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Studies on the incorporation of a covalently bound disubstituted phosphate residue into *Azotobacter vinelandii* flavodoxin *in vivo*

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Previous studies have shown the flavodoxin from *Azotobacter vinelandii* (strain OP, Berkeley) to contain a covalently bound disubstituted phosphate residue [Edmondson & James (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 3786–3789]. Phosphorylation of the protein *in vivo* was investigated by the addition of [^{32}P]phosphate to cells grown under N_2-fixing conditions, under conditions of *nif*-gene repression and under conditions of *nif*-gene de-repression. Rocket immunoelectrophoresis of cell extracts showed an approx. 5-fold decrease in the concentration of flavodoxin expressed in cells grown in the presence of NH_4^+ as compared with those grown under N_2-fixing conditions. A similar increase in flavodoxin concentration was observed on *nif*-gene de-repression. Incorporation of [^{32}P]phosphate occurs only into newly synthesized flavodoxin, as observed on SDS/PAGE of immunoprecipitates of cell extracts. Western blots demonstrated no observable precursor forms of flavodoxin. These data provide conclusive evidence for the phosphorylation of *Azotobacter* strain OP flavodoxin *in vivo* and suggest that the covalently bound phosphate residue does not exchange with cellular phosphate pools. Thus the role of this phosphodiester cross-link is proposed to be structural rather than regulatory.

INTRODUCTION

Flavodoxins are a class of low-M_r low-potential FMN-containing proteins that function as electron carriers in a number of metabolic functions in bacteria [1]. In general, their synthesis is induced to replace ferredoxin functionally in response to conditions of iron limitation in the growth medium. The flavodoxin from *Azotobacter vinelandii* appears to be an exception in that it is a constitutive protein in the cell and its synthesis is relatively unaffected by the iron content of the growth medium. Its synthesis (*nifF* expression) is affected, however, by the nitrogen content of the medium, as is the synthesis of the other proteins associated with the N_2-fixation pathway in *Azotobacter* [2–4]. The control of flavodoxin synthesis by the nitrogen content of the medium and the demonstration that it can transfer electrons to the Fe protein in support of nitrogenase activity has led to the general view of its physiological function as an electron-transfer protein in cellular N_2 fixation [5]. Recent studies on *nifF*$^-$ mutants of *Azotobacter vinelandii* have shown that N_2 fixation still occurs, but at a decreased rate, compared with wild-type cells [4].

Previous ^{31}P-n.m.r. studies on *Azotobacter* (strain OP, Berkeley) flavodoxin [6] demonstrated the presence of a single covalently bound disubstituted phosphate group in this protein. Chemical degradation studies [7] showed the covalently bound phosphate residue to be in a diester linkage between a serine and a threonine residue in the protein. Further evidence for this unusual structure has been found by means of two-dimensional multiquantum ^{1}H–^{31}P-n.m.r. experiments [8]. Experiments to test whether this unusual covalently bound phosphate group exists in other known flavodoxins have to date been negative. Of the known flavodoxins only those from *Rhodospirillum rubrum* [9] and from *Klebsiella* [10,11] have not been tested, at least in our laboratory. Although a number of flavoenzymes and metalloflavoenzymes have been shown to be phosphorylated [6,12], only *Aspergillus niger* gluycose oxidase has been shown to contain a disubstituted phosphate residue [13], although the amino acid residues involved in the phosphate linkage have not been characterized. The phosphate residue, however, has been shown to be protein-bound rather than bound to the carbohydrate portion of the molecule.

In general, protein phosphorylation has been found to be involved with regulation of cellular activity in proteins from both prokaryotes and eukaryotes. Structural information on these systems has shown the mode of phosphorylation to involve monosubstitution of the covalently attached phosphate group (*O*-phosphoserine, *O*-phosphothreonine or *O*-phosphotyrosine) and, in cases involving a disubstituted phosphate group, the process involves adenylation of a specific residue (cf. ref. [14]) or *O*-phosphopantetheinyl-serine, as found in the acyl-carrier protein of bacteria, yeasts and higher plants [15]. The chemical and n.m.r. data available to date on *Azotobacter* flavodoxin show that the covalently bound phosphate residue is not due to adenylation of an amino acid side chain.

The work to date suggests, but does not prove, that this covalently bound disubstituted phosphate residue in *Azotobacter* flavodoxin has a structural rather than a regulatory role. Arguments against a regulatory role include (1) the finding of 1 mol of acid-precipitable P/mol of protein at various stages of cell growth and (2) the fact that, if the role of the phosphate group were regulatory, it might be expected to be in a monosubstituted linkage or an adenylated linkage.

In an effort to probe the mechanism of incorporation and possible role of this unusual phosphate linkage, we report in the present paper studies of the phosphorylation of *Azotobacter* flavodoxin *in vivo*. The results demonstrate that (1) only newly synthesized protein is phosphorylated, (2) there does not appear to be any exchange *in vivo* of protein-bound phosphate with exogenously added phosphate and (3) protein biosynthesis appears to precede its phosphorylation. These findings support the notion that the role of the covalently bound phosphate group in *Azotobacter* flavodoxin is structural rather than regulatory. A preliminary report of this work was presented at the 1988 Meeting of the American Society of Biological Chemistry and Molecular Biology, Las Vegas, NV, U.S.A.

MATERIALS AND METHODS

Materials

Azotobacter vinelandii (strain OP, Berkeley) flavodoxin was purified as described previously [6,16] from cells grown under N_2-fixing conditions. Rabbit polyclonal antiserum raised against

Azotobacter flavodoxin was prepared by Pel-Freez Biologicals, Rogers, AR, U.S.A., and was further purified by chromatography on a flavodoxin affinity column before use [17]. Pre-immune serum was used as a control in all experiments requiring antiserum. Protein concentrations were determined by the biuret procedure [18], with BSA (Sigma Chemical Co., St. Louis, MO, U.SA.) as a standard. $K_2H^{32}PO_4$ (200 mCi/mmol) was purchased from Amersham International, Arlington Heights, IL, U.S.A.

Cell growth

Azotobacter vinelandii (strain OP, Berkeley) was grown batchwise in liquid medium containing Burk's salts and 2% (w/v) sucrose as the sole carbon source [19]. Under growth conditions where *nif*-gene repression was required, a filter-sterilized solution of ammonium acetate was added to the sterile medium to a final concentration of 28 mM [3]. All incubations were performed aerobically with agitation at 30 °C.

Radiolabelling of flavodoxin with ^{32}P

Azotobacter vinelandii was grown to mid-exponential phase either in nitrogen-free medium or in the presence of 28 mM-ammonium acetate. At mid-exponential growth stage the cells were centrifuged down, washed twice with distilled water to remove NH_4^+ and resuspended to the original cell density in Burk's medium containing 0.1 mM-phosphate. After resuspension, 76 μCi of [^{32}P]phosphate was added to the growth medium. All time measurements are relative to the zero-time point of the addition of [^{32}P]phosphate to the medium. Ammonium acetate was added to a final concentration of 28 mM to certain samples in order to maintain repression of the synthesis of proteins associated with N_2 fixation. Cells were incubated at 30 °C and samples were removed at 45 min, 90 min and 180 min. After being washed twice in 50 mM-Tris/HCl buffer, pH 7.5, the cells (0.05 g wet wt.) were suspended in 1.0 ml of the above buffer and were broken by sonication for three 15 s periods at 0 °C with the micro-tip probe of a Heat Systems Ultrasonics model W-385 sonicator. The resulting cell debris was removed by centrifugation at 12000 *g* for 15 min. Cell-free extracts thus obtained were analysed either directly by rocket immunoelectrophoresis or by SDS/PAGE after immunoprecipitation as described below.

Electrophoretic techniques

SDS/PAGE was performed by the procedure of Laemmli [20]. Proteins were detected by autoradiography and/or by staining with Coomassie Brilliant Blue. Western blotting was carried out by the procedure of Burnette [21] with BA85 nitrocellulose filters (pore size 0.45 μm; Schleicher and Schuell, Keene, NH, U.S.A.). Alkaline-phosphatase-conjugated goat anti-(rabbit IgG) antibody (Sigma Chemical Co.) was used as second antibody in Western blotting. Rocket immunoelectrophoresis was performed at 4 °C in 1% (w/v) agarose (Seakem grade LE; FMC Bioproducts, Rockland, ME, U.S.A.) in barbital hydrochloride buffer, pH 8.6, by using published procedures [22].

Immunoprecipitation of flavodoxin

Pilot experiments involving manipulation of antigen/antibody ratios and times of reactions established the following protocol for immunoprecipitation of flavodoxin. Anti-flavodoxin antibody was preincubated for 60 min at 25 °C with the following proteinase inhibitors: aprotinin (final concentration in the reaction mixture 2 trypsin-inhibitor units/ml), benzamidine hydrochloride (2 mM) and phenylmethanesulphonyl fluoride (2 mM). The treated antiserum was clarified by centrifugation at 12000 *g* in a Microfuge for 15 min. Cell-free extracts of *Azotobacter vinelandii* (100 μg of protein) prepared after incubation in [^{32}P]phosphate-containing Burk's medium were incubated with 15 μl volumes of antiserum for 15 h at 20 °C. To each mixture was added 5 μl of goat anti-(rabbit IgG) antibody and samples were incubated at 20 °C for 6 h. Immunoprecipitates were harvested by centrifugation at 12000 *g* for 15 min and washed once with 200 μl of 100 mM-NaCl. Washed immunoprecipitates were resuspended in the sample buffer of Laemmli [20].

RESULTS

In order to study the incorporation of phosphate into flavodoxin, it was essential to be able to control the level of protein synthesis. Previous work [23] has shown (in A.T.C.C. strain 478) that flavodoxin synthesis is 10-fold greater in cells grown under N_2-fixing conditions than from cells grown in the presence of NH_4^+. Bennett *et al.* [4] also demonstrated, using a *nifF–lacZ*-gene-fusion strain of *Azotobacter vinelandii* (strain OP), that flavodoxin synthesis (as measured by β-galactosidase activity) increased 2-fold on de-repression of the *nif* genes. In the present study, in order to maintain a high specific radioactivity of [^{32}P]phosphate incorporation, cells were incubated on low concentrations of phosphate. Flavodoxin synthesis was manipulated by alterations in the NH_4^+ content of the medium.

Two cell cultures were grown in the presence of NH_4^+ and one culture was grown in nitrogen-free medium. At the mid-exponential phase of growth cells were harvested and subjected to the following conditions. For culture I, *nif*-gene repression in cells grown in the presence of NH_4^+ was continued by resuspension in NH_4^+-containing medium (these cells are referred to below as *nif*-gene-repressed cells). For culture II, cells grown in nitrogen-free medium were resuspended in a nitrogen-free medium (these cells are referred to below as N_2-fixing cells). For culture III, cells from a *nif*-gene-repressed culture were de-repressed by suspension in a nitrogen-free medium (these cells are referred to below as *nif*-gene-de-repressing cells). In *nif*-gene-de-repressing cells flavodoxin synthesis should be greatly enhanced as a result of de-repression of *nif*-gene expression, whereas in *nif*-gene-repressed cells flavodoxin synthesis should remain low. N_2-fixing cells served as a control sample in which flavodoxin synthesis occurs both before and after cell manipulation.

Rocket immunoelectrophoresis of crude cell extracts was performed to determine flavodoxin concentrations in the three conditions of cell growth. As shown in Fig. 1 (*a*)(i), only N_2-fixing cells grown continuously in nitrogen-free medium exhibit an observable flavodoxin content 45 min after transfer to the low-phosphate media. Similarly, small amounts of radioactivity are observed on autoradiography of this rocket [Fig. 1(*b*)(i)], which suggests that no exchange of phosphate occurs between mature flavodoxin and cellular phosphate pools. Incubation of cells for longer periods of time [90 min and 180 min; see Figs. 1(*a*)(ii) and 1(*a*)(iii)] shows that in *nif*-gene-de-repressing cells that have been de-repressed for N_2 fixation extensive flavodoxin synthesis has taken place by 90 min and has not apparently increased further by 180 min [Figs. 1(*a*)(ii) and 1(*a*)(iii), lanes 3]. In fact, the extents of expression are comparable with the amount observed in N_2-fixing cells grown continuously in nitrogen-free medium [Figs. 1(*a*)(ii) and 1(*a*)(iii), lanes 2]. Autoradiography [Fig. 1(*b*)] shows that [^{32}P]phosphate incorporation into flavodoxin has occurred in the de-repressed cells, with the levels of intensity corresponding to increased concentrations of newly formed flavodoxin after exposure of the cells to [^{32}P]phosphate. These results would support the view that

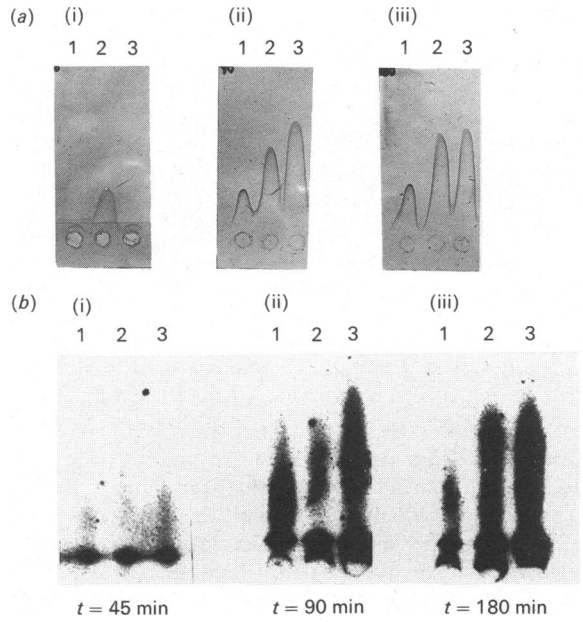

Fig. 1. Rocket immunoelectrophoresis of crude cell extracts prepared from *Azotobacter vinelandii* grown under conditions of N_2-fixing, *nif*-gene repression and *nif*-gene de-repression

Panel (*a*) shows rockets visible after staining with Coomassie Blue. Panel (*b*) is an autoradiogram of the stained rockets. (i), (ii) and (iii) correspond to extracts prepared from cells removed at $t = 45$ min, 90 min and 180 min respectively after the addition of [^{32}P]phosphate. Lanes 1, extract of *nif*-gene-repressed cells; lanes 2, extract of N_2-fixing cells; lanes 3, extract of *nif*-gene-de-repressed cells. Each lane was loaded with 10 μg of protein.

[^{32}P]phosphate incorporation takes place only on synthesis of flavodoxin and that, once synthesized, the covalently bound phosphate does not exchange with cellular phosphate pools. These considerations would exclude a regulatory role for the covalently bound phosphate residue.

To identify the phosphorylated protein further as flavodoxin and to probe for any possible precursor forms of flavodoxin, SDS/PAGE was performed on immunoprecipitates from extracts of cells grown as described above (Fig. 2). Both protein staining and autoradiography showed a band corresponding to the M_r of flavodoxin in *nif*-gene-de-repressing cells. This band increased in intensity up to 180 min under both methods of detection [Figs. 2(*a*) and 2(*b*), lanes 3]. Of interest is the finding that flavodoxin in N_2-fixing cells exhibited a flavodoxin band detectable by protein staining but not by autoradiography. Only small amounts of ^{32}P radioactivity incorporated into the protein were observed 180 min after the addition of [^{32}P]phosphate to the cells [Fig. 2(*b*)(iii), lane 2]. These data demonstrate more definitely that phosphate incorporation occurs upon flavodoxin synthesis and that no phosphate exchange is observed with the mature protein. As expected, the concentrations of flavodoxin (as judged both by protein staining and by autoradiography) were considerably higher in *nif*-gene-de-repressing cells than in *nif*-gene-repressed cells.

In addition to flavodoxin, a number of other proteins (both phosphorylated and non-phosphorylated) are observed in the immunoprecipitates. Their presence may be due to the association of flavodoxin with other proteins by non-covalent forces that are strong enough to remain with the protein on immunoprecipitation and subsequent washings. These experimental results demonstrate the importance of multiple approaches in the analysis of immunoprecipitates that may contain substantial

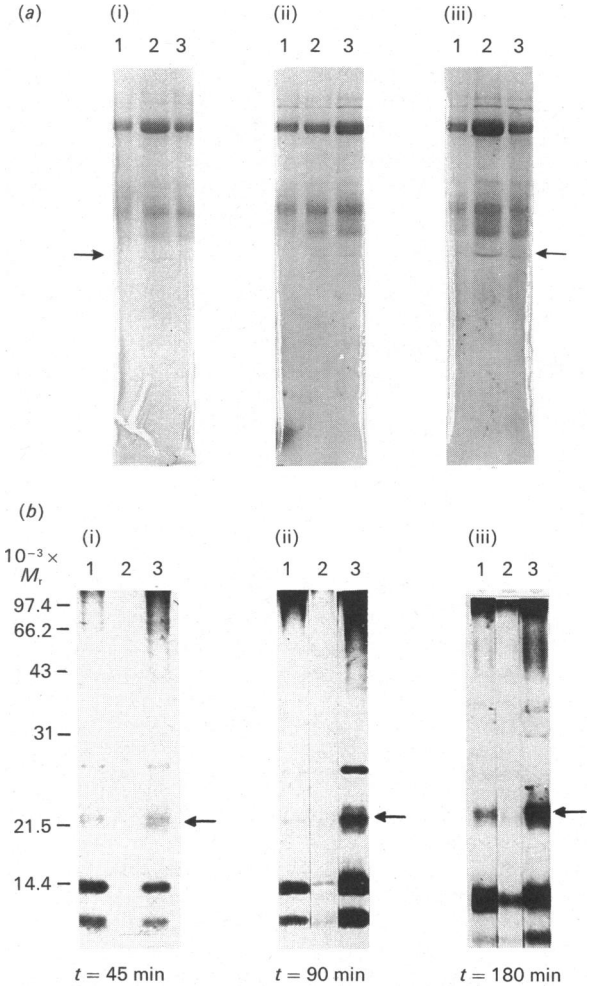

Fig. 2. SDS/PAGE analysis of flavodoxin synthesis and phosphorylation

Azotobacter vinelandii cells were labelled with [^{32}P]phosphate as described in the Materials and methods section, cell-free extracts were prepared and 100 μg of protein was immunoprecipitated with flavodoxin-specific antibody. Immunoprecipitates were analysed by SDS/15%-PAGE followed by staining with Coomassie Blue (*a*) and by autoradiography (*b*). (i), (ii) and (iii) show the immunoprecipitates of extracts obtained from cells removed at $t = 45$ min, 90 min and 180 min respectively after the addition of [^{32}P]phosphate. Lanes 1, immunoprecipitate of extract from *nif*-gene-repressed cells; lanes 2, immunoprecipitate of extract from N_2-fixing cells; lanes 3, immunoprecipitate of extract from *nif*-gene-de-repressing cells. The arrows show the position of flavodoxin. The positions of M_r markers are shown to the left.

impurities. The protein-staining band at M_r 55000 is probably due to the heavy chain of the IgG [Fig. 2(*a*)]. Prominent ^{32}P-containing bands that do not stain with Coomassie Blue are observed at M_r 14400 and at a lower M_r (out of the range of our M_r markers) [Fig. 2(*b*)]. These bands are observable with N_2-fixing cells [Fig. 2(*b*)(iii), lanes 2 and 3], but in smaller amounts. A protein band at M_r 27000–28000 is observed in *nif*-gene-de-repressing cells with maximal extents of phosphorylation at 90 min. At longer times (180 min) this band is decreased in intensity with a concomitant increase in flavodoxin concentration (Fig. 2). One possible explanation for the transitory behaviour of this higher-M_r protein is that it could be a precursor form of flavodoxin rather than a separate protein that was associated with the flavodoxin–antibody complex.

To investigate this point further, the SDS/polyacrylamide gel

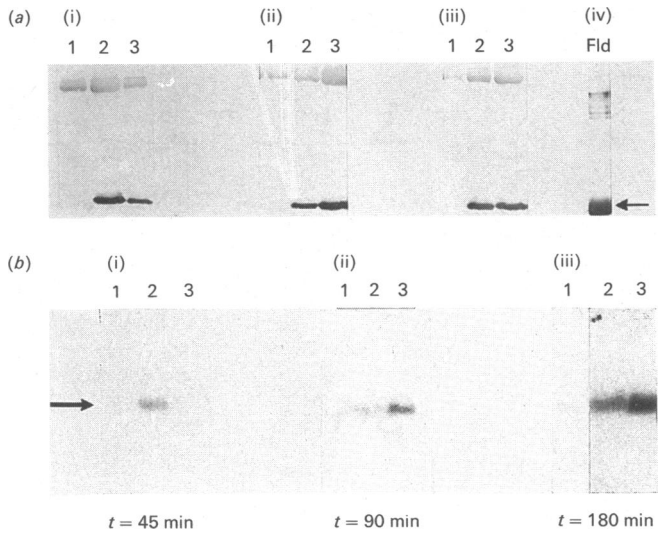

Fig. 3. Western-blot analysis of flavodoxin synthesis and phosphorylation

Immunoprecipitates of cell extracts were subjected to SDS/PAGE followed by Western blotting. (i), (ii) and (iii) represent immunoprecipitates of extracts obtained from cells removed at $t = 45$ min, 90 min and 180 min respectively after addition of [^{32}P]phosphate. Panel (*a*) shows proteins detected on nitrocellulose by alkaline-phosphatase staining after treatment with antibody. Panel (*b*) shows an autoradiogram of a Western-blot filter. Lanes 1, immunoprecipitate of extract from *nif*-gene-repressed cells; lanes 2, immunoprecipitate of extract from N_2-fixing cells; lanes 3, immunoprecipitate of extract from *nif*-gene-de-repressing cells. The lane marked 'Fld' in (iv) in panel (*a*) contains purified flavodoxin. The arrows show the position of flavodoxin.

was subjected to Western blotting (Fig. 3). As shown in Fig. 3(*a*), only a single band corresponding to native flavodoxin was observed under any conditions when transferred proteins were detected by alkaline-phosphatase-conjugated goat antibody to rabbit IgG. These data demonstrate that the transient phosphoprotein band at M_r 27000–28000 is not in fact a precursor form of flavodoxin, and also demonstrate that there are no non-phosphorylated higher-M_r precursor forms of the protein during its synthesis. [The high-M_r band observed in Fig. 3 is probably due to alkaline-phosphatase-conjugated anti-(rabbit IgG) antibody binding to IgG present in the immunoprecipitation reaction.]

Autoradiograms of the Western blot demonstrate that the ^{32}P-labelling occurs after flavodoxin synthesis [Fig. 3(*b*)]. Although subject to further quantification, comparison of the labelling patterns on autoradiographs with the antibody-detected protein stain intensity suggests that phosphorylation takes place after the polypeptide chain is formed. Thus incorporation of the covalently bound disubstituted phosphate residue into *Azotobacter* flavodoxin appears to be a post-translational rather than a co-translational process. The absence of significant signal apart from flavodoxin upon autoradiography of the Western blot may be due to other ^{32}P-labelled bands being lipid-associated material that does not blot well under the experimental conditions used.

DISCUSSION

The presence of a phosphodiester linkage between two amino acid residues in a protein is, at present, a unique feature of *Azotobacter* flavodoxin. The data presented here demonstrate unequivocally that *Azotobacter* (strain OP, Berkeley) is phosphorylated *in vivo* and that the covalently bound phosphate group remains with the protein, as expected, on gel electrophoresis under denaturing conditions. These results, plus the extensive chemical and n.m.r. data on this flavodoxin, should dispel any doubts that have been expressed in the literature [24,25] regarding the reality of the covalently bound disubstituted phosphate as an integral structural entity in this protein. The results presented here also demonstrate that the phosphorylation event is associated only with newly synthesized protein and that no observable exchange of protein-bound phosphate occurs with cellular phosphate pools. These data suggest that the reason for phosphodiester incorporation into the protein is to fulfil a structural rather than a regulatory role. At present, this putative structural role is not well-defined. ^{31}P-n.m.r. data demonstrate that the phosphodiester linkage is on the surface of the protein in that it is susceptible to paramagnetic line-broadening [7]. Other n.m.r. data [26] showed that complexing of the flavodoxin to cytochrome *c* does not influence the susceptibility of the covalently bound phosphate to paramagnetic line-broadening. Thus a reasonable conclusion is that the covalently bound phosphate is not involved directly in the electrostatic complex of flavodoxin and cytochrome *c*. It is worth noting that *Azotobacter* flavodoxin is one of the larger of the known flavodoxins and does not contain any disulphide bonds. Perhaps one role of this phosphodiester link is to stabilize the structure of the protein in lieu of a disulphide bond, since the probability of the latter being reduced in the low-potential environment of the flavodoxin [E_m (semiquinone/hydroquinone) = −500 mV) may be quite high. Further work is required to investigate this possibility.

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