In Vitro Binding of Riboflavin to Subcellular Particles from Maize Coleoptiles and Cucurbita Hypocotyls

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Abstract. Saturable and reversible in vitro binding of [14C]riboflavin was found to occur on subcellular, sedimentable particles from maize coleoptiles and Cucurbita hypocotyls. The KD was ca. 6 μM, the pH optimum was near 6.0, and the number of binding sites amounted to 0.1-0.5 μM on a fresh-weight basis. When the reducing agent dithionite was present, riboflavin binding increased - the KD was 2.5 μM, and the pH optimum above 8.0. The binding was specific: flavin mononucleotide (FMN) and flavin adenosinedinucleotide (FAD) bound less tightly to these sites than riboflavin and another major soluble flavin, the previously described riboflavin-analog “FX”, occurring in grass coleoptiles. These flavin-binding sites were localized on vesicles derived from plasmalemma and endoplasmic reticulum by analyzing sucrose and metrizamide density gradients and marker enzymes.

Key words: Cucurbita - Endoplasmic reticulum - Light (blue) receptor Plasmalemma - Riboflavin binding - Zea.

Introduction

The action spectrum of phototropism in grass coleoptiles or in Phycomyces, indicates a flavoprotein or carotenoid as the photoreceptor pigment. Since carotenoidless mutants of Phycomyces (less than 0.1% of the carotenoid content of the wildtype) have been shown to be fully sensitive to blue light (Presti et al., 1977), it is most likely that the photoreceptor contains a flavin. Other evidence favoring a flavin as the photoreceptor has been summarized by Presti and Delbrück (1978). The fine structure of the action spectrum in the blue region indicates that the photoactive flavin for tropism must be bound, located in a hydrophobic environment, or both. The latter hypothesis is further supported by studies with polarized light in lower (Jaffe, 1958; Jaffe and Etzold, 1962; Jesaitis, 1974) and higher plants (Zurzycki, 1972), implicating a dichroic, oriented photoreceptor localized at or near the plasmalemma.

In corn, the amount of tightly bound, non-mitochondrial flavoprotein is relatively low (Jesaitis et al., 1977), while there is abundant free flavin. In Avena, the riboflavin analog “FX” constitutes the major flavin component in the cell (Zenk, 1967b), while in maize, riboflavin constitutes two-thirds and “FX” one-third of the soluble flavin (Hertel, unpublished).

In this paper we hypothesize that the above-described bound and oriented flavin photoreceptor may result from a reversible interaction of a soluble, extractable flavin with a specific membrane-binding site. We present evidence for the existence of a specific binding site for riboflavin. Furthermore, we show that the site is localized on plasma membrane and endoplasmic reticulum fractions from corn coleoptiles and Cucurbita hypocotyls.

Material and Methods

Chemicals and Radiochemicals

Dithioerythritol, sodium dithionite, sucrose, buffers, and riboflavin were obtained from E. Merck, Darmstadt, FRG. Stock solutions of riboflavin were kept at 80 μM in water or buffer at -20 °C. FMN, FAD, uridine-5-diphosphoglucose (ammonium salt), and reduced β-nicotinamide dinucleotide (NADH) were purchased.
from Boehringer GmbH, Mannheim, FRG; cytochrome c from Serva, Feinbiochemica, Heidelberg, FRG; metrizamide (2-(3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzimido)-2-deoxy-D-glucose) from Dr. Molter GmbH, Heidelberg, FRG; 2-[14C]riboflavin 1.18·10^8 Bq mmol⁻¹ from Amersham-Buchner, Braunschweig, FRG; and 2,3,4,5-[3H]naphthylphthalamic acid (NPA) 6.23·10^11 Bq mmol⁻¹ from CEA-France, Gif-sur-Yvette, France. NPA and auxin analogs were obtained as described by Jacobs and Hertel (1978).

Plant Material
Seeds of zucchini squash (Cucurbita pepo L. cv. Black Beauty, Asgrow Seed Company, Kalamaozo, MI, USA) were planted in moist vermiculite and grown for six days at 24 °C in total darkness. Seeds of maize (Zea mays L., WF9 x Bear 38, Bear Hybrid Corn Co., Decatur, IL, USA) were soaked in water and grown on wet paper for five days in a dark room at 24 °C, receiving 2 h of red light every night. Sections of zucchini hypocotyls 2-3 cm long, beginning just below the apical hook, were harvested on ice at daylight. Maize coleoptiles were removed from the primary leaf above the node. Oats (Avena sativa L.) were also planted in moist vermiculite and grown in total darkness at 24 °C. They were harvested after five days for flavin extraction from the shoots.

Particle Preparation
All procedures were carried out at 2-4 °C in daylight. Hypocotyl sections or coleoptiles were chopped with razor blades in a volume of extraction medium (0.25 M sucrose, 50 mM tris-acetate, pH 8, 1 mM EDTA, 0.1 mM MgCl₂) equal in mls to the gram weight of the plant material. The chopped material was ground in a mortar and squeezed through a narrow mesh nylon cloth. The remaining cake was ground in a second, equal volume of extraction medium, again squeezed through nylon, and the filtrates from each squeezing were combined. This crude filtrate (about pH 7) was centrifuged at 5,000 g for 10 min (with exception of the experiment in Table 4), the pellet being discarded. When an unfractionated, particulate preparation was desired, the supernatant from the above precentrifugation was centrifuged at 200,000 g for 20 min in a Beckman Ti50 rotor (Beckman Instruments, Fullerton, CA). The supernatant was discarded and the pellets (5-200 KPa) were resuspended in a glass homogenizer in 1 ml of binding assay medium/g fresh weight of experimental tissue. Since the material could be kept indefinitely when frozen at −70 °C without loss in RBF-binding activity, large batches of unfractionated particles were prepared, resuspended at 6 g fresh tissue weight equivalents/ml in gradient medium (see below) plus 0.25 M sucrose, deep-frozen and quickly rethawed just before use.

Flavin Extraction
A crude flavin extract was obtained following the procedures of Zenk (1967a), both from oats and from corn. The total flavin content was determined as described below.

Fractionation and Density Gradients
In experiments employing differential centrifugation, the pooled nylon filtrate was centrifuged sequentially in angle rotors (Sorvall SS 34 to 40,000 g, Spinco Ti50 for higher forces). Pellets were resuspended in a glass homogenizer in gradient medium (10 mM tris-acetate, 1 mM EDTA, 0.1 mM MgCl₂, 1 mM KCl, pH 7), an aliquot was removed for enzyme assays, and the remainder of each fraction was diluted with the appropriate binding assay medium to 1 ml particulate suspension/g fresh weight of experimental tissue.

To fractionate particulate matter by density gradient centrifugation, the supernatant from the precentrifugation was layered over density gradients made by one of two different methods. In the first, solutions of gradient medium with 15% and 45% (w/w) sucrose were employed. Linear gradients (15-45%, 20 ml) were constructed, each over 2 ml 45% sucrose, in the bottom of cellulose nitrate centrifuge tubes. A layer of 2 ml 15% sucrose was pipetted onto the top of each gradient. In the second method, solutions of gradient medium containing 28% metrizamide (Rickwood and Birnie, 1975) or a mixture of 5% metrizamide and 5% sucrose were employed. Linear gradients (5%+5% to 28%, normally 20 ml) were constructed over 2 ml 28% metrizamide in the bottom of the centrifuge tubes. A layer of 1.5 ml 5% metrizamide +5% sucrose was pipetted onto the top of each gradient. A second layer of 0.5 ml 9% sucrose in gradient medium was pipetted over the 5%+5% layer.

After both methods of density-gradient preparation, the supernatant from the precentrifugation (approximately 16 ml) was layered over the gradient and the tubes were centrifuged at 100,000 g for 180 min (sucrose gradients) or 50-60 min (metrizamide gradients) in a SW 27 rotor (Beckman Spinco L2-50B ultracentrifuge). After centrifugation, fractions of a prescribed volume were collected with a gradient fractionator and the sucrose or metrizamide density was determined with a hand-sugar refractometer. The fractions were subsequently tested for RBF binding and enzyme activities.

Binding Assays
Specific, i.e., saturable, [3H]NPA binding was determined as described by Ray (1977) and Dohrmann et al. (1978). The assay for [14C]riboflavin binding to pelletable material was similar to that for auxin binding (see Ray et al. 1977). Unfractionated or gradient-fractionated subcellular particles were resuspended in re-suspension medium containing 0.25 M sucrose, 10 mM MOPS and 5 mM MgSO₄, adjusted to pH 6. When a pH higher than 6.5 was needed, MOPS and tris were added, keeping the total buffer molarity at 25 mM. The binding test was started with the addition of [14C]RBF at 1 or 2·10⁻⁷ M. This labeled suspension was distributed to individual tubes. To each tube, one-tenth volume on non-radioactive riboflavin or flavin extracts at different concentrations (0-40 μM) in buffer or water was added. When reducing conditions were required, Na-dithionite was dissolved to 1 M in water immediately before the test and diluted into the assay samples to yield 5·10⁻³ M. Normally, the pelleting test was done with 2 ml samples in a Ti50 Rotor Spinco centrifuge at 186,000 g for 20 min at 4 °C. Some routine assays were centrifuged in 1 ml Ependendorf vessels in a Sorvall SM 24 rotor — with adaptors — at 40,000 g for 45 min. After centrifugation, the supernatant was decanted and an aliquot was counted. The ends of the centrifuge tubes containing the pellets were cut off, put directly into the scintillation fluid (Bray’s solution), and the radioactivity counted ("cpm in pellet"). Saturable or specific [14C]RBF binding is the difference in radioactivity between a sample containing only the radioactive RBF and the cpm in a parallel sample to which saturating amounts of unlabeled RBF had also been added. The data can also be presented as “% saturable (or specific) binding” (see Tables 1, 2 and 4) when the saturable cpm in the pellets are expressed as percent of the total cpm left in the supernatant.