COMPLEX FORMATION BETWEEN FERREDOXIN-NADP⁺-OXIDOREDUCTASE AND FLAVODOXIN

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Introduction

Although the interaction between ferredoxin-NADP⁺-oxidoreductase (FNR) and ferredoxin has been studied extensively (1,2), little information is available on the interaction of FNR with flavodoxin since the initial demonstration (3) that the two proteins can form a tight complex via electrostatic interactions. Further study of the FNR-flavodoxin complex is of interest in view of sequence studies of mammalian Cytochrome P₄₅₀ reductase which shows the C-terminal portion to exhibit homology with FNR and the N-terminal to exhibit sequence homology with flavodoxin (4). Detailed studies of the interaction of these two flavoproteins may provide insights into the general question of flavin-flavin electron transfer reactions. We report here studies of the interaction of Azotobacter flavodoxin with Anabaena variabilis FNR and the properties of the covalent, crosslinked complex of these two proteins. Both organisms share a common function in that they are both capable of nitrogen fixation. In addition, ³¹P NMR studies are reported on the interaction of spinach FNR with Azotobacter flavodoxin.

Results and Discussion

The binding of Azotobacter flavodoxin to Anabaena FNR can be readily monitored by absorption spectral perturbations in the visible spectral region as a result of alterations in the vibronic bands of the FMN and FAD coenzymes on complex formation. Increasing the ionic strength of the medium abolishes the observed perturbations which suggests the complex to involve predominately electrostatic interactions. A plot of spectral titration data is shown in Fig.1. Analysis of these data show a 1:1 stoichiometry and a K_d value of $7\mu\text{M}$. Thus, flavodoxin binding to FNR is only 2-fold weaker than the binding constant determined for ferredoxin (5).

Fig.1. Spectral titration of the binding of Azotobacter flavodoxin to Anabaena FNR. Aliquots of flavodoxin were added to a solution of FNR in 50 mM phosphate, pH 7.0 and difference absorption spectra were measured after each addition. The inset is a Scatchard Plot of the data.

Treatment of the FNR-flavodoxin complex with N-ethyl-3-(3-dimethylaminopropyl)carbodiimide resulted in the formation of

a covalently crosslinked complex of the two proteins as judged by SDS-gel electrophoresis. The crosslinked complex could be separated from unreacted proteins by gel filtration chromatography. Flavin analysis of the purified complex showed 1.23 moles FAD and 0.83 moles FMN per mole of complex (M.W.=59,000). The data in Table 1 shows a comparison of catalytic properties of flavodoxin crosslinked FNR with native FNR.

Table 1. Comparison of Catalytic Activities of FNR with Flavodoxin-Crosslinked FNR.

Activity	FNR	FNR-Flavodoxin Complex
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A) Diaphorase		
K_m Fe(CN) 6^{-3}	230 μ M	450 μ M
K_m NADPH	66 μ M	73 μ M
Turnover Number	31,000 min $^{-1}$	16,000 min $^{-1}$
B) NADPH-Cytochrome c Reductase Activity		
Turnover Number (- Ferredoxin)	109 min $^{-1}$	170 min $^{-1}$
Turnover Number (+ Ferredoxin)	14,000 min $^{-1}$	170 min $^{-1}$
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The results show the K_m for NADPH is unaffected on complex formation with flavodoxin while the K_m value for ferricyanide is increased by a factor of two. No stimulation of cytochrome c reductase activity by ferredoxin is observed for the complex and suggests that the bound flavodoxin occupies the same site as ferredoxin.

To further probe the interaction of FNR with Azotobacter flavodoxin, 31 P NMR studies were performed on the non-covalent complex. Spinach FNR was used in these studies since it was possible to obtain sufficient quantities of enzyme required for such experiments. The pyrophosphate resonances of the FAD moiety of FNR are observed in the -7 to -12 ppm

region (Fig.2). The two phosphorus resonances due to the FMN and covalent phosphate of flavodoxin in the complex are identical in chemical shift with those of the free form (6.3 and 1.3 ppm, respectively) (6). No significant alterations in chemical shift of any of the phosphorus resonances are evident on complex formation. Addition of Mn^{+2} to the solution results in line broadening of only the covalent phosphate resonance of the bound flavodoxin and has no affect on the linewidths of the flavin phosphate resonances. These data show the coenzyme phosphorus groups to be inaccessible to solvent while the covalent disubstituted phosphorus group of the flavodoxin is still exposed to solvent in the complex as is the situation for the free flavodoxin (6). Reduction of the FMN of the flavodoxin to its semiquinone form results in paramagnetic line broadening of only the FMN phosphate resonance but not

Fig.2. 81 MHz ^{31}P NMR spectra of spinach FNR (0.8 mM) alone and in the presence of an equal molar quantity of Azotobacter flavodoxin. All samples were in 50 mM Tris-acetate, pH 8. All spectra were accumulated using 1H broadband decoupling and 4K Hz spectral width. Flavodoxin semiquinone was generated by the addition of 0.5 moles dithionite/mole flavodoxin under an argon atmosphere.

of the FAD pyrophosphate resonances of FNR (Fig.2). These data show the FMN ring to be greater than 10 Å from the side chain of the FAD moiety of FNR in the complex. The resonance due to the covalent phosphorus group of flavodoxin is split on FMN semiquinone formation. This is observed both in the free and complexed forms and suggests the covalent phosphorus group is sensing two different environments which do not rapidly interconvert on the NMR time scale.

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