Studies on the Binding and Function of Flavin Phosphates with Flavin Mononucleotide-dependent Enzymes*

(Received for publication, July 19, 1965)

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SUMMARY

The binding and function of FMN, 3,6,7-trimethyl-9-((1'-o-ribityl)isoalloxazine 5'-phosphate (3-methyl-FMN), 6,7-dichloro-FMN, and 6,7-dimethyl-9-(5'-hydroxypentyl)isoalloxazine 5'-phosphate (5'-hydroxypentyl-FMN) has been investigated with reduced nicotinamide adenine dinucleotide phosphate dehydrogenase and NADPH cytochrome c reductase from yeast, NADH oxidase from a pseudomonad, pyridoxamine phosphate oxidase from liver, and glycolate reductase from yeast, NADH oxidase from a pseudomonad, pyridoxamine phosphate oxidase from liver, and glycolate oxidase from spinach. Considerable variation has been observed in the quantitative behavior of the flavin phosphates with the differing apoenzymes through measurements of $V_{max}$ and $K_m$ or $K_I$.

The earlier postulation that the 3-imino group of the isooalloxazine is a primary point of attachment of the flavin phosphate to an FMN-dependent apoenzyme must now be modified, especially for the NADPH dehydrogenase ("old yellow enzyme") which has been considered to be the prototype of flavin coenzyme-apoenzyme interaction. Present observations with 3-methyl-FMN indicate a lack of absolute dependence on the 3-imino group and suggest a rather diffuse interaction through mutual polarization or partial charge transfer between relatively large portions of the isooalloxazine structure and perhaps aromatic amino acid residues in the proteins.

The generally better binding than function of 6,7-dichloro-FMN indicates that the relatively poor function of similar analogues may be expected with substituted changes in position 6 and 7 which are of the approximate size of methyl groups but through contribution to the electromeric structure of the isooalloxazine markedly change the oxidation-reduction potential.

The optimal requirement for a full complement of secondary hydroxyl groups in the configuration on the side chain in position 9 is apparent from the generally decreased enzymatic activity seen with 5'-hydroxypentyl-FMN. However, there is no absolute dependence on interaction of the enzymes with each secondary hydroxyl group of a flavin phosphate, but rather a steric relationship which favors proper disposition of coenzyme to apoenzyme.

"Old yellow enzyme," reduced nicotinamide adenine dinucleotide phosphate dehydrogenase from yeast, has been considered as the prototype of flavin coenzyme-apoenzyme interaction (1). The attachment of flavin mononucleotide to the apodehydrogenase has been suggested to involve electrostatic bonding of the 5'-phosphate and an interaction at the imino group in position 3 of the flavin, presumably by hydrogen bonding to a hydroxyl group in a tyrosine residue (2). The conclusion concerning the participation of the 3-imino group was based mainly on the following observations. First, the fluorescence of FMN is quenched upon binding to the apodehydrogenase (3, 4) as was reported to occur by methylation of the flavin at position 3 (5). However, it was shown later that such methylation does not abolish fluorescence, and, although there is no longer the possibility for hydrogen bonding, fluorescence quenching can occur through complexation with many reagents which also quench the fluorescence of FMN (6). In fact, we have found 3-methyl-FMN' to be nearly as fluorescent as FMN (7). Thus a direct causal relationship of the 3-imino group to the fluorescence phenomenon seems dubious. Second, it was reported that 3-methyl-FMN cannot react with the apodehydrogenase to form a catalytically active holoenzyme (8). Our current findings tend to qualify this report as well.

More generally, the influences that different portions of the FMN molecule exert on binding and function with various FMN-dependent enzymes heretofore had not been assessed adequately (1, 9), although the general specificity of NADPH cytochrome c reductase for flavin phosphates has been examined (10). The effect that secondary hydroxyl groups of the side chain in position 9 of flavins is apparent from the generally decreased enzymatic activity seen with 5'-hydroxypentyl-FMN. However, there is no absolute dependence on interaction of the enzymes with each secondary hydroxyl group of a flavin phosphate, but rather a steric relationship which favors proper disposition of coenzyme to apoenzyme.

*This investigation was supported by the National Institutes of Health as grants to D. B. McCormick (AM-04585-05) and to L. D. Wright (HE-0413307).
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‡This paper is the continuation of investigations concerned with flavins by D. B. McCormick, to whom requests for reprints and inquiries should be addressed.

1 Abbreviations used are: 3-methyl-FMN, 3,6,7-trimethyl-9-((1'-o-ribityl)isoalloxazine 5'-phosphate; 6,7-dichloro-FMN, 6,7-dichloro-9-((1'-o-ribityl)isoalloxazine 5'-phosphate; 5'-hydroxypentyl-FMN, 6,7-dimethyl-9-(5'-hydroxypentyl)isoalloxazine 5'-phosphate.
9 of the flavin have on binding was uncertain as was the over-all contribution of substituents in positions 6 and 7, which markedly influence the oxidation-reduction potential.

The present investigation compares the behavior of FMN and three analogues in five FMN-dependent systems. The 3-methyl-FMN was tested to elucidate specifically the importance of the 3-amino group in binding. The 5'-hydroxypentyl-FMN and 6,7-dichloro-FMN were selected to delineate generally the influence of the secondary hydroxyl groups of the side chain on binding and the substituents in the benzeneoid portion of the ring on oxidation-reduction function, respectively. The extent to which these FMN analogues substitute for or competitively displace the natural coenzyme is demonstrated with NADPH dehydrogenase and NADPH cytochrome c reductase from yeast, NADH oxidase from a pseudomonad, pyridoxamine phosphate oxidase from liver, and glycolate oxidase from spinach.

**EXPERIMENTAL PROCEDURE**

**Materials**

Common biochemicals were obtained from Sigma; common organic chemicals were from Eastman.

**Flavin Phosphates**—For the synthesis of 3-methylriboflavin, methylalloxan, prepared by oxidation of theobromine (11), was condensed with 2-amino-4,5-dimethyl-N-(1'-n-ribityl)aniline (12) according to Kuhn et al. (13). Criteria for purity of the product included melting point, chromatographic behavior, and absorption and fluorescence spectra, all of which were identical with those of a sample of 3-methylriboflavin obtained from Merck Sharp and Dohme. 6,7-Dichlororiboflavin was synthesized by condensation of 2-amino-4,5-dichloro-N-(1'-n-ribityl)aniline with alloxan (14). 5'-Hydroxypentylflavin was synthesized according to Chassy, Arsenis, and McCormick (15).

The 5'-phosphate ester of each of the flavins was prepared by phosphorylating the terminal hydroxymethyl group with dichlorophosphoric acid by the procedure of Flexser and Farkas (16). The products were applied to Whatman No. 3MM paper, and the pure FMN analogues were eluted after development of the chromatograms in ascending n-butyl alcohol-acetic acid-water (5:2:3, v/v/v).

**Enzymes**—NADPH dehydrogenase, purified from brewers' yeast according to Theorell and Åkeson (17), was kindly supplied by Dr. C. A. Vestling from the State University of Iowa. NADPH cytochrome c reductase was purified from brewers' yeast by the method of Haus, Horecker, and Hogness (18). NADH oxidase, purified from a camphor-degrading pseudomonad according to Trudgill, DuBus, and Gunsalus (19), was supplied by Drs. P. W. Trudgill and L. C. Gunsalus from the University of Illinois. Pyridoxamine phosphate oxidase was purified from rabbit liver by the method of Wada and Snell (20). Glycolate oxidase was purified from spinach by the method of Zelitch and Ochoa (21).

Each of these flavoproteins was resolved to an apoenzyme by treatment with acid-ammonium sulfate essentially as described by Warburg and Christian (22). Individual modifications are noted in the reports describing purification. Their activities when reconstituted with FMN are given in "Results."

**Methods**

NADPH dehydrogenase was assayed in cuvettes which contained 25 μmoles of potassium phosphate buffer (pH 7.4), varying amounts of flavin phosphates as indicated, and 0.32 mg of apodehydrogenase in 1.15 ml. The contents were stirred, and the reactions were initiated with 0.34 μmole of NADPH in 0.08 ml. The linear absorbance decrease at 340 μm due to oxidation of NADH was measured against a water blank at room temperature for 1 min in a Gilford model 2000 recording spectrophotometer.

NADPH cytochrome c reductase was assayed in cuvettes which contained 20 μmoles of potassium phosphate buffer (pH 7.5), 1 mg of cytochrome c, varying amounts of flavin phosphates as indicated, and 0.08 mg of aporeductase in 2.9 ml. The contents were stirred and allowed to stand at room temperature for 5 min. The reactions were initiated with 5 μmoles of NADPH in 0.1 ml. The linear absorbance increase at 550 μm due to reduction of cytochrome c was measured against a blank without cytochrome c for 5 min in the recording spectrophotometer.

NADH oxidase was assayed in cuvettes which contained 60 μmoles of Tris-HCl buffer (pH 7.2), varying amounts of flavin phosphates as indicated, and 0.5 μmole of NADH in 2.65 ml. The contents were stirred, and the reactions were initiated with 0.22 μg of apooxidase in 0.05 ml. The linear decrease in absorbance at 340 μm due to oxidation of NADH was measured against a water blank at room temperature for 3 min in the recording spectrophotometer.

Mixtures for assaying pyridoxamine phosphate oxidase were in Erlenmeyer flasks which contained 0.5 μmole of Tris-HCl buffer (pH 8), 1 μmole of pyridoxamine 5-phosphate, varying amounts of flavin phosphates as indicated, and 0.1 mg of apooxidase in a total volume of 3.5 ml. The flask were stoppered, and the contents were incubated at 37° for 50 min. The reactions were terminated with 0.3 ml of 100% (w/v) trichloracetic acid. The pyridoxal 5-phosphate formed was allowed to react with 2% (w/v) phenylhydrazine hydrochloride in 0.2 ml of 10% sulfuric acid, the mixture was allowed to stand for 10 min, and the color developed was read at 410 nm against a reagent blank.

Glycolate oxidase was assayed in jacketed cells at 37° which contained 100 μmoles of potassium phosphate buffer (pH 8), varying amounts of flavin phosphates as indicated, and 0.06 mg of apooxidase in 1.75 ml. The reactions were initiated with 20 μmoles of potassium glycolate in 0.25 ml. The linear period of oxygen consumption was measured for 1 min with a Gilson model K oxygraph with the polarizing voltage set at −0.65 volt and the electrode oscillator at 120 cps.

**RESULTS**

**NADPH Dehydrogenase**—The extent to which varying concentrations of flavin phosphates reactivated NADPH apodehydrogenase is shown by the data in Fig. 1. Although the partial restoration of activity with low concentrations of FMN appears much greater than that with corresponding concentrations of the analogues, the maximum coenzymeric activities relative to FMN (Km = 2.2 × 10−7 m) are 69% for 3-methyl-FMN (Km = 1.4 × 10−7 m), 56% for 5'-hydroxypentyl-FMN (Km = 4.8 × 10−4 m), and 39% for 6,7-dichloro-FMN (Km = 1.6 × 10−4 m). Thus, methylation of the 3-amino group elicits a less marked decrease in activity than loss of the secondary hydroxyl groups from the ribityl side chain which in turn is less critical than the alteration of oxidation-reduction potential effected by substituent changes in positions 6 and 7. The smallest Km value found with FMN may indicate greater affinity relative to the analogues.
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FIG. 1. Reactivation of NADPH apodehydrogenase by flavin phosphates. Oxidation of NADPH was followed by decreases in absorbance at 340 μM per min as described in “Methods.” Specific activity of the FMN-restored holoenzyme was 129 μmoles of NADPH oxidized per min per mg.

5'-Hydroxypentyl-FMN

0.06 -

6,7-Dichloro-FMN

2 4 6

Flavin Phosphate (M x 10^6)

FIG. 2. Reactivation of NADPH cytochrome c aporeductase by flavin phosphates. Reduction of cytochrome c was followed by increases in absorbance at 550 μM per 5 min as described in “Methods.” Specific activity of the FMN-restored holoenzyme was 20 μmoles of cytochrome c reduced per 5 min per mg.

5'-Hydroxypentyl-FMN

Flavin Phosphate (M x 10^5)

FIG. 3. Reactivation of NADH apooxidase by flavin phosphates. Oxidation of NADH was followed by decreases in absorbance at 340 μM per 3 min as described under “Methods.” Specific activity of the FMN-restored holoenzyme was 540 μmoles of NADH oxidized per 3 min per mg.

NADPH Cytochrome c Reductase—The extent to which varying concentrations of flavin phosphates reactivate NADPH cytochrome c aporeductase is shown by the data in Fig. 2. At high concentrations, 5-hydroxypentyl-FMN seems to have better coenzymatic activity than FMN itself. The maximum coenzymatic activities relative to 5'-hydroxypentyl-FMN (K_m = 2.9 x 10^-7 M) are 52% for FMN (K_m = 7.7 x 10^-4 M), 43% for 6,7-dichloro-FMN (K_m = 2.5 x 10^-7 M), and 91% for 3-methyl-FMN (K_m = 2.1 x 10^-7 M). Loss of the secondary hydroxyl groups on the side chain does not result in drastic changes in the coenzymatic function at low concentrations, although it seems to increase activity at higher concentrations. The chloro substituents in positions 6 and 7, which alter the potential, cause less decrease in activity than methylation of the 3-imino group. The smallest K_m value found with FMN may indicate greater affinity relative to the analogues.

NADH Oxidase—The extent to which varying concentrations of flavin phosphates reactivate NADH apooxidase is shown by the data in Fig. 3. Although the partial restoration of activity with low concentrations of FMN appears much greater than that with corresponding concentrations of the analogues, the maximum coenzymatic activities relative to FMN (K_m = 3.8 x 10^-6 M) are 72% for 6,7-dichloro-FMN (K_m = 3.9 x 10^-6 M), and 33% for 5'-hydroxypentyl-FMN (K_m = 3.1 x 10^-6 M). Substituent alterations in positions 6 and 7 appear less critical than the loss of the secondary hydroxyl groups on the side chain. The 3-methyl-FMN is a competitive inhibitor with a K_i value of 1.3 x 10^-3 M as calculated from the data shown in Fig. 4.

FIG. 4. Inhibition of FMN reactivation of NADH apooxidase by 3-methyl-FMN. The 3-methyl-FMN was added at 9 x 10^-6 M final concentration.
Fig. 5. Reactivation of pyridoxamine phosphate apooxidase by flavin phosphates. Formation of pyridoxal phosphate was followed by increases in absorbance of the phenylhydrazone at 410 nm as described under "Methods." Specific activity of the FMN-restored holoenzyme was 250 mmoles of pyridoxal phosphate formed per 30 min per mg.

Comparison of this $K_i$ with the smaller $K_m$ values for FMN and the other analogues indicates that 3-methyl-FMN may not be as avidly bound by the apoprotein.

Pyridoxamine Phosphate Oxidase—The extent to which varying concentrations of flavin phosphates reactivation pyridoxamine phosphate apooxidase is shown by the data in Fig. 5. The maximum enzymatic activities of the analogues relative to FMN ($K_m = 1.9 \times 10^{-4} M$) are 56% for 3-methyl-FMN ($K_m = 7.1 \times 10^{-4} M$) and 6% for 5'-hydroxypentyl-FMN ($K_m = 1.9 \times 10^{-4} M$). Methylation of the 3-imino group elicits an intermediate decrease in activity, whereas loss of the secondary hydroxyl groups on the side chain results in a marked decrease in activity. As shown by the data in Fig. 6, 6,7-dichloro-FMN is a competitive inhibitor with a $K_i$ value of $1.8 \times 10^{-4} M$, indicating binding as good as that with FMN to the protein.

Glycolate Oxidase—The extent to which varying concentrations of flavin phosphates reactivation glycolate apooxidase is shown by the data in Fig. 7. Restoration of activity by FMN ($K_m = 3.6 \times 10^{-4} M$) is much greater than that with the same concentrations of the analogues. Either methylation of the 3-imino fluorescence was examined under ultraviolet light. The flavin phosphate was recovered as unaltered 3-methyl-FMN ($R_F = 0.21$) with no detectable FMN ($R_F = 0.12$).
group or loss of the secondary hydroxyl groups of the side chain results in very marked decreases in activity. The 6,7-dichloro-FMN is a competitive inhibitor with a $K_i$ value of $7.0 \times 10^{-5}$ M, as calculated from the data shown in Fig. 8. The latter analogue may bind to the apoenzyme almost as well as FMN, with the loss of coenzymatic activity mainly attributable to the unfavorable oxidation-reduction potential.

**DISCUSSION**

The information provided by the present study illustrates the considerable variation in the means by which different FMN-dependent enzymes bind and function with FMN or its analogues. Since flavin phosphates bind and function with all of the apoenzymes much more effectively than do the corresponding free flavins, one common site of attachment must directly involve the anionic phosphate group of such coenzymes. However, the previously held suppositions on those other positions in the flavin structure which are primarily concerned with binding to any FMN-dependent enzyme must now be considerably modified.

The earlier postulation that the 3-imino group of the isosalloxazine is a primary point of attachment of the flavin to the protein (2) cannot be substantiated by the present results to occur in all systems. In fact, of the five apoenzymes investigated, only glycolate apoxide is very poorly restored to catalytic activity by 3-methyl-FMN but is also essentially inactive with 5'-hydroxypentyl-FMN. Methylating the 3-imino position causes the apooxidase also can be demonstrated by the analogue competitively inhibiting restoration of activity with FMN. Additional confirmation of the lack of absolute binding of the 3-imino group to some apoflavoproteins comes from a study with egg white flavoprotein, wherein the decrease in binding of 3-methylriboflavin compared with riboflavin is again less than when relatively minor changes are made in the side chain (23).

As already mentioned, part of the reason for assuming a primary role played by the 3-imino group has been due to the erroneous conclusions of earlier workers that either enzymatic (3, 4) or chemical (5) interaction at this position quenched fluorescence of the flavin. Since more recent work (6, 7) has established the lack of specific relationship of the 3-imino group to the fluorescence phenomenon, the quenching of fluorescence of FMN or its analogues upon binding to an apoenzyme must be attributed to other causes. Probably the quenching results mainly from the mutual polarization or partial charge transfer between large portions of the structure of flavin and aromatic amino acid residues, e.g. tyrosine, in the proteins. Such general effects have been shown to be of importance in the combination of 3-methylriboflavin (9) as well as riboflavin (24) with many compounds which similarly quench the fluorescence of FMN and FAD inter- and intramolecularly, respectively (7, 25). These observations together with the present ones may suggest that a rather diffuse interaction of the isosalloxazine nucleus and enzyme occurs.

In agreement with our previous studies with NADPH cytochrome c reductase (10), 6,7-dichloro-FMN was found to be less coenzymatically active than FMN with all the enzymes presently tested. The oxidation-reduction potential for FMN ($-0.20$ volt) is considerably more negative than that for the dichloro analogue ($-0.11$ volt) which upon binding to the apoenzyme may shift the potential of the resulting holoenzyme to one less favorable for catalytic function when equilibrium conditions are similar. Values for $K_m$ or $K_i$ would suggest that binding of 6,7-dichloro-FMN is comparable in magnitude to FMN for most of those enzymes investigated. This general disparity in binding to function may be expected for other flavins with substituent changes in positions 6 and 7 which are of the approximate size of methyl groups but through contribution to the electronic structure of the isosalloxazine markedly change the oxidation-reduction potential (10, 26).

The optimal requirement for a full complement of secondary hydroxyl groups in $\alpha$ configuration on the side chain at position 9 is apparent from the generally decreased coenzymatic activity seen with 5'-hydroxypentyl-FMN which, as pointed out above, is even less satisfactory than the 3-methyl analogue with certain of the enzymes. This finding confirms and augments other observations that alterations in the side chain decrease activity of flavin phosphates with NADPH dehydrogenase (8) and NADPH cytochrome c reductase (10). Similar restrictions on configuration (27, 28), length (15, 27), and degree of hydroxylation (15) of the side chain of flavin substrates have been demonstrated with liver flavokinase. There is no absolute dependence on interaction of the enzymes with each secondary hydroxyl group of a flavin phosphate, but rather a steric relationship which favors proper disposition of coenzyme to apoenzyme.

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Studies on the Binding and Function of Flavin Phosphates with Flavin Mononucleotide-dependent Enzymes
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