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Substrate specificity of riboflavin hydrolase from *Pseudomonas riboflavina*

Riboflavin is known to be decomposed by several species of bacteria[1] and by plants[2]. Except in *Pseudomonas RF*[3], which extensively degrades the isoalloxazine structure of the vitamin, most of the systems were shown to cleave only the ribityl side chain and leave lumichrome as product. By using cell-free extracts of the bacterium *Pseudomonas riboflavina*, YANAGITA AND FOSTER[4] were able to show that riboflavin was hydrolyzed to lumichrome and ribitol, and the name of riboflavin hydrolase (EC 3.5.99.1) was introduced. A similar hydrolase has also been extracted from plant tissues and shown to require reduced glutathione and Mg^{2+} for maximal activity[2]. Though the stoichiometry and cofactor requirements have been elucidated, both substrate specificity and mechanism of the reaction remain unclear. The present study was undertaken to circumscribe more completely the substrate specificity of the riboflavin hydrolase system from *P. riboflavina*.

D-Riboflavin [6,7-dimethyl-9-(1'-D-ribityl)isoalloxazine] was obtained from Eastman Organic Chemicals. [2-^{14}C]Riboflavin was from Nuclear-Chicago Corporation. Other flavins were supplied by the Merck, Sharp and Dohme Laboratories or synthesized as reported previously[5].

P. riboflavina (ATCC 9526) was cultured aerobically for 24 h at 30° on a riboflavin–yeast extract–salts medium[4]. The bacteria were harvested with a Servall continuous-flow centrifuge, and washed twice with 0.9% NaCl solution. Cells were suspended in approx. 20 vol. of 0.1 M potassium phosphate buffer (pH 7) and ruptured by sonic oscillation for 5 min with a Branson sonifier. The mixture was then centrifuged at 900 × *g* for 30 min to remove the unbroken cells and large fragments. The turbid supernatant solution was centrifuged again at 10 000 × *g* for 30 min to obtain most of the enzymatic activity in the sedimented particles. This particulate fraction was washed and resuspended in the phosphate buffer for use as enzyme preparation. Aliquots were diluted as required for assay.

Reaction mixtures generally contained 0.1 mmole potassium phosphate buffer (pH 7), 1 mμmole flavin, and 0.5 ml of enzyme preparation in 2.5 ml total volume. The mixture was incubated in 25-ml erlenmeyer flasks at 30° for 30 min in a gyrotatory shaker. The reactions were stopped by adding 2.5 ml of ethanol containing 5% acetic acid, and the mixtures were centrifuged to remove the particulate matter. Controls were run in the same manner, except that the ethanol–acetic acid solution was added before the enzyme preparation. Generally, the amount of flavin remaining was measured with an Aminco-Bowman spectrophotofluorometer, in which the activating wavelength was set at 450 mμ and the fluorescence read at 520 mμ. The amount of [^{14}C]riboflavin remaining and the formation of [^{14}C]lumichrome were determined by applying aliquots of the ethanol–acetic acid extracts to Whatman No. 1 paper, developing the chromatograms in *n*-butanol–acetic acid–water (4:1:5, v/v/v, upper phase), and quantitating the radioactivity with an Actigraph III radiochromatogram scanner. Lumichrome was also detected by means of an ultraviolet lamp, and the amount quantitated by measuring its fluorescence with a Densicord recording densitometer.

The stoichiometry and progress of riboflavin degradation catalyzed by ribo-

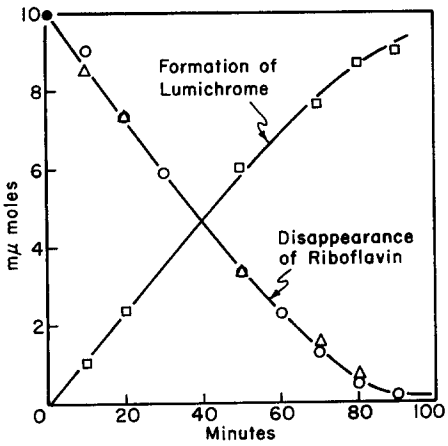

Fig. 1. Stoichiometry and progress of the reaction. Symbols are: ○, fluorescence assay of riboflavin remaining; △, chromatographic assay of [^{14}C]riboflavin remaining; □, chromatographic assay of [^{14}C]lumichrome formed.

flavin hydrolase is shown in Fig. 1. The reaction follows a near-linear course until the latter stages when substrate becomes limiting. There is a stoichiometric relationship between riboflavin degradation and lumichrome formation. No other compounds were detected.

The substrate activities of flavins relative to riboflavin are listed in Table I. The significant but decreased activities of iso- and dichlororiboflavin point up the preference for the 6,7-dimethylisoalloxazine structure of the natural vitamin. Also

TABLE I

RELATIVE SUBSTRATE ACTIVITIES OF FLAVINS

Flavin	*Relative activity*	*Flavin*	*Relative activity*
D-Riboflavin	100	D-Alloflavin	5
Isoriboflavin	31	D-Erythroflavin	6
Dichlororiboflavin	16	DL-Glyceroflavin	5
3-Methylriboflavin	2	6′-Hydroxyhexylflavin	9
L-Lyxoflavin	26	5′-Hydroxypentylflavin	14
L-Deoxylyxoflavin	8	4′-Hydroxybutylflavin	17
D-Lyxoflavin	3	3′-Hydroxypropylflavin	7
D-Araboflavin	13	2′-Hydroxyethylflavin	6
D-Galactoflavin	4	Formylmethylflavin	5
D-Sorboflavin	1	Lumiflavin	7
D-Dichlorosorboflavin	3		

the system is more reactive toward a D-ribityl side chain as shown by the lower substrate activities of the polyhydroxyl chains of L-lyxo- and D-araboflavin and the ω-hydroxyalkyl chains of 5′-hydroxypentyl- and 4′-hydroxybutylflavin.

The v_{max} and K_m values of some representative flavins are listed in Table II. The system has a high ability to catalyze the degradation of riboflavin and requires

TABLE II

SUBSTRATE PROPERTIES OF REPRESENTATIVE FLAVINS

Flavin	v_{max} *(mμmoles decomposed per 30 min)*	K_m *($M \times 10^7$)*
D-Riboflavin	8.4	16.0
Isoriboflavin	1.0	1.3
L-Lyxoflavin	0.9	0.6
5′-Hydroxypentylflavin	0.4	0.7
D-Erythroflavin	0.2	0.8

higher substrate concentration to saturate the enzyme to attain the relatively higher v_{max}. This might be responsible for the high K_m value observed. It should be noted that substrate concentration is very critical in the assay and most flavins show substrate inhibition above an optimum.

Finally, it should be pointed out that there is no obvious correlation between the mechanism involved in the hydrolase-catalyzed cleavage of flavins and that involved in their photodecomposition, as has been erroneously suggested by others[2]. The former enzymic reaction entails the splitting off of the intact side chain. In the present study, formation of lumichrome was proved in the degradation of L-lyxoflavin, D-araboflavin, and 5′-hydroxypentylflavin as well as riboflavin. The corresponding 5,6-dimethylalloxazine was demonstrated to arise from isoriboflavin[4]. On the other hand, photochemical cleavage leads to intermediates with partially degraded side chains, even in the case of ω-hydroxyalkyl flavins which exhibit different kinetics during photolytic cleavage[6].

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