Synthesis of 10-(8-Aminooctyl)flavin and Its Use in Affinity Chromatography

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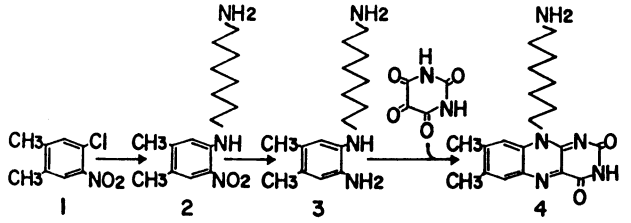
Synopsis. 7,8-Dimethyl-10-(8-aminooctyl)isoalloxazine was synthesized by coupling *N*-(8-aminooctyl)-4,5-dimethyl-1,2-benzenediamine with alloxan. The flavin was immobilized to epoxy activated gel to use for affinity chromatography. The gel was effective for purification of riboflavin-binding protein from egg white and flavokinase from rat liver.

10-(ω-Aminoalkyl)flavins (7,8-dimethylisoalloxazines) are readily bound covalently to many kinds of solid supports, for example, epoxy-, CNBr-, or *N*-hydroxysuccinimide-activated ones, with the alkylamino group. The immobilized flavin would be useful for affinity chromatography, technology using flavin-flavin binding protein complexes, study on flavoenzyme mechanism, preparation of flavoenzyme functioned materials, and others. The only example of such a flavin to be reported is 7,8-dimethyl-10-(2-aminoethyl)isoalloxazine. This was synthesized by reductive amination of 10-(formylmethyl)flavin, a periodate oxidation product of riboflavin, with sodium cyanotrihydroborate and ammonium chloride.[1] This synthetic route, however, is an unusual one for an (ω-aminoalkyl)flavin. In this report, we describe a more convenient synthetic method for this kind of flavin, as is shown in Scheme 1.

1-Chloro-2-nitro-4,5-dimethylbenzene (**1**) was coupled with 1,8-octanediamine to give 1-(8-aminooctylamino)-2-nitro-4,5-dimethylbenzene (**2**), which was catalytically hydrogenated with Raney's catalyst. Addition of strong acid was necessary to complete the reduction. The reduced compound **3** was coupled with alloxan in the usual way to give 7,8-dimethyl-10-(8-aminooctyl)isoalloxazine (**4**).

The epoxy-activated Cellulofine, a cellulose gel, was prepared and was bound with **4** as described by Sundberg and Porath.[2] The flavin content of the gel so prepared was approximately 17 μmol of the flavin/mL of the hydrated gel, and was relatively high compared with many kinds of flavin immobilized gel prepared by Merrill and McCormick (16.4—1.4 μmol mL^{-1}).[3]



Scheme 1.

The flavin immobilized gel so obtained was used for affinity chromatography to show the high efficiency of the gel. Many kinds of flavin affinity chromatographic materials have already been prepared which have various features including ease or difficulty of preparation, high or low flavin content, high or low efficiency, etc.[4] An important factor is the position of immobilization of the flavin; for example, only gels immobilized at position 10 of flavin were effective for the purification of flavokinase from rat liver; gels immobilized at position 3 and 8 were negative.[3] Consequently, two proteins were chosen for purification; riboflavin-binding protein (RBP) from egg white, since this protein has been used for many studies and is easy to obtain, and flavokinase from rat liver, since the gel seems to be suitable for the purification of this protein as described above.

RBP, partially purified as described in the experimental section, was subjected to affinity chromatography and the elution pattern was shown in Fig. 1. RBP was bound to the gel and selectively eluted with a buffer containing 20 μM (M=mol dm^{-3}) riboflavin.

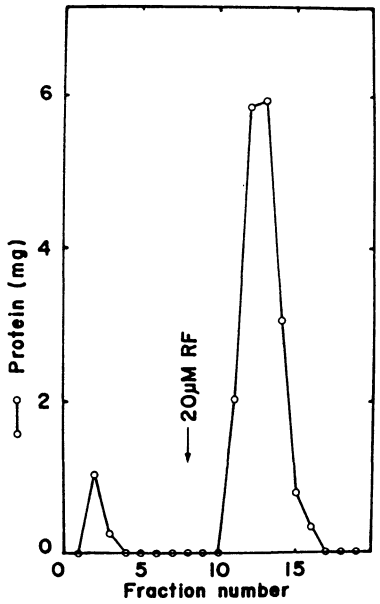


Fig. 1. Elution pattern for affinity chromatography of riboflavin-binding protein. Partially purified RBP was applied on a flavin-immobilized column and the column was washed. The bound RBP was eluted from the column with a phosphate buffer containing 20 μM riboflavin. The amount of protein in each fraction was determined according to Lowry et al.[7] and plotted.

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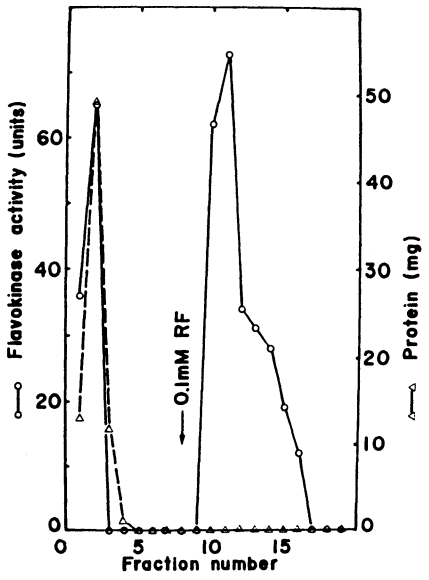

Fig. 2. Elution pattern for affinity chromatography of flavokinase from rat liver. A pooled sample from Sephadex G-75 chromatography was applied on an affinity chromatography and the column was washed. The bound flavokinase was eluted from the column with 100 μM riboflavin solution. The amount of protein (△) and flavokinase activity (○) in each fraction were plotted. Protein concentrations were determined according to Smith et al.[8]

The purified sample was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and gave a single band (data not shown).

A crude extract of rat liver was fractionated with ammonium sulfate, and a 55 to 75% saturation fraction was chromatographed on a Sephadex G-75 column. Fractions containing flavokinase activity were collected and applied on the affinity column. Flavokinase with high specific activity was eluted with 0.1 mM riboflavin solution as shown in Fig. 2, though approximately 30% of the total eluted activity were found in the washings similarly to the affinity column reported by Merrill and McCormick.[5] Flavokinase binds irreversibly to fresh gel, so that a better yield was obtained after using several times.

These results indicate that the affinity column so prepared is effective for the purification of many proteins such as riboflavin carrier protein and flavokinase from many different origins.

Experimental

UV spectra were recorded on a Hitachi 320 recording spectrophotometer; 400 MHz ^{1}H NMR spectra were taken with a JEOL NMR spectrometer model JMM GX 400. Melting points were measured with a Yanaco MP micro melting point apparatus.

1-(8-Aminooctylamino)-2-nitro-4,5-dimethylbenzene (2). 1-Chloro-2-nitro-4,5-dimethylbenzene (**1**) was synthesized according to the procedure of Adams et al.[6] from 4,5-dimethyl-2-nitroaniline (Aldrich). A mixture consisting of 5.0 g of **1** and 25.0 g of 1,8-octanediamine was heated at 100 °C for 2 h and cooled to room temperature. The brown syrup obtained was then dissolved in 250 mL CH_2Cl_2 and washed five times with water. Anhydrous $MgSO_4$ was added to the organic phase and filtered off. The filtrate and washings were combined and evaporated in vacuo. The residue, dissolved in CH_2Cl_2, was applied on a silica-gel column (Wakogel C-200, 3 ×25 cm) and developed with a mixture of CH_2Cl_2 and methanol (8 : 2). Fractions from the second large red band were collected and evaporated. The syrupy residue was dissolved in a minimum amount of toluene and **2** was crystallized by gradually adding hexane. Fine orange crystals were filtered off, washed with hexane and dried over $CaCl_2$ in vacuo. Yield 7.0 g (88%): mp 68—70 °C; ^{1}H NMR ($CDCl_3$) (*exchangeable H) δ=1.34 (6H, m, CH_2), 1.44 (2H, m, CH_2), 1.51 (2H, m, CH_2), 1.71 (2H, m, CH_2), 2.17 (3H, s, CH_3), 2.27 (3H, s, CH_3), 2.62* (2H, s(broad), NH_2), 2.74 (2H, t, J=7 Hz, 8′CH_2), 3.26 (2H, td, J=5 and 7 Hz, 1′CH_2), 6.61 (1H, s, φ-H), 7.91 (1H, s, φ-H), 7.97* (1H, s(broad), NH). Found: C, 65.60; H, 9.20; N, 14.04%. Calcd for $C_{16}H_{27}N_3O_2$: C, 65.49; H, 9.28; N, 14.32%.

7,8-Dimethyl-10-(8-aminooctyl)isoalloxazine (4). 1.0 g of **2** was dissolved in 30 mL acetic acid and hydrogenated over 5% palladium on carbon (400 mg) at 100 °C under atmospheric pressure. When the color of the solution changed from orange-red to colorless (approximately 1 h later), 3 mL concentrated hydrochloric acid was added to the reaction mixture and hydrogenation was continued for a further 1 h. The catalyst was filtered off and washed with acetic acid. The combined filtrate and washings were evaporated and 30 mL water was added to the residue. The solution was evaporated to dryness to remove acetic acid. This procedure was repeated twice. The residue was dissolved in 30 mL water and DEAE-cellulose (OH^- form, suction dried weight, 20 g) was added to the solution and the suspension stirred gently for 10 min. The cellulose was filtered off and washed with water. The combined filtrate and washings were evaporated. Because the triamine so obtained was readily oxidized, it was subjected to the next reaction without isolation. The residue, dissolved in 30 mL methanol, was added to a hot solution of 0.5 g alloxan monohydrate and 10 g boric acid in 20 mL methanol. The solution was heated at 60 °C for 2 h and evaporated. The syrupy residue was crystallized from a solution of 2 g boric acid in 20 mL ethanol to yield 1.0 g (43%) of **4** as pentaborate. The crystals were dried over P_2O_5 at 150 °C overnight for elemental analysis: mp over 300 °C; ^{1}H NMR (DMSO-d_6) δ=1.32 (6H, m, CH_2), 1.47 (2H, m, CH_2), 1.57 (2H, m, CH_2), 1.71 (2H, m, CH_2), 2.40 (3H, s, CH_3), 2.52 (3H, s, CH_3), 2.75 (2H, m, 8′CH_2), 4.58 (2H, t, J=8 Hz, 1′CH_2), 7.81 (1H, s, φ -H), 7.90 (1H, s, φ-H), 8.06* (2H, s(broad), NH_2), 11.31* (1H, s, 3NH). UV (50 mM phosphate buffer pH 7.0) 223 nm (ε_{mM} 27.3), 266 (31.5), 368 (8.86), 443 (10.7); (6 M HCl) 265 (28.9), 392 (18.0), (0.1 M NaOH) 270 (36.3), 352 (10.3), 447 (10.8); Found: C, 41.71; H, 4.97; N, 12.43%. Calcd for $C_{20}H_{27}N_5O_2 \cdot 1.3H_2B_4O_7$: C, 41.86; H, 5.19; N, 12.20%.

Preparation of Flavin Immobilized Gel. Cellulose gel (Cellulofine, Chisso) was epoxy-activated using 1,4-butanediol diglycidyl ether according to Sundberg and Porath,[2] and allowed to react with **4**. 2.7 g of **4** was dissolved in a mixture of 50 mL of dioxane and 50 mL of 0.2 M carbonate buffer, pH 10.5, and 120 mL epoxy-activated Cellulofine gel (hydrated) was added to the solution. The slurry was stirred gently at 60 °C for 24 h. The yellow stained gel was filtered off and washed with hot water, dioxane, acetic acid and 20% NaCl solution repeatedly until greenish yellow fluorescence was not observed in the washings. Finally, the gel was washed with buffer for affinity chromatography. The nitrogen contents in the dried samples of modified and unmodified Cellulofine were 0.66 and 0.06% respectively. It was calculated from these data that the dry and hydrated gel contained approximately 86 μmol of flavin/g and 17 μmol of

flavin/mL respectively.

Affinity Chromatography. RBP from Egg White. RBP was partially purified from two eggs according to Nishina[9] before affinity chromatography; RBP was bound to 20 g DEAE-cellulose at pH 4.3 and then eluted with 0.1 M acetate buffer, pH 3.6. The collected fractions were rechromatographed on a Sephadex G-25 column (90×2.6 cm) using a glycine-HCl buffer (pH 3.15) to remove riboflavin. Protein was precipitated from the eluate by adding ammonium sulfate to 85% saturation. The precipitate was dialyzed against a 50 mM phosphate buffer (pH 7.0). The dialyzed sample (3 mL) was applied on a column of the flavin immobilized gel (15×1.0 cm), and the column was washed with the same buffer. RBP was eluted from the column with the phosphate buffer containing 20 μM riboflavin.

Flavokinase from Rat Liver. A buffer containing 0.1 M 3-morpholino-1-propanesulfonic acid (MOPS) and 0.1 mM $ZnSO_4$, pH 7.0 was used throughout. Flavokinase activity was determined as follows; each assay mixture was prepared adding 100 μL sample to 300 μL of a buffer containing 100 μM riboflavin, 1 mM ATP, 0.1 mM $ZnSO_4$, and 0.1 M MOPS, pH 8.0. After 30 min incubation at 37 °C, 50 μL of the mixture was assayed using HPLC; a Cosmosil 10C18 packed column (4.6×150 mm, Nacalai Tesque) and 50 mM phosphate buffer, pH 6.25, containing 0.5% pyridine, 9% ethanol, were used for separation. FMN synthesis was expressed in units of flavokinase activity; one unit produces 1 nmol FMN/h under the conditions described above.

Three rat livers (approximately 25 g) were homogenized in 75 mL buffer and the supernatant was fractionated by adding ammonium sulfate. A 55 to 75% saturation precipitate was dissolved in a small amount of buffer and applied on a Sephadex G-75 column (30×2.6 cm). After development, the fractions containing flavokinase activity were pooled and approximately one third of the volume was applied on the flavin affinity column (10×1.0 cm) again and the column was washed with the buffer. Fractions with high flavokinase activity were obtained by elution with the buffer containing 100 μM riboflavin.

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