorus due to the FMN coenzyme and *no covalently bound phosphorus* (Table I). These data are consistent with our previous suggestion (Boylan & Edmondson, 1990) that phosphodiester incorporation into *Azotobacter* flavodoxin is a posttranslational process. Presumably, either *E. coli* does not have the enzyme(s) necessary for this post-translational modification or, alternatively, the flavodoxin is not a good substrate if such enzymes are present. As a control to demonstrate the lack of protein phosphorylation observed is *not* due to alterations in the flavodoxin gene as a result of

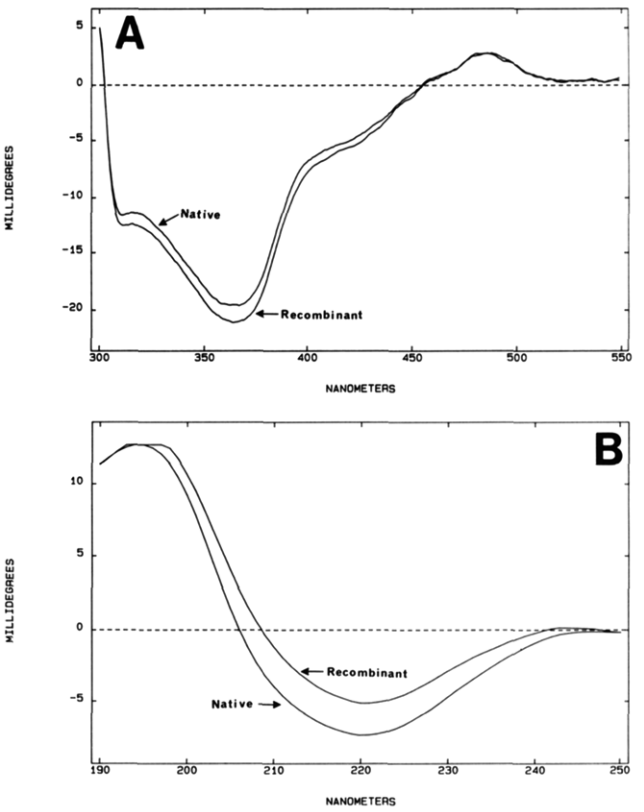

FIGURE 3: Circular dichroism spectra of native and recombinant *Azotobacter* flavodoxins in the visible and far-ultraviolet spectral regions. (A) Spectra in the 600–300-nm spectral region. The concentration of each flavodoxin sample is 45.7 μM in 50 mM Tris–acetate, pH 8.0, and each spectrum was measured in 10-mm quartz cells. (B) Far-ultraviolet spectra were measured in 1-mm quartz cells in 5 mM Tris–acetate, pH 8.0. The concentration of the flavodoxin samples used was 4.57 μM.

manipulations, the gene was transformed into strain DJ 130, a derivative of *Azotobacter vinelandii* (strain OP) in which the *nifF* gene was fused to the *lacZ* gene of *E. coli* near the N-terminal portion of the flavodoxin gene (Bennett et al., 1988) (see Materials and Methods). Transformation resulted in reinsertion of the flavodoxin gene into the bacterial chromosome. Isolation of the flavodoxin expressed from a large-scale growth of these cells under nitrogen-fixing conditions and analysis of the phosphorus content showed 1 mol of covalent phosphorus per mole of protein. This control experiment shows that the lack of covalent phosphorylation on expression of the flavodoxin gene in *E. coli* not to be due to any alterations in gene structure as a result of manipulations.

Spectral and Oxidation–Reduction Properties of the Recombinant Dephospho Flavodoxin. The possession of large quantities of pure dephospho flavodoxin permitted a number of experiments to compare its properties with the native phospho form, which should provide insights into the role of the phosphodiester linkage in the structure and redox properties of the flavodoxin. Circular dichroism spectroscopy has been used (Edmondson & Tollin, 1971) to monitor the environment of the optically inactive isoalloxazine ring of the bound FMN coenzyme in its asymmetric binding site in the apoprotein. The visible CD spectra of phospho and dephospho flavodoxin are shown in Figure 3A. The spectra are quite similar in shape and intensity, which shows that the binding site of the FMN coenzyme is only slightly perturbed (as judged by the small increase in intensity of the negative band at 365 nm for the dephospho flavodoxin). More significant alterations in CD properties are observed on comparison of the two flavodoxins

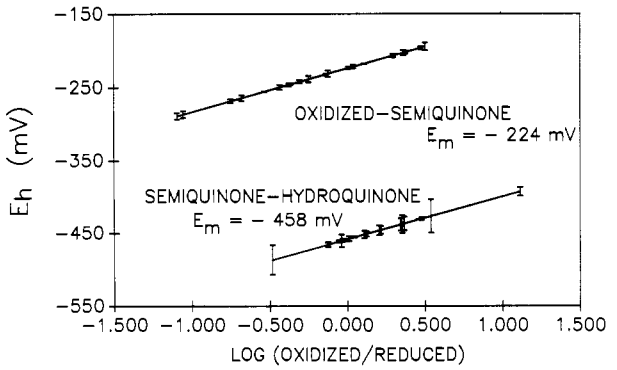

FIGURE 4: Nernst plot of oxidation–reduction potentials for the oxidized/semiquinone and semiquinone/hydroquinone couples of the FMN coenzyme of recombinant flavodoxin. All potentials were determined by using a Pt electrode and a Ag/AgCl reference electrode with the following mediator dyes: methyl viologen (2 μM) (E_m = –446 mV), 1,1′-propylene-2,2′-bipyridylium (10 μM) (E_m = –550 mV), anthraquinone-2-sulfonate (2 μM) (E_m = –225 mV), and indigo disulfonate (0.2 μM) (E_m = –170 mV). Mediator titrants used for the spectrocoulometric titrations were 1,1′-butylene-2,2′-bipyridylium (100 μM) (E_m = –640 mV) and potassium ferrocyanide (100 μM). Levels of flavodoxin reduction were determined spectrophotometrically. The buffer used in these experiment was 50 mM Tris–acetate, pH 8.0.

in the far-UV spectral region (Figure 3B). The negative band at 220 nm is more intense in the case of the native phospho flavodoxin than for the dephospho recombinant protein. These spectral data show an influence of the phosphodiester linkage on the structure of the protein and suggest its role to be structural.

Since *Azotobacter* flavodoxin is best known for the stability of its neutral FMN semiquinone and exhibits the lowest semiquinone/hydroquinone oxidation–reduction potential of any known flavoprotein, it was of interest to determine if any of the properties of the FMN semiquinone would be altered by the structural alterations in the protein observed on loss of the phosphodiester linkage. Illumination of an anaerobic solution of recombinant flavodoxin in the presence of 5-deazariboflavin and EDTA (Massey & Hemmerich, 1978) resulted in the formation of near-stoichiometric quantities of the neutral FMN semiquinone (data not shown). Continued illumination resulted in the further reduction of the flavodoxin to its hydroquinone form. These data show that the protein still stabilizes the formation of the FMN semiquinone and that the one-electron potentials are sufficiently separated to permit the essential stoichiometric one-electron reduction of the flavin without any observable comproportionation. The oxidation–reduction potentials were determined for the recombinant flavodoxin by using the spectrocoulometric method described by Stankovich (1981) and the Nernst plots of these data are shown in Figure 4. Reversible one-electron potentials were observed for each redox couple with the oxidized/semiquinone potential determined to be –224 mV and the semiquinone/hydroquinone potential measured to be –458 mV at pH 8.0. The oxidized/semiquinone couple determined for the recombinant dephosphoprotein is quite similar to that measured for the phospho form [–215 mV, Watt (1979), Williamson and Edmondson (unpublished data)] at pH 8.0. The semiquinone/hydroquinone couple measured for the dephosphoprotein is ~40-50 mV *higher* than the value measured for the native protein [–515 mV, Watt (1979); –500 mV, Williamson and Edmondson (unpublished data)] also at pH 8.0. This difference in potential is out of the range of experimental uncertainty and demonstrates that the presence of the phosphodiester linkage does influence the structure of the protein

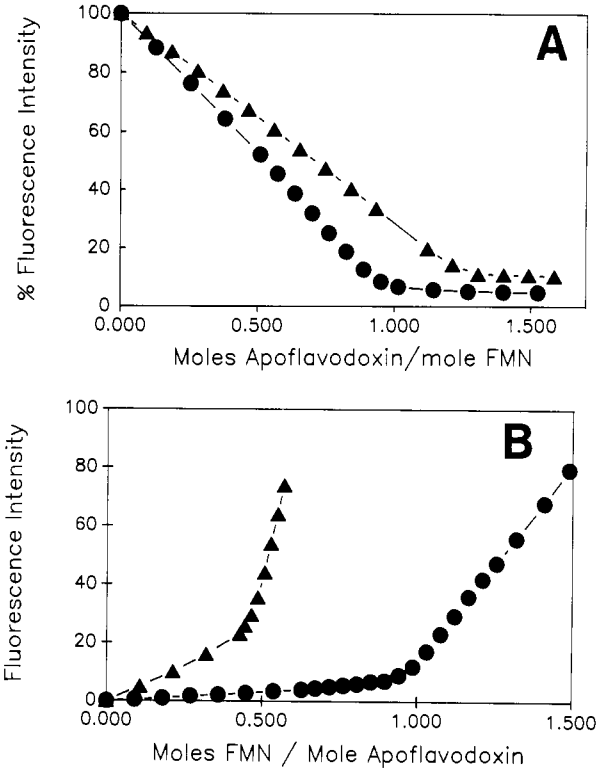

FIGURE 5: Fluorescence titrations to monitor the binding of FMN to the apoflavodoxins from native and recombinant flavodoxins. (A) Fluorescence quenching of a 1 μM solution of FMN in 10 mM potassium phosphate–0.1 mM EDTA, pH 7.1, upon the addition of microliter aliquots of apoflavodoxin [34.7 μM recombinant apoflavodoxin (▲) and 43.75 μM phospho apoflavodoxin (●)]. (B) FMN fluorescence on the addition of microliter aliquots of 34.3 μM FMN to dilute solutions of apoflavodoxins [1.16 μM recombinant apoflavodoxin (▲) and 1.46 μM phospho apoflavodoxin (●)]. The same buffer was used as in (A). All data points are corrected for dilution and all titrations were carried out at 25 °C in a darkened room.

such that the low-potential FMN redox couple is altered. From thermodynamic considerations, we calculate the FMN hydroquinone is bound with approximately 2-fold greater affinity to the recombinant dephospho flavodoxin than to the native protein.

Spectral and Flavin Binding Properties of Recombinant, Dephospho Apoflavodoxin. A property of *Azotobacter* flavodoxin that is shared by all other flavodoxins studied is the facile preparation of a stable apoprotein (Edmondson & Tollin, 1971), which reversibly binds FMN and many of its analogues. Lower molecular weight apoflavodoxins, as a rule (D'Anna & Tollin, 1972; Mayhew, 1971), do not bind riboflavin, whereas the higher molecular weight flavodoxins (e.g., *Azotobacter* flavodoxin) bind riboflavin with ~10^2–10^3 lower affinity than FMN (Mayhew & Ludwig, 1975). Apoflavodoxins were prepared from both the native and recombinant proteins by using the procedure described previously (Edmondson & Tollin, 1971). The apoflavodoxins were tested for their FMN binding capacity by monitoring the quenching of FMN fluorescence on the addition of aliquots of protein (Figure 5A). Recombinant apoflavodoxin is capable of binding FMN as judged by the quenching of FMN fluorescence in the titration shown in Figure 5A. The stoichiometry of recombinant apoflavodoxin required to reach an end point in the titration was 1.24 mol of protein/mol of FMN, whereas the stoichiometry exhibited by the native apoflavodoxin is 1:1 in a parallel experiment. These data suggest the presence of a fraction of the recombinant apoflavodoxin preparation (~20%) that has lost its ability to rebind FMN. The fraction

of the preparation with this lost ability increased with time after apoflavodoxin preparation (in contrast to the native apoflavodoxin) such that essentially all binding ability was lost after storage overnight at 4 °C. Under the same conditions, the native apoflavodoxin retained its FMN binding ability for several days. These data demonstrate that loss of the covalent, disubstituted phosphate from the protein results in a much more labile or unstable apoprotein.

The instability of recombinant apoflavodoxin is more apparent when a dilute solution of the protein (~1 μM) is titrated with a solution of FMN and the fluorescence due to the flavin is monitored (Figure 5B). The end point of the titration is observed at 0.47 mol of FMN/mol of recombinant apoflavodoxin as compared to a value of 0.97 for the phospho apoflavodoxin. In addition, the fluorescence observed for FMN bound to the dephospho apoflavodoxin is higher than that observed for the native protein or when a solution of FMN is titrated with a concentrated solution of recombinant apoflavodoxin. These data demonstrate that, on dilution, recombinant apoflavodoxin rapidly loses its FMN binding capacity, in contrast to the native phospho apoflavodoxin, and that the fraction of bound FMN exhibits an increase in fluorescence emission relative to that exhibited by the FMN bound by the phospho apoflavodoxin. Fluorescence experiments with unresolved recombinant flavodoxin showed no major differences from that of the native flavodoxin. Thus, in the experiment in Figure 5B, the recombinant apoflavodoxin fraction capable of binding FMN did not bind it in a manner identical with that of phospho apoflavodoxin or in the manner exhibited on addition of concentrated recombinant apoflavodoxin to an FMN solution. Due to the observed instability of the recombinant apoflavodoxin in dilute solutions, no quantitative studies on the binding affinity were carried out since these experiments would have required even more dilute solutions.

No binding of riboflavin by the recombinant apoflavodoxin was observed as judged by fluorescence quenching measurements under conditions where the native apoflavodoxin bound riboflavin with an affinity similar to that observed previously (Edmondson & Tollin, 1971; MacKnight et al., 1973) and where the recombinant apoflavodoxin bound FMN (cf. Figure 5A). A small perturbation of the CD spectrum of 60 μM riboflavin was observed on the addition of an equal quantity of recombinant apoprotein (estimated by the concentration of FMN combining sites), which suggests some weak interaction; however, this aspect was not investigated any further.

Previous CD studies of D'Anna & Tollin (1972) have shown that those apoflavodoxins that do not bind riboflavin undergo significant alterations in their far-UV CD spectral properties on resolution of the FMN moiety from the protein. In contrast, the changes in CD spectral properties on removal of FMN from those apoflavodoxins that do bind riboflavin are smaller. These experiments would suggest that an apoflavodoxin would bind riboflavin if the conformation of the apoprotein were not altered appreciably in comparison to the holoprotein. These considerations would suggest that the reason the dephospho apoflavodoxin does not bind riboflavin strongly is that the conformation of the apoprotein is altered appreciably on FMN resolution and that this alteration in conformation does not occur in the phospho flavodoxin. Previous CD spectral data on the native holoprotein and the apoprotein (Edmondson & Tollin, 1971) support this latter suggestion. The far-UV CD spectra shown in Figure 6 show that the recombinant apoflavodoxin exhibits a quite different spectrum than the phospho apoflavodoxin. The spectrum of the recombinant apoprotein shows (compared to that of the

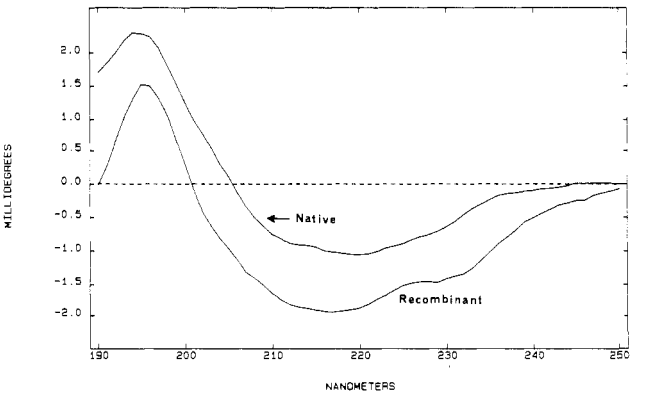

FIGURE 6: Comparison of far-ultraviolet circular dichroism spectrum of recombinant apoflavodoxin with that of apoflavodoxin prepared from native protein. Apoflavodoxin spectra were measured at 1 μM concentrations in 5 mM Tris–acetate, pH 8.0, in 1-mm quartz cells.

phospho apoflavodoxin) a diminished intensity in the 195-nm region, an increase in ellipticity in the 220-nm region, and a significant peak in the 220-nm region. These spectral differences are more marked than the differences observed in the far-UV region for the respective holoproteins (Figure 3B). Taken together, these combined flavin binding and CD spectral studies demonstrate that the phosphodiester linkage has a much larger influence on protein structure when the FMN coenzyme has been removed than when it is bound.

Redox Level of Recombinant Flavodoxin in E. coli. During initial stages of this work, we observed the recombinant flavodoxin band on the fractionation of crude cell extracts to DEAE columns was a greenish yellow color, which suggested its redox level in *E. coli* to be a reduced form rather than the oxidized form. To investigate this point further, the flavodoxin gene was expressed in *E. coli* under control of the *tac* promoter, which permitted manipulation of expressed flavodoxin levels on the addition of IPTG to a cell culture (see Materials and Methods). The cells isolated after exposure to IPTG were noticeably blue in color, which suggested that the flavodoxin expressed is at its neutral semiquinone redox level. ESR spectral data on whole cells and on cell extracts (Figure 7) confirm this suggestion. The $g = 2$ ESR signal is much more intense in cells and in extracts prepared from those cells exposed to IPTG than those grown in its absence. The peak-to-peak line width of the ESR signal is 20 G, which has been shown to be a characteristic of protein-bound neutral flavin semiquinones (Palmer et al., 1971). The intensity of the ESR signal does not change very much on cellular disruption, which is expected because the reactivity of the flavodoxin semiquinone with oxygen is slow. Taken together, these properties support the assignment of this ESR signal to the flavodoxin in its semiquinone form. Whether all of the expressed flavodoxin is in the semiquinone form remains to be determined. These data and consideration of the measured oxidation–reduction potentials for the two one-electron couples (Figure 4) demonstrate that the intracellular oxidation–reduction potential of this strain of *E. coli* grown under the conditions described is in the range −280 to −420 mV. In principle, one could monitor alterations in intracellular redox status by manipulations of extracellular redox substrate concentrations and monitor the intracellular potential by ESR spectroscopy.

Discussion

This study represents the second example of expression of a foreign flavodoxin gene in *E. coli* using recombinant DNA methodology with the first example being *Desulfovibrio vulgaris* flavodoxin (Krey et al., 1988). Recombinant *D.*

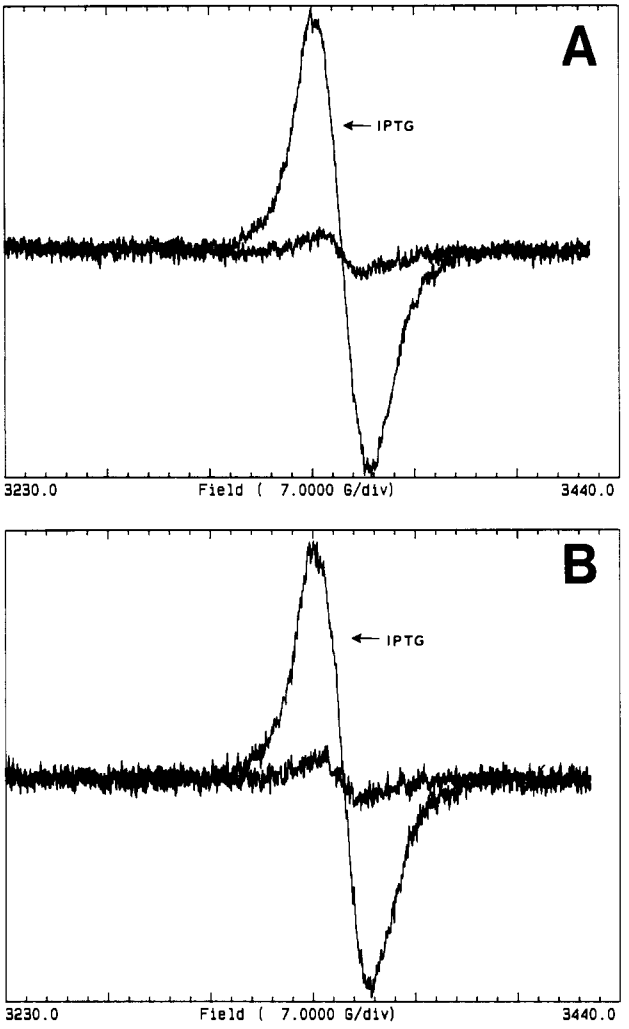

FIGURE 7: Electron spin resonance spectra of *E. coli* cells and cell extracts with and without IPTG induction of the flavodoxin gene (see Materials and Methods). (A) Cells were pelleted by centrifugation and resuspended in a equal volume of 100 mM HEPES, pH 7.5, and the resulting suspension was placed in 3-mm quartz ESR tubes and frozen in liquid nitrogen. The following spectral conditions were used: microwave power, 2 mW; modulation amplitude, 2 G; gain, 6.3×10^5; temperature, 82 K. (B) Cells from the same growths and the same quantity used in (A) were suspended in a equal volume of the buffer used in (A) and sonicated for 45 s by using a microtip probe at 0 °C. Cellular debris was removed by centrifugation and the supernatants were placed into quartz 3-mm ESR tubes and frozen. The spectral conditions used are as described for (A).

vulgaris flavodoxin is also expressed in a form with properties similar to that of the wild-type flavodoxin in that it has the same molecular weight, contains FMN, can be reduced to the semiquinone level, and contains the expected N-terminal amino acid (Krey et al., 1988). *D. vulgaris* flavodoxin apparently does not contain a covalent phosphodiester linkage (Favaudon et al., 1980; Vervoort et al., 1986) and thus the recombinant protein might be expected to exhibit properties identical with the native wild-type protein.

The spectral and redox potential data reported in this study show that the disubstituted phosphate in *Azotobacter* flavodoxin does influence the structural properties of the protein and the protein–flavin interactions that impart some of the unusual properties to the flavin such as the very low oxidation–reduction potential for the semiquinone/hydroquinone couple. Much more dramatic effects are observed for the role of the phosphodiester linkage on the structural stability and integrity of the apoprotein. Two dominant areas where effects are observed are (a) the stability of the apoprotein as measured by its FMN binding ability and (b) inability of the apoprotein to bind riboflavin. These results would support a general view of this cross-link as having a structural role in the protein, much as a disulfide bond is thought to function in protein structures in general. Oxidation–reduction potential data presented here demonstrate the ratio of FMN binding constants is unaffected for the oxidized and semiquinone forms on loss of the phosphodiester linkage but the FMN hydroquinone binding affinity is actually increased (relative to that of the semiquinone form) by a factor of 2. Preliminary studies show the recombinant flavodoxin to exhibit activity as an electron carrier from the light-5-deazaflavin system to the nitrogenase complex as monitored by the reduction of ethylene to acetylene (we are grateful to Dr. V. Morgan and Dr. L. Mortenson, University of Georgia, for assistance with these assays). Further work needs to be done to quantitatively compare the efficiency of the recombinant flavodoxin with that of the native flavodoxin in this assay.

Although the sequence location of this phosphodiester linkage has not yet been established, the similar mobilities observed on SDS–PAGE gels for the phospho and dephospho forms of the flavodoxin suggest the serine and threonine residues involved to be relatively near one another; otherwise the two proteins would exhibit different electrophoretic mobilities. Drummond (1986) has suggested, on the basis of computer graphics analysis, three very probable linkages: Ser^{9}–Thr^{54}, Ser^{129}–Thr^{130}, or Ser^{157}–Thr^{160}. The electrophoresis data would eliminate the first of the above possibilities but not the other two. Similarly, the four less probable possibilities listed in his paper would be eliminated in that they involve Ser/Thr residues remote from one another and would therefore be expected to influence the mobility of the phospho form on SDS gels.

If it turns out that the phosphodiester linkage does indeed link either adjacent Ser–Thr residues (e.g., Ser^{129}–Thr^{130}) or those residues separated by only two other residues (e.g., Ser^{157}–Thr^{160}), then it will be very interesting to study further how such a linkage could lead to such major alterations in protein structural stabilization. Although such a phosphodiester cross-link has not been detected in other high molecular weight flavodoxins (from other organisms) tested in our laboratory, it does not necessarily follow that other types of intramolecular cross-links do not exist. Unpublished work in this laboratory demonstrates the flavodoxin isolated from *Azotobacter vinelandii* (strain 478) does not contain a covalently bound phosphate but exhibits properties more similar to the phospho flavodoxin than to the recombinant dephospho form. Work is currently under way to determine whether or not a non-phospho cross-link is present in this flavodoxin, and, if so, examination of flavodoxins from other organisms for such linkages would be called for.

The availability of large quantities of the dephospho flavodoxin permits a number of new approaches not heretofore possible. It will now be possible to gain new insights into the role of this cross-link in protein structure by a number of physical and spectroscopic approaches. Furthermore, the dephospho protein will be quite useful as a substrate to assay for any enzyme(s) involved in phosphorylation and subsequently as a probe for the mechanism of phosphodiester incorporation. Studies along these lines are currently under way in our laboratory.

ACKNOWLEDGMENTS

The authors thank S. Husein for her assistance in experiments on the characterization of the flavodoxin expressed on transformation of the gene into the mutant strain of *Azoto-*

bacter vinelandii (DJ 130). We also thank Dr. Dennis Dean, Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, for providing the cloned flavodoxin gene and for the mutant form of *Azotobacter* that does not express flavodoxin.

References

Allen, G. (1981) *Lab. Tech. Mol. Biol. 9*, 28–42.
Bartlett, G. A. (1959) *J. Biol. Chem. 234*, 466–468.
Bennett, L. T., Jacobsen, M. R., & Dean, D. R. (1988) *J. Biol. Chem. 263*, 1364–1369.
Boylan, M. H., & Edmondson, D. E. (1990) *Biochem. J.* (in press).
Brosius, J., & Holy, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6929–6933.
D'Anna, J. A., & Tollin, G. (1971) *Biochemistry 10*, 57–64.
D'Anna, J. A., & Tollin, G. (1972) *Biochemistry 11*, 1073–1080.
Dagert, M., & Ehrlich, S. D. (1979) *Gene 6*, 23–28.
Deistung, J., Cannon, F. C., Cannon, M. C., Hill, S., & Thorneley, R. N. F. (1985) *Biochem. J. 231*, 743–753.
Dickerson, J. L., Kornuc, J. J., & Rees, D. C. (1985) *J. Biol. Chem. 260*, 5175–5178.
Dreef-Tromp, C. M., Erkelens, C., van der Marel, G. A., & van Boom, J. H. (1989) *J. Am. Chem. Soc. 111*, 6470–6471.
Drummond, M. H. (1986) *Eur. J. Biochem. 159*, 549–553.
Edmondson, D. E., & Tollin, G. (1971) *Biochemistry 10*, 124–132.
Edmondson, D. E., & James, T. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3786–3789.
Edmondson, D. E., & James, T. L. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) pp 111–118, Elsevier, Amsterdam.
Favaudon, V., LeGall, J., & Lhoste, J. M. (1980) in *Flavins and Flavoproteins* (Yagi, K., & Yamano, T., Eds.) pp 373–386, Japan Scientific Societies Press, Tokyo.
Frost, J. W., Bender, J. L., Kadonaga, J. T., & Knowles, J. R. (1984) *Biochemistry 23*, 4470–4475.
Gomez-Moreno, C., Sancho, J., Fillat, M., Pueyo, J. J., & Edmondson, D. E. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 335–339, W. de Gruyter & Co., Berlin.
Gornall, A. G., Bardawill, C. J., & Davids, M. M. (1949) *J. Biol. Chem. 177*, 751–766.
Hinkson, J. W., & Bulen, W. A. (1967) *J. Biol. Chem. 242*, 3345–3351.
Johnson, J. L., London, R. E., & Rajagopalan, K. V. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6493–6497.
Krey, G. D., Vanin, E. F., & Swenson, R. P. (1988) *J. Biol. Chem. 263*, 15436–15443.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Live, D. H., & Edmondson, D. E. (1988) *J. Am. Chem. Soc. 110*, 4468–4470.
MacKnight, M. L., Gillard, J. M., & Tollin, G. (1973) *Biochemistry 12*, 4200–4206.
Massey, V., & Hemmerich, P. (1978) *Biochemistry 17*, 9–17.
Mayhew, S. G. (1971) *Biochim. Biophys. Acta 235*, 289–302.
Mayhew, S. G., & Ludwig, M. L. (1975) *Enzymes (3rd Ed.) 12*, 57–118.
Mayhew, S. G., & Strating, M. J. J. (1975) *Eur. J. Biochem. 59*, 539–544.
Owen, P. (1985) in *Enterobacterial Surface Antigens: Methods for Molecular Characterization* (Korhonen, T. K. K., Dawes, E. A., & Makela, P. H., Eds.) pp 207–242, Elsevier, Amsterdam.
Page, W. J. & von Tigerstrom, M. (1979) *J. Bacteriol. 139*, 1058–1061.
Palmer, G., Muller, F., & Massey, V. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 123–150, University Park Press, Baltimore.
Rock, C. O., & Cronan, J. E. (1981) *Methods Enzymol. 71*, 341.
Stankovich, M. T. (1980) *Anal. Biochem. 109*, 295–308.
Tanaka, M., Haniu, M., Yasunobu, K. T., & Yoch, D. C. (1977) *Biochemistry 16*, 3525–3537.
Tollin, G., Brown, K., De Francesco, R., & Edmondson, D. E. (1987) *Biochemistry 26*, 5042–5048.
Vervoort, J., van Berkel, W. J. H., Mayhew, S. G., Müller, F., Bacher, A., Nielsen, P., & LeGall, J. (1986) *Eur. J. Biochem. 161*, 749–756.
Vetter, H., & Knappe, J. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 433–446.
Vogel, H. J. (1984) in *Phosphorus-31 NMR: Principles and Applications* (Gorenstein, D. G., Ed.) pp 105–154, Academic Press, New York.
Watt, G. D. (1979) *Anal. Biochem. 99*, 399–407.
Williamson, G., & Edmondson, D. E. (1985) *Biochemistry 24*, 7790–7797.