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PREPARATION OF RIBOFLAVIN SPECIFICALLY LABELED IN THE 5'-HYDROXYMETHYL TERMINUS USING A VITAMIN B_2-ALDEHYDE-FORMING ENZYME FROM *SCHIZOPHYLLUM COMMUNE*

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Summary

A method is described for synthesis of riboflavin selectively labeled in the hydrogens at the 5'-hydroxymethyl position. In this method, a vitamin B_2-aldehyde-forming enzyme from *Schizophyllum commune* is used to specifically and completely oxidize the 5'-hydroxymethyl of riboflavin to the 5'-aldehyde. This reaction is monitored spectrophotometrically by the reduction of 2,6-dichlorophenolindophenol at 600 nm. Appearance of aldehyde product was directly quantitated by reverse-phase high-performance liquid chromatography. Product is extracted from the incubation mixture by phenol after saturation with $(NH_4)_2SO_4$ and then further purified by benzyl alcohol extraction. The 5'-aldehyde is reduced with appropriately labeled sodium borohydride to yield the vitamin specifically labeled in the 5'-hydroxymethyl group.

Key Words: **5'-labeled riboflavin, B_2-aldehyde, *S. commune* enzyme**

Introduction

There was no high-yield, direct chemical procedure available for selectively introducing labeled hydrogens into a given position in the D-ribityl side chain of riboflavin (vitamin B_2). At present only [G-^{3}H]riboflavin is commercially available. Yet the vitamin specifically labeled would be of value in elucidating certain aspects of its transport and metabolism. Fortunately *S. commune* and other basidiomycetes have been found to oxidize the terminal 5'-hydroxymethyl group of

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riboflavin to form the corresponding aldehyde and acid, initially called schizoflavins (1-4). A vitamin B_2-aldehyde-forming enzyme, which catalyzes oxidation of riboflavin primarily to the 5'-aldehyde, *viz.* 7,8-dimethyl-10-(2,3,4-trihydroxy-4-formylbutyl)isoalloxazine, has been purified (5) and more recently shown to be rather narrowly specific for the vitamin as oxidizable substrate (6). The enzyme can utilize the redox dye 2,6-dichlorophenolindophenol (DCIP) as the reducible substrate (electron acceptor). This provides a sensitive spectrophotometric method for assay of the reaction, since it was shown previously that the formation of B_2-aldehyde and reduction of DCIP proceeds in equimolar amounts (4); hence, the aldehyde product can be conveniently estimated by following rapid reduction of DCIP at 600 nm (7) with relatively slow air reoxidation of the colorless reduced dye. The B_2-aldehyde can be facilely reduced with sodium borohydride, as was outlined in a method to deutero-label the vitamin for ^{1}H-NMR spectral analysis (8).

The above findings have been optimized in the present method whereby crude enzyme is used to form milligram quantities of B_2-aldehyde which is reduced by appropriately labeled sodium borohydride to yield riboflavin specifically labeled in the 5'-hydroxymethyl position. This approach should also be applicable for preparation of the limited number of 5'-hydrogen-labeled analogs that are substrates for the enzyme (6). The numbered structure of riboflavin with the labeled hydrogens is illustrated in Figure 1.

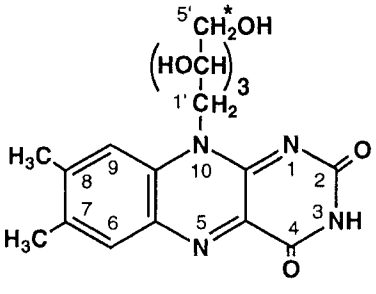

Figure 1: Numbered structure of riboflavin with labeled hydrogens indicated in the 5'-hydroxymethyl.

Experimental

Substrates and reagents

Riboflavin was purchased from Sigma Chemical Co. (St. Louis, MO). The 2,6-dichlorophenolindophenol (sodium salt) was from Aldrich Chemical Co. (Milwaukee, WI). Sodium borohydride can be obtained as the unlabeled compound from Aldrich, which is also a source for the deuterium-labeled compound (98 atom % D); the tritium-labeled compound is sold

by Amersham and can be obtained in aqueous 0.1 M NaOH with a specific radioactivity of 999 GBq/mmol (27 Ci/mmol). Culture medium (YM broth) and HPLC-grade acetonitrile were obtained from Fisher Scientific Co. (St. Louis, MO). Other common chemicals were of analytical grade from Mallinckrodt.

Cultivation of *Schizophyllum commune*

The organism was obtained from the American Type Culture Collection (No. 20165) and maintained on YM agar plates. Seed cultures were prepared by mincing pieces of agar-grown fungi in a Waring blender, transferring the mince into YM broth, and cultivating the liquid cultures aerobically on a reciprocal shaker for 2 days at 30° C. Large-scale cultures (6 x 2 liters) were prepared by 5% inoculations followed by shaking for 3 days. Mycelia were harvested by filtering the broth, washing the filter cake with distilled water and drying by lyophilization overnight.

Enzyme Preparation and Assay

Crude enzyme was prepared from the dried mycelia as described previously (6) with the exception that mycelia were broken by freezing in liquid nitrogen and grinding with a mortar and pestle prior to homogenization in 0.1 M sodium acetate buffer (pH 5.5). Enzyme activity was assayed by the rate of DCIP reduction monitored by the decrease in absorbance at 600 nm using a Spectronic 3000 Array Spectrophotometer (Milton Roy) with the Rate Analysis Software Program. One unit of enzyme activity was defined as the amount of enzyme which forms 1 μmole of B_2-aldehyde or reduces 1 μmole of DCIP per minute at 25° C as calculated from its molar extinction coefficient which approximates 7.6×10^3 at pH 5.5.

Chromatographic Analyses

The product formed was confirmed by conventional ascending paper chromatography using Whatman No. 1 paper and ethyl acetate: pyridine:water (10 : 3 : 3, v/v/v, upper phase) as a solvent system to differentiate riboflavin (R_F = 0.43) from the 5'-aldehyde (R_F = 0.55) (9). A more rapid check on conversion of substrate to product can be done by thin layer chromatography using relatively short strips (5 cm x 7.5 cm) of silica gel on polyester (Aldrich, 250 μm) with chloroform:acetonitrile: dimethylformamide (5 : 3 : 2, v/v/v) as solvent to resolve riboflavin (R_F = 0.38) from the 5'-aldehyde (R_F = 0.53). For HPLC a Shimadzu LC-600 system equipped with a rheodyne injector was linked to a Shimadzu SPD-6A UV spectrophotometric

detector for monitoring absorbance (270 nm) or a Gilson 121 fluorometer equipped with 305 - 395 nm excitation filter and a 475 - 650 nm emission filter (Gilson Medical Electronics, Middleton, WI) for monitoring fluorescence. A μBondapak C18 column (0.39 x 30 cm, 10 μm) was used for the analyses. The mobile phase was 80 : 20 (v/v) of 5 mM ammonium acetate (pH 6) : acetonitrile at a flow rate of 1 ml/min.

Results and Discussion

The B_2-aldehyde obtained by enzymatic conversion of riboflavin using DCIP as an electron accepting cosubstrate was recovered from the reaction mixture by phenol and benzyl alcohol extractions. The aldehyde is then subjected to reduction by $NaBH_4$ with or without labeled hydrogens to yield correspondingly labeled riboflavin which is then extracted with benzyl alcohol and reextracted into water. Purity can be checked with ascending paper chromatography (4,9) or by reverse phase HPLC as described below.

Operational steps and determinations based on a typical one-liter enzyme reaction mixture are described in the following.

Formation of B_2-aldehyde

The mixture initially contained 213 μM (80 mg) riboflavin and 448 μM (130 mg) of 2,6-dichlorophenolindophenol (sodium salt) with 0.25 units of enzyme per liter of 50 mM sodium acetate buffer, pH 5.5.

Monitoring the reaction

The mixture is stirred in the dark for approximately 24 hours at 30° C. It should be noted here that the stoichiometry of the two substrates is 1 : 1 (7), but the excess of DCIP used insures completion of reaction, as illustrated by the data in Figure 2 where a further addition of DCIP was made after beginning with an amount only equimolar to riboflavin.

The time required for completion of the reaction was estimated based on enzyme activity, but to be certain of this, DCIP reduction was monitored spectrophotometrically over 24 hours. More reoxidation of reduced DCIP occurs with longer time, but this does not affect flavin aldehyde formed. An example of results obtained during enzyme-catalyzed oxidation of riboflavin with reduction of DCIP followed by the gradual air reoxidation of the reduced DCIP is shown in Figure 3.

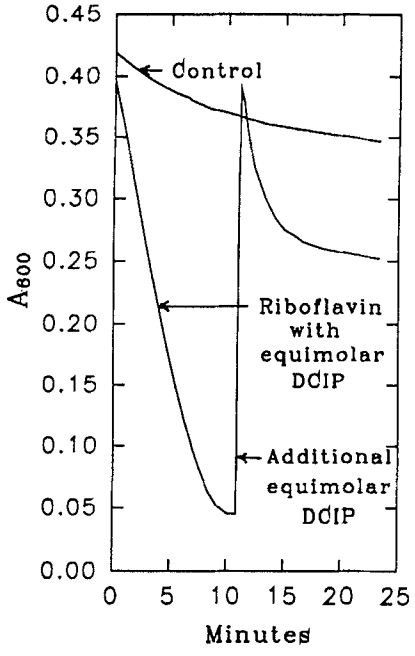

Figure 2: Determination of DCIP concentration helpful for reaction completion. The initial reactions in standard cuvets contained 50 μM DCIP plus or minus (control) 50 μM riboflavin and 0.012 units of enzyme in 50 mM sodium acetate, pH 5.5, in 3 ml total volume. After 10 min of incubation at 25° C, when the reduction seemed to be complete, a second portion of DCIP equivalent to the initial one was added as shown.

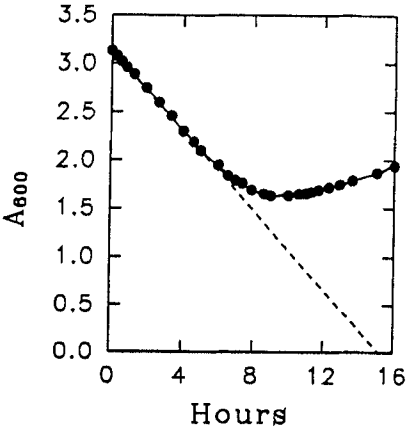

Figure 3: Reduction and reoxidation of DCIP during long-time incubation. The reaction mixture contained 213 μM Riboflavin, 448 μM DCIP and 2.5 x 10^{-4} units of enzyme per ml in 50 mM sodium acetate buffer, pH 5.5 at 30° C. The dashed line indicates an extrapolation toward maximal 5'-aldehyde formation.

Formation of the B_2-aldehyde during the reaction is maximal (~95%) after ~16 hours of incubation under the given conditions when aliquots were examined by paper chromatography. More quantitative assessment of the disappearance of riboflavin and appearance of B_2-aldehyde was made using HPLC. A typical resolution of a substrate/product mixture is shown in Figure 3.

Isolation of B_2-aldehyde

Product B_2-aldehyde was obtained from the reaction mixture by simplifying the phenol extraction method used by Tachibana and Murakami (4) followed by benzyl alcohol extraction

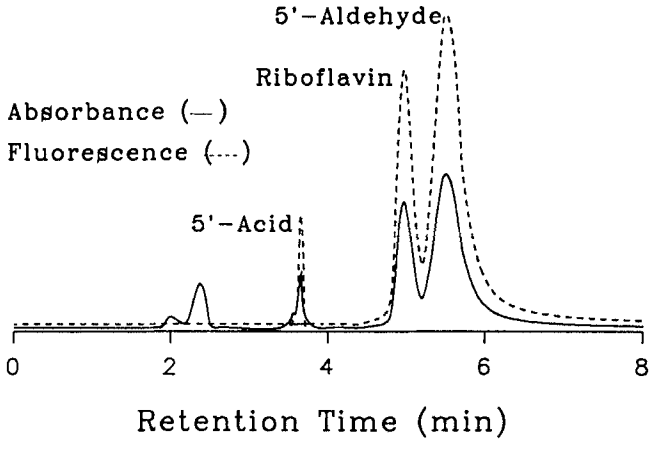

Figure 4: HPLC Profile of ~100-μL (0.2 μg) mixture of riboflavin and the 5'-aldehyde with traces of the 5'-acid and an impurity derived through the benzyl alcohol phase of extraction of an incubation mixture of riboflavin with the enzyme.

modified from Williamson and Edmondson (8). All operations were done in dim (or red) light to avoid photodecomposition of flavin. The reaction mixture (1 liter) is saturated with $(NH_4)_2SO_4$ and flavin is extracted into one-tenth volume (100 ml) of aqueous phenol (~90% phenol and 10% water) by vigorous shaking in a separatory funnel. The lower phase is discarded and 150 ml of water and 150 ml of diethyl ether is added to the upper phenol phase. This mixture is shaken to extract phenol into a top ether phase and leave flavin in the bottom aqueous phase. This aqueous fraction is extracted a second time with 150 ml of diethyl ether and then reduced in volume (~50 ml) by rotary evaporation under reduced pressure below 50° C. The concentrated aqueous flavin solution still contains significant salt. Flavin is extracted into 200 ml of benzyl alcohol and the lower phase washed once with 50 ml of water. Two volumes (400 ml) of diethyl ether and 1 volume (200 ml) of water are added to the benzyl alcohol fraction. This mixture is then shaken to force flavin back into the aqueous phase which is washed twice with 100-ml portions of ether to remove traces of benzyl alcohol. The final aqueous solution is reduced in volume by rotary evaporation under partial vacuum below 50° C and the concentrate lyophilized to obtain solid B_2-aldehyde.

Yields of the flavin aldehyde are typically about 25% (based on starting riboflavin) with purity greater than 90%. Extraction with phenol after ammonium sulfate saturation of the initial reaction results in a better yield than starting with benzyl alcohol, but flavin extracted by benzyl alcohol exhibits higher purity. Therefore, the combined extraction using phenol initially to

concentrate flavin from large volumes and then using benzyl alcohol later to remove the residual $(NH_4)_2SO_4$ seems to be optimal for obtaining a relatively salt-free product in reasonable time and yield.

Reduction of B_2-aldehyde to 5'-hydrogen-labeled riboflavin

The conversion of flavin aldehyde to riboflavin specifically labeled in the 5'-hydroxymethyl position can be done essentially as described for the deuterium-labeled compound (8). The known chemistry is that the labeled hydrogen of product derives from a hydride ion that is added to the aldehydic carbon and the second hydrogen in the generated alcoholic function derives from solvent. Hence, one can vary the extent of label by changing the ratios of labeled and unlabeled reductant borohydride. Typically 0.1 mmole (38 mg) of flavin aldehyde is reduced with 20-fold excess of sodium borohydride (20 mg) containing labeled hydrogen, e.g. 25 mCi (925 MBq) of $[^3H]NaBH_4$ in 1.25 ml of 0.1 M NaOH stirred into 125 ml of flavin in sodium phosphate buffer (pH 8.0) at 25° C for 30 min in dim light. The tritiated riboflavin product (Sp. Act. = 12.5 mCi or 460 MBq/mmole) is isolated by benzyl-alcohol extraction (50 ml) generally as described above. Approximately 30 mg of product representing 79% flavin recovery and a 4% radiochemical (tritium) yield is obtained. If traces of impurities are present, final purification can be achieved by preparative chromatography on Whatman No. 3 paper using descending ethyl acetate: pyridine: water (10:3:3, v/v/v, upper phase) or other solvents found useful in ascending, analytic paper chromatography (4) or by preparative HPLC. The band of pure 5'-labeled riboflavin is eluted from the paper with minimum water and the eluate rotoevaporated to dryness in the dark.

Acknowledgements

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