BINDING AND OXIDATION-REDUCTION OF MONOAMINE OXIDASE-TYPE 8α-(S-PEPTIDYL)FLAVINS WITH AZOTOBACTER (SHETHNA) FLAVODOXIN

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SUMMARY. Binding of 8α-(S-peptidyl)flavins, the type of covalently attached flavin within the active site of mitochondrial monoamine oxidase, can occur within the FMN-binding site of Azotobacter flavodoxin. Association results in typical hypochromicity of the visible absorbance and a bathochromic shift. There is a decrease in fluorescence of protein but not flavin and a generation of negative ellipticity in the near-UV band in the CD spectra. Bound peptidyl flavins are reduced with EDTA and light to blue semiquinone complexes. Reduction to the hydroquinone level can be effected with excess free flavin during photoreduction or with excess dithionite. No significant monoamine oxidase activity could be detected. In common with other flavodoxins, the FMN-binding site of Azotobacter flavoprotein affixes the coenzyme within a flexible tryptophanyl-containing pocket that allows the benzenoid edge of the isoalloxazine ring to project toward solvent.

Flavodoxins are relatively simple flavoproteins, which bind one FMN per single polypeptide chain. Earlier work (1) had shown the probable involvement of tryptophanyl residues in the binding site of the first-recognized flavodoxin, which was isolated and characterized from Clostridium pasteurianum (2-4). Complex association between flavin and amino acid residues was clearly established by the x-ray crystallographic studies of the small flavodoxins from Desulfovibrio vulgaris (5,6) and Clostridium MP (7), wherein the flavin was shown to be in a cleft with the dimethyl benzenoid portion of FMN oriented towards solvent. Chemical and spectroscopic investigations of the flavodoxins from Peptostreptococcus elsdenii (8,9) and Rhodospirillum rubrum (10-12) have broadened our understanding of

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the general similarities in this group of flavoproteins.

With the realization that the Shethna flavoprotein from Azotobacter vinelandii has flavodoxin activity (13) and functions as a component in nitrogen fixation by this organism (14), studies have been made to elucidate the FMN-binding site of this flavodoxin as well. Circular dichroism (CD) spectroscopy has demonstrated the sensitivity to interactions of the ribityl chain and ring portions of FMN (10). Sequencing of the N-terminal portion of flavodoxins from Azotobacter and other organisms has established the significant homology that exists in those amino acid residues that hydrogen bond to the ribityl phosphate moiety (15). Again, a tryptophanyl residue has been implicated with complexing the isoalloxazine portion on the basis of fluorescence properties of the native flavoprotein (16) and that modified by N-bromosuccinimide oxidation (17,18). Some structural variations that can be tolerated in the flavin have been noted (11,19).

In this report, it is demonstrated that Azotobacter flavodoxin can bind and allow oxidation-reduction of such 8α-(S-peptidyl)flavins as occur within the active site of monoamine oxidase (20,21).

MATERIALS AND METHODS. Flavodoxin was isolated from A. vinelandii (Wisconsin 'O') by the method of Hinkson and Bulen (22). Apoprotein was prepared according to Edmondson and Tollin (23). Semiquinone flavoprotein was prepared by illumination of holoenzyme in the presence of EDTA by the method of Massey and Palmer (24). Monoamine oxidase activity was assessed with benzylamine, essentially as described by Tabor et al. (25).

The 8α-[S-(N-acetyl)-L-cysteinyl or cysteinyl-L-tyrosyl]flavins* were synthesized by condensation of acetyl cysteine or acetyl cysteinyl tyrosine with 8α-bromotetraacetylriboflavin, followed by hydrolytic removal of the ribityl acetyl functions, as described by Falk et al. (26). Sulfone was prepared by oxidation with excess peracetic acid. The 5'-phosphates were made by phosphorylating the peptidyl riboflavins with excess chlorophosphoric acid, as described by Flexser et al. for FMN (27), and the compounds chromatographically purified, as was done for FMN by Moffatt and Khorana (28).

Absorption spectra were recorded with a Coleman Hitachi 124 double-beam spectrophotometer with a Beckman recorder. Fluorescence measurements were made with assembled instrumentation [(16), for flavin] and an Aminco-Bowman spectrophotofluorometer (for protein). CD spectra were taken with a Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment.

*Abbreviations for these compounds are: AcCysRb, AcCysTyrRb, AcCys(SO_2)TyrRb, and AcCysTyrFMN.

RESULTS AND DISCUSSION. Typical absorption spectra resulting from addition of AcCysRb, AcCysTyrRb, and AcCysTyrFMN to Azotobacter apoflavodoxin are shown, in comparison to the free flavins, in Fig. 1. With the flavoquinone holoenzyme so-generated, there is a hypochromic effect in the 450-nm region, a shifting to longer wavelengths, and slightly greater resolution of the vibronic structure. These changes are quite similar to those seen upon binding riboflavin or the more tightly associating FMN and reflect the interaction of the flavin in the less-polar binding site of the protein (10). Also, as found with the native (FMN) flavodoxin, only trivial changes occur in the near-UV band upon binding the 8α-(S-peptidyl)flavins shown. Positions of maximal absorbance in this region remain essentially the same as for free flavin and are at 372 nm for riboflavin and FMN,

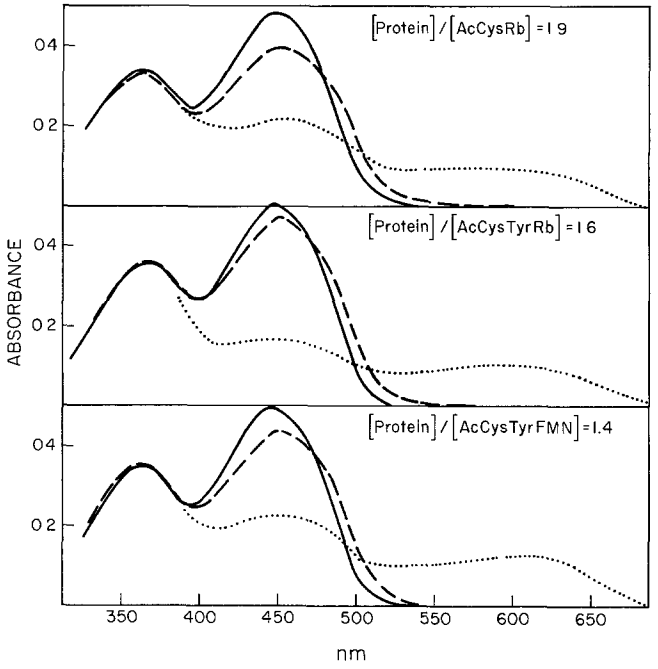

Fig. 1. Absorption spectra of peptidyl flavins alone (——) and with oxidized (— — —) and semiquinone (----) Azotobacter flavodoxin. Absorption of 4×10^{-5} M flavin, with and without protein, was measured at 28° in 25 mM sodium phosphate, pH 7. For semiquinone spectra, samples with 50 mM EDTA were irradiated anaerobically at 25°.

366 nm for 8α-thioether-linked flavins, and 353 nm for the AcCys(SO_2)TyrRb. Hypsochromic shifts in the second absorption band of such peptidyl flavins are attributed to restriction of resonance stabilization of the excited state by the electron-withdrawing substituents in the 8α-position (29).

Upon light irradiation of the anaerobic flavodoxin complexes with EDTA as photoreductant at pH 7, the typical 'blue' flavosemiquinone spectra are generated. As also shown in Fig. 1, there is a decrease in absorbance near 450 nm, with corresponding increase in long-wavelength absorption centered near 600 nm. This, too, is indicative of the 'productive' flavin-site binding also found with the native holoenzyme. Similar reduction of the free flavins does not produce significant semiquinone, as such neutral radicals disproportionate rapidly and extensively to yield oxidized and fully reduced flavin (30,31). When excess (free) flavin is present during the photoreduction of the holoflavodoxin, or when dithionite is used as chemical reductant, the flavohydroquinone spectrum is achieved, although this is not the case with the natural, FMN-containing protein.

The stabilities of the semiquinone complexes toward molecular oxygen are greater with FMN, or even peptidyl FMN, than with riboflavin or peptidyl riboflavins, as seen by data in the table. However, the more-rapid oxidation of the complexes with the 8α-S-linked flavins than found with the FMN or riboflavin counterparts indicates the 'looseness' with which the bulkier flavins fit in the flavin-binding site. Again, though, the particular influence that the protein exerts upon the redox capacity of the flavins is generally characteristic of the dehydrogenase rather than oxidase type. Even with the attendant, more-positive E_m values characteristic of the S-cysteinyl and S-cysteinsulfonyl flavins (32), they do not have measurable monoamine oxidase activity when bound in the flavodoxin system.

As shown in Fig. 2, there is a decrease in protein tryptophanyl fluorescence upon binding of peptidyl FMN to Azotobacter apoflavodoxin, though the extent of quenching is not so great as measured at comparable ratios of FMN to protein (16). The association constant for AcCysTyrFMN can be estimated to be somewhat greater

Table I. Rate of Air Oxidation of Azotobacter Flavodoxin Semiquinones*

Flavin in complex	Half-time (min)
FMN	700
Riboflavin	2.0
AcCysTyrFMN	2.6
AcCysTyrRb	0.7
AcCysRb	0.2

*Conditions were as described in Fig. 1 for generation of the flavoprotein semiquinones by addition of flavin to a molar excess of apoprotein in 25 mM phosphate containing 50 mM EDTA as photoreductant. Oxidation of semiquinone at 35° was followed by the decrease at 600 nm upon resaturation with air.

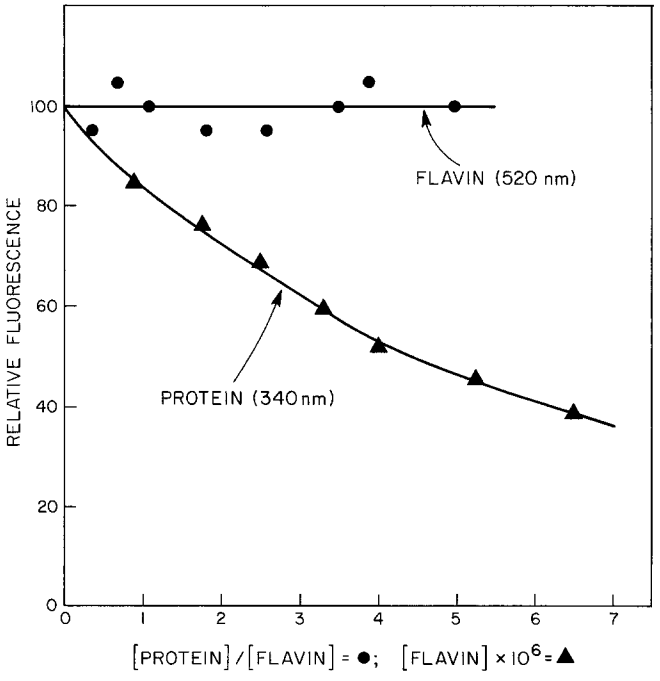

Fig. 2. Fluorescence titration of Azotobacter apoflavodoxin with AcCysTyrFMN. Flavin fluorescence was measured by 360-nm activation of 1.2×10^{-6} M flavin with varying protein concentration at 25° in 25 mM sodium phosphate, pH 7. Protein fluorescence was measured by 280-nm activation of 9.3×10^{-7} M (initial) to 5.6×10^{-7} (final) protein with varying flavin concentration at 27° in 25 mM sodium phosphate, pH 7.

than 10^5 M^{-1}, which can be compared to a value in the order of 10^8 M^{-1} with FMN (23). Unlike with FMN, there is no significant, concommitant change in the flavin fluorescence of AcCysTyrFMN upon binding. As characteristic of 8α-thioether-linked flavins, the free peptidyl flavin has about 2% of the fluorescence of free FMN in the aqueous buffer (26). The fluorescence of such 8α-S-peptidyl flavins increases markedly when they are in more nonpolar (less aqueous) media. Hence, such mutual quenching interaction as occurs between the active-site tryptophanyl residue and the peptidyl FMN is offset in the latter by the increased hydrophobicity of the binding environment.

The CD spectra generated upon binding any of the 8α-S-peptidyl flavins are qualitatively similar to that observed with FMN in the near-UV to visible region. A comparison of such spectra of free and bound AcCysRb and bound FMN are shown in Fig. 3. In all cases, upon binding of flavin or flavin phosphate, the position of maximum negative ellipticity is centered near the λ_{max} for absorbance of fla-

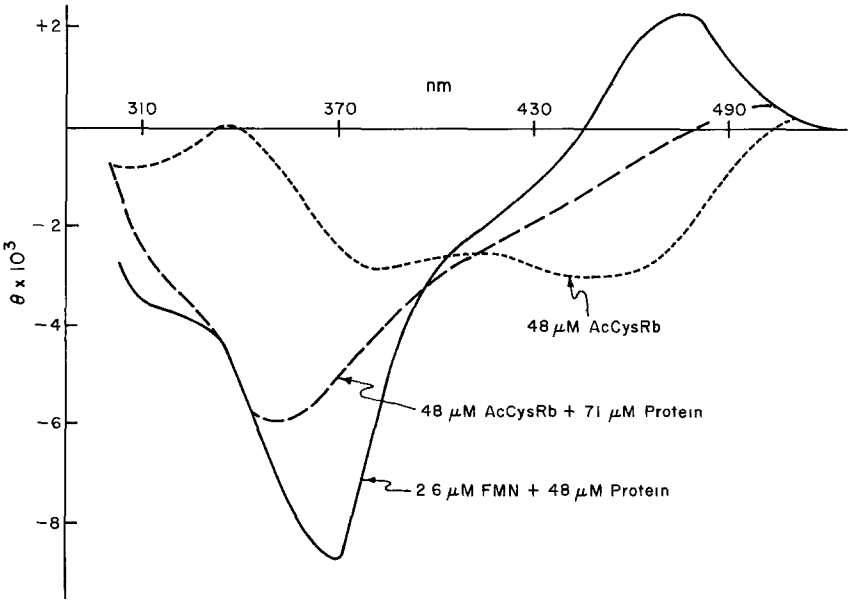

Fig. 3. CD spectra of Azotobacter flavodoxin complexes with AcCysRb in comparison to the native holoenzyme at 36° in 25 mM sodium phosphate, pH 7.

vin in the near-UV, while ellipticity becomes more positive toward the visible band. Quantitatively larger changes are seen with the tighter FMN complex than with the peptidyl flavin complexes.

From all of the foregoing, it appears that the flavin-binding site of the Azotobacter flavodoxin, in common with other flavodoxins, should be envisioned as a somewhat flexible cleft that allows the dimethyl benzenoid end of FMN to stick out toward solvent. Further, it is clear that the protein moiety determines that the flavin system will exhibit dehydrogenase-like, rather than oxidase-like, behavior.

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