SPECIFICITY OF FLAVIN ADENINE DINUCLEOTIDE PYROPHOSPHORYLASE FOR FLAVIN PHOSPHATES AND NUCLEOSIDE TRIPHOSPHATES*

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The biosynthesis of flavin adenine dinucleotide (FAD) in plant and animal tissues has been observed by several investigators (1). The overall mechanism of FAD biosynthesis was elucidated by Schrecker and Kornberg (2) who partially purified an enzyme from yeast which catalyzes the reversible synthesis of FAD and inorganic pyrophosphate from riboflavin-5'-phosphate (FMN) and adenosine-5'-triphosphate (ATP). That a similar means for FAD synthesis occurs in animal tissues was demonstrated by Watarai et al. (3) who found that liver preparations catalyze the incorporation of FMN (P^{32}) into FAD without loss of specific radioactivity of the phosphate. DeLuca and Kaplan (4) showed the synthetic activity for FAD in liver is located in the soluble fraction of the cytoplasm and is largely masked by FAD-destroying enzymes which can be partially removed during purification of the FAD pyrophosphorylase.

Heretofore a study has not been made on the specificity of FAD pyrophosphorylase for both interacting substrates, flavin phosphate and nucleoside triphosphate. Since several riboflavin analogues are partially able to support growth in organisms (1) and, via catalysis by flavokinase, are converted to analogues of FMN (5-7), the conversion of the latter to functional analogues of FAD should be sought. Moreover the presence of FAD pyrophosphorylase and nucleoside triphosphates in the soluble fraction of the cell, but lack of biosynthetic analogues of FAD in which the adenine moiety is replaced by other purines or pyrimidines, would suggest essentially absolute specificity for ATP

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and should bear investigation. The present study demonstrates that FAD pyrophosphorylase from rat liver exhibits a relative specificity for the flavin phosphate and an apparent absolute specificity for ATP.

Materials and Methods

Riboflavin, FMN, FAD, and ribo-and deoxyribofuranoside-5'-triphosphates of purines and pyrimidines were the highest purity available from Sigma Chemical Company. Isoriboflavin (5,6-dimethyl-9-(1'-D-ribityl)isoalloxazine and D-araboflavin (6,7-dimethyl-9-(1'-D-arabityl)isoalloxazine) were from the Merck Sharp an Dohme Laboratories. 6-Methylriboflavin (6-methyl-9-(1'-D-ribityl)isoalloxazine) and D-Erythroflavin (6,7-dimethyl-9-(1'-D-erythrityl)isoalloxazine) were prepared from their corresponding substituted o-nitroanilines and aldoses by the method of Kuhn et al. (8). 2',3',4'-Trideoxyriboflavin (6,7-dimethyl-9-(5'-hydroxypentyl)-isoalloxazine) and 2'-deoxyglyceroflavin (6,7-dimethyl-9-(3'-hydroxypropyl)isoalloxazine were synthesized via displacement reactions with the appropriate amino-alcohol and 1,2-dimethyl-4,5-dinitrobenzene as described by Weygand et al. (9). The monophosphate ester of each of the flavins was prepared by phosphorylation of the terminal hydroxymethyl group with dichlorophosphoric acid according to Flexser and Farkas (10). The FAD pyrophosphorylase from rat liver was partially purified as described by DeLuca and Kaplan (4).

Mixtures for assessing substrate reactivity contained 0.5 mM FMN or other flavin phosphate, 1 mM ATP or other nucleoside triphosphate, 1 mM $MgCl_2$, 25 mM potassium phosphate buffer (pH 7.5), and 2.5 mg. of protein in 3 ml. total volume. Incubation was carried out in the dark at 37° for 60 min. The reaction was terminated by heating the mixtures in a boiling water bath for 3 min. The solutions were saturated with $(NH_4)_2SO_4$, the mixtures filtered, and the flavin compounds in the filtrates extracted with 0.25 ml. of liquid (warmed) phenol. 50 Lambda aliquo of the phenol layer were analyzed for FAD or its analogues by the paper chromatographic method of Giri (11), with n-butyl alcohol: acetic acid: water (4:1:5) as a solvent system. The chromatograms were examined under an ultraviolet light, and the paper strips containing FAD compounds were cut out and eluted with 3 mM potass

phosphate buffer, pH 7. FAD compounds in the eluates were determined fluorometrically according to the method of Yagi (12). Flavins, flavin phosphates, and appropriate standards also were applied to Whatman No. 1 paper and the fluorescent spots observed under ultraviolet light after development of the chromatograms in ascending solvents as described by Huennekens and Felton (13). Secondary confirmation of purity and mobility of the flavin compounds was obtained in this manner (14).

Results and Discussion

The R_F values of flavins, flavin phosphates, and flavin adenine dinucleotides are shown in Table I.

TABLE I

Paper Chromatography of Flavin Compounds

Compound	R_F values in ascending solvents*		
	S_1	S_2	S_3
Riboflavin	0.30	0.30	0.80
" -5'-phosphate	0.53	0.12	0.17
" adenine dinucleotide	0.35	0.05	0.23
Isoriboflavin	0.28	0.30	0.79
" -5'-phosphate	0.50	0.11	0.17
" adenine dinucleotide		0.05	
6-Methylriboflavin	0.40	0.25	0.80
" -5'-phosphate	0.64	0.09	0.19
" adenine dinucleotide		0.04	
6,7-Dibromoriboflavin**	0.18	0.44	0.70
" -5'-phosphate	0.45	0.17	0.19
" adenine dinucleotide		0.06	
D-Araboflavin	0.31	0.32	0.80
" -5'-phosphate	0.60	0.12	0.20
" adenine dinucleotide		0.05	
D-Erythroflavin	0.33	0.38	0.86
" -4'-phosphate	0.64	0.15	0.28
" adenine dinucleotide		0.05	
2',3',4'-Trideoxyriboflavin	0.32	0.65	0.95
" -5'-phosphate	0.56	0.23	0.35
" adenine dinucleotide		0.07	
2'-Deoxyglyceroflavin	0.25	0.57	0.90
" -3'-phosphate	0.57	0.18	0.31
" adenine dinucleotide		0.06	

* Solvents systems: S_1 = 5% aqueous Na_2HPO_4; S_2 = n-butyl alcohol: acetic acid: water (4:1:5, upper phase); S_3 = phenol: n-butyl alcohol: water (16:3:10, lower phase).

** This flavin and its nucleotides exhibit a diminished fluorescence efficiency.

Each of the dinucleotides reported is observed as the enzymatic product in the FAD pyrophosphorylase reaction with ATP and the respective flavin phosphate as substrates. Thus the pyrophosphorylase has some capacity for catalyzing the condensation of the adenylate moiety of ATP with variously substituted flavins bearing an orthophosphate ester grouping on the side chain at position 9 of the alloxazine ring. However, when FMN was included with other nucleoside triphosphates replacing ATP, no FAD analogues could be detected. Ribo-and deoxyribofuranoside-5'-triphosphates with inosine, guanine, cytosine, uracil, and thymine as the base were all inactive. This apparent absolute specificity of FAD pyrophosphorylase for ATP would explain why no analogues in which adenine is replaced by other purines or pyrimidines are found biosynthetically even though both enzyme and nucleoside triphosphates occur together in the soluble fraction of the cytoplasm.

The quantitative assays of enzymatically synthesized flavin adenine dinucleotides are shown in Table II.

TABLE II

Enzyme-Catalyzed Formation of Flavin Adenine Dinucleotides

Flavin monophosphate added (500 μM)	Flavin adenine dinucleotide formed (μM)
Riboflavin-5'-phosphate	10.3
Isoriboflavin-5'-phosphate	28.8
6-Methylriboflavin-5'-phosphate	7.5
6,7-Dibromoriboflavin-5'-phosphate	3.2
D-Araboflavin-5'-phosphate	1.2
D-Erythroflavin-4'-phosphate	1.2
2',3',4'-Trideoxyriboflavin-5'-phosphate	2.2
2'-Deoxyriboflavin-3'-phosphate	1.0

Under the assay conditions employed, isoriboflavin-5'-phosphate is an even better substrate than the natural one, FMN. As demonstrated earlier (5,6), isoriboflavin-5'-phosphate is not formed in the flavokinase reaction and does not inhibit the normal phosphorylation of riboflavin. Also it has been shown that isoriboflavin is an effective antagonist to riboflavin *in vivo* and inhibits the binding of FMN to TPNH cytochrome c reductase *in vitro* (15). Taken altogether these findings suggest that isoriboflavin may competitively displace FMN, both as substrate for

FAD pyrophosphorylase and as coenzyme. The observed conversions of the 5'-phosphates of 6-methylribo-,6,7-dibromoribo-, and D-araboflavins to their FAD analogues is supported by their known flavokinase-catalyzed phosphorylations (5-7) and biological activities (1). Similarly the formation of a flavin adenine dinucleotide from D-erythroflavin-4'-phosphate is supported by the recent syntheses and investigation of the biological activities of this flavin and its phosphate ester (16). The reactivity of the flavins with ω-hydroxyalkyl side chains has not yet been investigated in other systems.

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