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Covalently bound non-coenzyme phosphorus residues in flavoproteins: ^{31}P nuclear magnetic resonance studies of *Azotobacter* flavodoxin

(phosphodiester linkage/covalent phosphorus in flavoproteins/pepsinogen/flavin mononucleotide phosphate)

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ABSTRACT **In addition to the 5′-phosphate ester on its flavin mononucleotide (FMN) moiety, flavodoxin from *Azotobacter vinelandii* contains 2 moles of tightly bound phosphate. One non-coenzyme phosphate group is covalently bound to the protein, as it remains with the protein on acid precipitation, whereas the other phosphate is released. The invariance of the ^{31}P nuclear magnetic resonance chemical shift of the covalently bound phosphate (−0.8 ppm relative to 85% phosphoric acid) with pH, even in the presence of protein denaturants, implies it is in a diester linkage to the protein. Because no evidence could be found for the presence of covalently bound sugars, nucleotides, or phospholipids, it is suggested that the phosphate residue forms a diester linkage with two hydroxyl amino acids in the protein. The only other suggestion of a phosphodiester linkage in proteins is from previous studies on pepsin and pepsinogen [Perlmann, G. E. (1955) *Adv. Prot. Chem.* 10, 1–30]. The observed changes in ^{31}P chemical shift with pH show that the covalent phosphorus in pepsinogen has ionization properties of a monoester rather than a diester. The ^{31}P resonance of the FMN phosphate occurs at −5.6 ppm in native *Azotobacter* flavodoxin. No ionization of the protein-bound FMN phosphate is observed since the chemical shift does not change appreciably in the pH range of 5.5–9.5. The chemical shift data suggest, but do not prove, that the coenzyme phosphate in its protein-bound form is dianionic. Chemical analysis of several other flavoenzymes from a variety of sources shows the presence of covalently bound phosphorus in quantities stoichiometric with the flavin content in most of the enzymes tested. Thus, the presence of covalent phosphorus in flavoenzymes may be a general phenomenon with currently unknown catalytic significance.**

Flavodoxins are low-potential electron-transfer flavoproteins which have been found in several sources of bacteria and algae. They have been intensively studied by a variety of chemical and physical techniques (for a recent review, see ref. 1) because of their ready availability, stability, and small size. X-ray crystallographic studies of *Clostridium MP* flavodoxin by Ludwig and colleagues (2, 3) and of *Desulfovibrio vulgaris* flavodoxin by Jensen and coworkers (4, 5) have provided an intimate description of the binding of the flavin mononucleotide (FMN) coenzyme in its three oxidation–reduction forms (oxidized, semiquinone, and hydroquinone) to the apoprotein. The results of these studies show the flavin molecule to be "buried" in the protein with only a benzenoid portion of the flavin ring system exposed to the solvent.

Although the flavodoxin from *Azotobacter vinelandii* has been extensively studied by spectral and chemical methods (6–10), its structure has not been investigated by x-ray crystallography. Structural information, therefore, must be inferred from comparison of its physical and chemical properties with those flavodoxins, the structures of which have been elucidated by x-ray crystallography. To provide direct information on the properties of the phosphate of the bound FMN in *Azotobacter* flavodoxin, we initiated ^{31}P nuclear magnetic resonance (NMR) studies.

In the course of these studies, we found that this flavodoxin contains 2 mol of tightly bound phosphate in addition to the phosphate ester of the FMN coenzyme. As documented in this paper, one phosphate residue is covalently bound to the protein, while the other can be released on acid treatment. Chemical and ^{31}P NMR studies strongly suggest the covalent phosphorus to be bound to the protein in a phosphodiester linkage.

MATERIALS AND METHODS

A culture of *A. vinelandii* (strain OP) was a gift from D. Yoch (University of California, Berkeley). The cells were grown under nitrogen-fixing conditions with sucrose as the sole carbon source. Flavodoxin was isolated from the crude cell extract by the method of Hinkson and Bulen (11), by use of either 25 mM sodium phosphate (pH 7.0) or 50 mM Tris acetate (pH 7.8) with the following modifications. The supernatant solution obtained from precipitation with 75% saturated $(NH_4)_2SO_4$ was applied to a DEAE-cellulose column equilibrated with 75% saturated $(NH_4)_2SO_4$ in the appropriate buffer and chromatographed according to the general principles described by Mayhew and Howell (12). After elution and concentration, the flavodoxin fraction was separated from salt and from any possible dimer (13) by chromatography on Sephadex G-100.

Porcine pepsinogen was obtained from Sigma and further treated by chromatography on Sephadex G-100. The following flavoenzymes were gifts from the following colleagues: *Megasphaera elsdenii* flavodoxin, V. Massey (University of Michigan); *Clostridium MP* flavodoxin, M. Ludwig (University of Michigan); microsomal NADPH-cytochrome *c* reductase, B. S. S. Masters (University of Texas, Dallas); soluble cardiac succinate dehydrogenase, B. A. C. Ackrell (this department); L-amino acid oxidase, C. Coles (this department); and thiamine dehydrogenase, C. Gomez-Moreno (this department).

Enzyme samples were prepared for NMR spectral experiments by lyophilization and were dissolved in ≈90% ^2H_2O. Paramagnetic metal impurities that would interfere with spectral properties were removed by passage of the enzyme solutions through small Chelex columns. Values of pH were uncorrected for the deuterium isotope effect at the glass electrode.

Total phosphorus concentrations were determined by the method of Bartlett (14). Absorption spectra were measured with a Cary 14 spectrophotometer interfaced to a Nova 2/4 minicomputer, utilizing the hardware and software supplied by On-Line Instrument Systems (Athens, GA). ^{31}P NMR spectra

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were obtained at 40.5 MHz on a Varian XL-100-15 spectrometer equipped with a Nicolet Fourier transform accessory and computer for data gathering and manipulation. Quadrature phase detection was used to enhance the signal-to-noise ratio. The spectrometer was locked on the deuterium resonance of ^2H_2O solvent in the sample. All chemical shifts were determined relative to an external standard of 85% phosphoric acid. All spectra were recorded at 25 ± 2°C, with 12-mm precision NMR tubes (Wilmad 514A-7PP).

RESULTS

Phosphorus Content of *Azotobacter* Flavodoxin. Our initial expectation was that the only phosphorus-atom present in *Azotobacter* flavodoxin would be that of the FMN coenzyme. Before embarking on the NMR spectral studies, we performed phosphate analyses to rule out any contamination of the enzyme samples with phosphate inasmuch as the flavodoxin was isolated in phosphate buffer and subsequently exchanged for Tris acetate buffer by chromatography on Sephadex. The analyses showed that *Azotobacter* flavodoxin contains 3 mol of phosphate per mol of protein (3.0 ± 0.06 SEM), regardless of whether phosphate or Tris buffers were used in the isolation procedure or whether different isolation procedures (11, 15) were used. Precipitation of the protein by treatment with 5% (wt/vol) trichloroacetic acid liberates the bound FMN, as well as a mole of non-FMN phosphorus. One mole of phosphorus (1.16 ± 0.05 SEM), however, remains with the protein even with extensive washing of the precipitate and is judged, therefore, to be covalently bound.

Attempts to release the bound phosphorus by extraction with various organic solvents were unsuccessful. This suggests that it does not arise from a tightly bound phospholipid that fails to dissociate from the protein moiety on acid precipitation. The flavodoxin gave a negative orcinol reaction, which shows the covalent phosphorus not to be a covalently bound sugar phosphate or nucleotide.

^{31}P NMR Spectra of the Holoprotein and Apoprotein. As expected from the phosphorus analytical data on the protein, three resonances are observed in the ^{31}P NMR spectrum of *Azotobacter* flavodoxin (Fig. 1). A single peak is seen at −5.6 ppm, a chemical shift characteristic for the dianionic form of phosphate. Two upfield peaks appear with chemical shift values of −0.8 and −0.2 ppm (Fig. 1). Thus, of the three phosphate residues in the native enzyme, it would seem that two are monoanionic and one dianionic. A comparison of the ^{1}H-coupled spectrum with the ^{1}H-decoupled spectrum (Fig. 1) shows that the resonances at −5.6 and −0.8 ppm are narrowed nearly 10 Hz with proton irradiation, suggesting that these two phosphate residues are in an ester linkage. Because of spectral overlap, the effect of proton decoupling on the linewidth of the −0.2 ppm resonance cannot be determined. The small peak apparent at −2 ppm was not present in most spectra and remains unidentified.

The three bound phosphates in *Azotobacter* flavodoxin do not ionize in the pH range of 5.5–9.5 since there was little or no effect of pH on the observed chemical shifts. The downfield resonance undergoes a 0.2 ppm change in chemical shift at pH 5.5 (from −5.6 to −5.4 ppm), as compared to that observed at pH 6.5 or higher. Flavodoxin begins to precipitate below pH 5.5, which precludes studies at lower pH values.

The information presented above necessitated the design of experiments to identify the phosphorus group responsible for each of the observed resonances. The most unequivocal method would be the stepwise removal of the phosphate residues from the flavodoxin and determination of the particular resonances that disappeared at each step. Precipitation of the protein with trichloroacetic acid removes the FMN as well as the noncovalent phosphate, leaving only the covalent phosphorus on the protein. This treatment produces an apoprotein capable of rebinding FMN (7). The ^{31}P NMR spectrum of the apoprotein is shown in Fig. 2. A single peak is observed at −0.9 ppm, corresponding to the covalently bound phosphorus. The apoprotein spectrum was obtained in 8 M urea since extensive aggregation and subsequent gel formation was observed in the absence of denaturants at the required protein concentration (1.15 mM). The chemical shift of the covalent phosphorus remains unchanged from that observed in the native protein even in the presence of 8 M urea at pH 8.0 (Fig. 2). These data show that this covalent residue is incapable of ionization to a form with a chemical shift characteristic of a phosphomonoester dianion and is probably attached to the protein via a phosphodiester linkage.

The problem of how to differentiate the FMN phosphorus resonance from the noncovalent phosphorus was simplified when it was found that aged flavodoxin preparations (3–4 months at −20°C) contained only 2 mol of phosphate after passage through a Sephadex G-25 column, one of which represents FMN and the other, the covalently bound phosphorus. The noncovalent, non-FMN phosphorus thus becomes labile on storage of the flavodoxin. The chemical group with which this noncovalent phosphate is associated in the native enzyme is not known; however, chemical analysis has shown that it is not inorganic phosphate, a sugar phosphate, a nucleotide, or a phospholipid. The ^{31}P NMR spectra of the aged protein

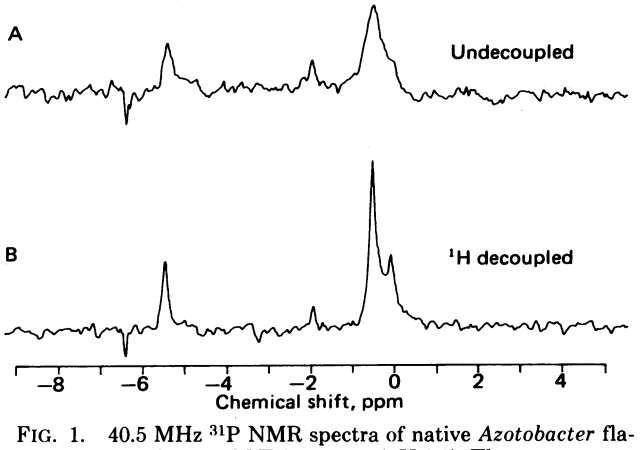

FIG. 1. 40.5 MHz ^{31}P NMR spectra of native *Azotobacter* flavodoxin (1.5 mM) in 0.1 M Tris acetate (pH 8.0). The spectra were obtained from 14,000 transients with a pulse repetition time of 0.34 sec and an exponential line broadening of 2.0 Hz. (*A*) Spectrum with no proton decoupling; (*B*) spectrum with broadband proton decoupling.

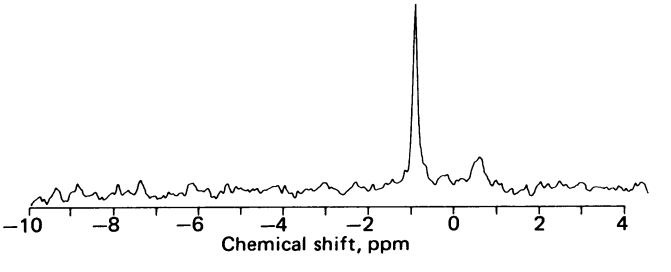

FIG. 2. 40.5 MHz ^{31}P NMR spectrum (proton-decoupled) of *Azotobacter* apoflavodoxin (1.15 mM) in 8 M urea/0.1 M Tris acetate (pH 8.0). The spectrum was obtained under conditions of Fig. 1 except that 11,000 transients were acquired.

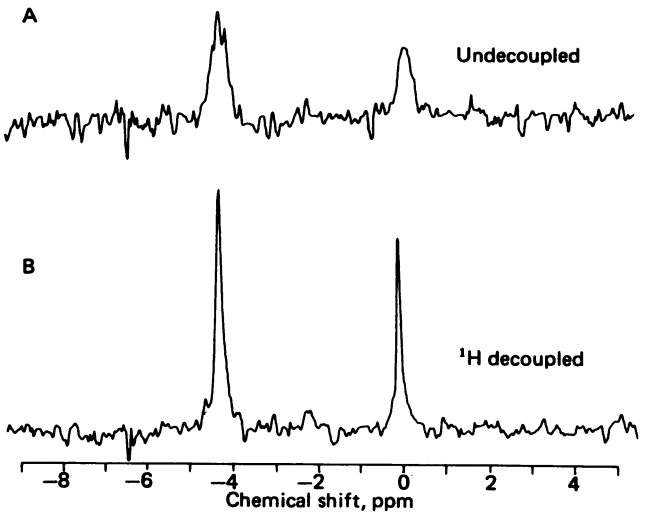

FIG. 3. 40.5 MHz ^{31}P NMR spectra of *Azotobacter* flavodoxin after denaturation in 3 M guanidine/0.1 M Tris acetate (pH 8.0). See Fig. 1 for spectral parameters. (*A*) Spectrum with no proton decoupling; (*B*) spectrum with broadband proton decoupling.

showed one peak at −5.6 ppm and one peak at −0.8 ppm. The resonance in the native protein at −5.6 ppm is, therefore, due to the FMN.

Denaturation of the flavodoxin in 3 M guanidine·HCl at pH 8.0 results in an intensification of the downfield phosphorus resonance, as well as small changes in chemical shift. The spectrum in Fig. 3 shows that the noncovalent, non-FMN phosphate dissociates from the protein and becomes dianionic (the pH of the medium is 8.0). As observed with the apoprotein (Fig. 2), the chemical shift of the covalent phosphate remains unaltered at pH 8.0 under denaturing conditions, as expected for a phosphodiester linkage. The ^{1}H-coupled spectrum (Fig. 3) shows significantly broader linewidths compared with the proton-decoupled spectrum. The splitting of the FMN phosphate resonance (−4.3 ppm) into a triplet by the 5′-CH_2 protons is apparent and suggests the FMN to be dissociated from the protein, since no splitting is observable in the ^{1}H-coupled spectrum of the native protein (Fig. 1). Confirmation of FMN dissociation from the protein is provided by the chemical shift of −4.3 ppm, which is the ^{31}P chemical shift found for a sample containing solely FMN at pH 8.0.

Covalent Phosphorus Content of Other Flavoenzymes. In view of the finding that *Azotobacter* flavodoxin is a phosphoprotein, it was of interest to determine if other flavodoxins and other flavoenzymes also contained covalently bound phosphorus. The data in Table 1 show that, in contrast to the *Azotobacter* flavodoxin, the flavodoxins from *M. elsdenii* and *Clostridium MP* do not contain covalent phosphorus groups. Covalent phosphorus groups are observed, however, in the enzymes L-amino acid oxidase, glucose oxidase, thiamine dehydrogenase, and liver NADH-cytochrome *c* reductase (Table 1). In each case the covalent phosphorus content is stoichiometric with the flavin content. Although these data do not show whether the structure of the covalent phosphorus present in these enzymes is a monoester or a diester, they do show that the presence of covalent phosphorus residues in flavoenzymes may be a more general occurrence than heretofore thought.

Table 1. Covalent phosphorus content of several flavoenzymes after acid precipitation

Enzyme	Source	Moles P_i/mole enzyme-flavin
M. elsdenii flavodoxin	Bacteria	0
Clostridium MP flavodoxin	Bacteria	0
L-Amino acid oxidase	Snake venom	1.1
Glucose oxidase	Mold	1*
NADPH-cytochrome *c* reductase	Mammalian liver	1.0
Thiamine dehydrogenase	Bacteria	1.0†
Succinate dehydrogenase	Mammalian heart	0†

* From ref. 16.

† Moles of phosphate found in addition to the 2 moles of phosphate contained in the covalently bound FAD.

DISCUSSION

The ^{31}P resonance peak at −5.6 ppm observed with *Azotobacter* flavodoxin has been assigned to the bound FMN phosphate. Because the ^{31}P chemical shift of the dianionic form of the FMN phosphate (pH 8.0) is at −4.3 ppm and that of the monoanionic form (pH 5.5) is at −0.9 ppm, the data suggest the protein-bound FMN phosphate to be dianionic. Recent data in the literature for other phosphoproteins and model compounds also support this suggestion (17, 18). ^{31}P NMR studies on alkaline phosphatase (18) have shown a covalent phosphorus residue with a chemical shift of −8.5 ppm at low pH. This anomalous downfield shift was attributed to strain in the P-O bond angles of the phosphoserine at the active site. Subsequent studies (19) on alkaline phosphatase confirmed that observation but noted that the presence of a bound metal ion in the enzyme contributed to the downfield shift. Because of the possibility that interaction of the FMN phosphate with the protein could induce distortion of the O-P-O bond angles, resulting in a downfield chemical shift (20), the assignment of a dianionic phosphate ester on the bound FMN of *Azotobacter* flavodoxin from chemical shift data alone cannot be considered unequivocal. Studies of FMN binding to *Azotobacter* apoflavodoxin (6) show the K_a to be independent of pH from 4.5 to 8.0. This would imply that either ionic form can be bound with equal affinity.

The finding of a covalent and of a tightly bound, noncovalent phosphorus residue in addition to FMN in *Azotobacter* flavodoxin was unexpected. The invariance of chemical shift with pH even in the presence of protein denaturants suggests strongly that the covalent phosphate is linked to the protein via a diester linkage. Because no evidence was found for sugars, nucleotides, or phospholipids, we propose the diester linkage to be between two hydroxyl amino acids on the protein.

Because the data indicate the presence of a phosphodiester linkage in *Azotobacter* flavodoxin, it was desirable to examine the ^{31}P NMR of another protein suspected of containing such a group. Previous enzymatic dephosphorylation studies on pepsin and pepsinogen suggested the covalent phosphorus to be linked to the protein in a diester linkage (21, 22); however, more recent dephosphorylation studies indicated, in contrast, a monoester linkage (23). The results unequivocally show the covalent phosphorus in pepsinogen to be in a monoester linkage because the ^{31}P chemical shift was found to be −0.4 ppm at pH 5.0 and −4.0 ppm at pH 8.0. A diester would not show the observed downfield ^{31}P chemical shift as the pH is increased from 5.0 to 8.0. The ^{31}P chemical shifts found for pepsinogen are identical to those of *O*-phosphoserine and are within 0.2 ppm of those found for the phosphoserine in phosvitin at low and at high pH values (16). Although the prosthetic group in citrate lyase has been shown to be attached to the enzyme via a phosphodiester linkage (24, 25), *Azotobacter* flavodoxin is the only known protein suspected of containing a phosphodiester between two amino acid residues of its polypeptide chain.

It remains for future work to isolate and to determine the amino acid sequence of the peptide containing the proposed phosphodiester structure in *Azotobacter* flavodoxin. Similarly, it remains for future work to determine whether the covalent and noncovalent phosphates play a role in the biological function of *Azotobacter* flavodoxin in mediating electron transfer to the nitrogenase complex (26, 27) or, alternatively, serve to maintain the structural integrity of the protein. The fact that only the scattering of the FMN phosphorus is observed in x-ray data from crystals of *Clostridium MP* and *D. vulgaris* flavodoxins (2–5) suggests that no other phosphorus groups are present in these flavodoxins. Indeed, chemical analysis of *M. elsdenii* and *Clostridium MP* flavodoxins shows only 1 mol of phosphorus per mole of protein, which is due to the bound FMN, while the respective apoproteins do not contain covalent phosphorus (Table 1). Previous far-ultraviolet circular dichroism data have shown large differences in protein folding between the holo- and apoproteins of *M. elsdenii* and *C. pasteurianum* flavodoxins, while small differences are observed for the flavodoxins from *A. vinelandii*, *Rhodospirillum rubrum*, and *D. vulgaris* (7, 28). It is quite possible that the presence of a phosphodiester linkage would stabilize the secondary structure of the protein in much the same way that disulfide bonds serve to stabilize protein structure. In this regard, none of the flavodoxins listed has intramolecular disulfide bonds (1).

From the data presented in Table 1, it appears that covalently bound phosphorus in flavoenzymes may be more common than has been heretofore recognized. The stoichiometric ratio of covalent phosphorus to flavin content (either FMN or FAD) suggests that its presence is unlikely to be coincidental. It remains for future work to determine the structure (monoester or diester linkage) and the role of these covalent phosphorus residues in enzymic catalysis.

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