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Uptake and Binding of Riboflavin by Membrane Vesicles of Bacillus subtilis

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Riboflavin uptake and membrane-associated riboflavin-binding activity have been investigated in Bacillus subtilis. The uptake and binding activity of the vitamin were found to be repressed coordinately by riboflavin present in the growth medium. The uptake of riboflavin has been shown to have properties of a carrier-mediated process, and membrane vesicles have been shown to demonstrate riboflavin counterflow and exchange. The membrane-associated binding activity for riboflavin has been solubilized with detergents, and a procedure for the partial purification of this component is described. The partially purified riboflavin-binding component has properties expected for a carrier involved in riboflavin uptake, as it shows saturation kinetics and is inhibited by riboflavin analogues. Evidence is also presented showing that reduced riboflavin binds to a greater extent than oxidized riboflavin, and the possible role of the reduced riboflavin in riboflavin uptake is discussed.

Key words: riboflavin, vitamin, Bacillus subtilis, binding activity, membrane vesicle

Although the transport of vitamins has not received nearly so much attention as the transport of other cellular nutrients, bacterial transport systems for a number of vitamins have been described [1]. Transport of folate and thiamine, as well as membrane-associated binding activity for these vitamins, has been investigated in the gram-positive organism Lactobacillus casei [2, 3]; thiamine transport has also been investigated in Escherichia coli [4]. Transport of vitamin B_{12} in E coli has been investigated and appears to involve an outer membrane receptor protein followed by slower, energy-dependent appearance of the vitamin within the cell [5, 6]. Transport systems for other vitamins such as lipoic acid [7], nicotinamide [8], vitamin B_6 [9, 10], and biotin [11, 12] have also been described.

Riboflavin transport had been previously investigated in the yeast Saccharomyces cerevisiae [13]; riboflavin was transported to a significant extent only in riboflavin-requiring strains of the yeast. Riboflavin-requiring strains of Bacillus subtilis have also been reported [14], and we have used one of these, B subtilis H52, to investigate riboflavin uptake by bacteria [15]. In B subtilis, riboflavin uptake was rapid and efficient even at

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low concentrations of riboflavin, with a K_m in the range of 5–20 nM. The uptake was consistent with a system whose properties are indicative of a carrier-mediated process: it is inhibited by substrate analogues, exhibits saturation kinetics, and is temperature-dependent. In addition, membrane-associated binding activity for riboflavin was found, and a binding component was demonstrated whose properties are consistent with its being part of the riboflavin uptake system in this organism. For example, the component had high affinity for the vitamin, with the K_D (10–30 nM) in the same range as the K_m for uptake.

Riboflavin uptake in intact B subtilis appeared to be intimately coupled to the action of flavokinase, as riboflavin was rapidly converted to FMN and FAD. The flavokinase of B subtilis has the unusual, perhaps unique, property of functioning only with reduced flavins and not with oxidized flavins, as is normally the case [16]. It was speculated that riboflavin might be reduced during the transport process and then acted upon by the flavokinase [16].

This report documents further evidence for the role of the membrane-associated riboflavin-binding component in vitamin transport. In addition, a partially purified preparation of the riboflavin-binding component is described. It is also shown that the binding component responds differently to reduced riboflavin than to oxidized riboflavin.

MATERIALS AND METHODS

Cells and Materials

Strain 168M, trp C_2 of B subtilis and a riboflavin-requiring mutant, H52, trp C_2, derived from it were obtained from Dr. A. Bacher, Technical University, Munich, Germany. [2-^{14}C]-Riboflavin (28.8 mCi/mmole) was purchased from Amersham/Searle. 5-Deazariboflavin and 2-thioriboflavin were gifts of Dr. Peter Hemmerich, University of Konstanz, Germany. Lumiflavin was the gift of Dr. Sandro Ghisla, University of Konstanz, and 7α-methylriboflavin was the gift of Dr. John P. Lambooy, University of Maryland.

Growth Conditions and Preparation of Membrane Vesicles

B subtilis H52 was grown and riboflavin uptake assayed as previously described [15]. Membrane vesicles were prepared essentially as described by Konings and co-workers [17, 18].

Riboflavin-Binding Assays

The binding of [2-^{14}C]-riboflavin to vesicles was measured either by Millipore filtration or by equilibrium dialysis as described previously [15]. Binding by solubilized extracts was determined by equilibrium dialysis.

Partial Purification of Riboflavin-Binding Component

Membrane vesicles of B subtilis H52 were stored in liquid nitrogen. The vesicles (464 mg) were thawed at 46°C, suspended in 95 ml of 10 mM Tris/Cl, pH 8.0, containing 50 mM NaCl and 1% (w/v) sodium cholate. The suspension was stirred at 2°C for 30 min and then centrifuged at 100,000g for 75 min. The cholate treatment solubilizes only a small amount of the riboflavin-binding activity while removing a significant amount of protein from the vesicles. The pellet is then suspended in 75 ml of 10 mM potassium phosphate, pH 7.0, containing 2% (w/v) Triton X-100, stirred for 30 min at 2°C, and then centrifuged for 75 min at 100,000g. The supernatant is chromatographed on a column

(2 × 25 cm) of DEAE-cellulose 52, equilibrated with the same buffer but containing only 0.2% Triton X-100. The column is washed with the buffer, then with 50 mM potassium phosphate, pH 7.0–0.2% Triton X-100. Most of the riboflavin-binding activity is eluted with the 50 mM potassium phosphate wash. Subsequent elutions with 100 mM and 500 mM potassium phosphate-Triton removes remaining protein, but little riboflavin-binding activity. The 50 mM potassium phosphate fractions containing riboflavin-binding activity are combined and dialyzed against 10 mM Tris, pH 8.4–0.2% Triton X-100. After dialysis the solution is concentrated with an ISCO electrophoretic concentrator (ISCO, Lincoln, Nebraska) at 3 ma in Tris buffer for 2 h as described by Allington et al [19]. The concentrated material is dialyzed against 10 mM potassium phosphate, pH 7.0–0.2% Triton X-100 and then applied to a Biogel HT hydroxylapatite column (1 × 15 cm) equilibrated in the same buffer. The column is washed with the same buffer, most of the riboflavin-binding activity being found in the effluent. Washing with 50 mM, 100 mM, and 500 mM potassium phosphate–0.2% Triton X-100, and finally with 500 mM phosphate–Triton containing 1 M NaCl, elutes the remaining protein, but with recovery of only an additional 25% of the riboflavin-binding activity. These are, therefore, discarded and only the initial fractions, containing the most highly purified riboflavin-binding activity are pooled. The preparation is stable for several days at 2°C.

One activity unit is defined as 1 pmole of riboflavin bound, specific binding activity as 1 pmole/mg of protein.

RESULTS

The ability of whole cells of B subtilis H52 to bind and transport riboflavin was dependent upon the concentration of riboflavin added to the growth medium (Fig. 1). The maximal activities for both binding and transport were found when the cells were grown in the presence of very low amounts of riboflavin (approximately 30 μg/liter or 80 nM). At higher concentrations of added riboflavin, both binding and transport activities were repressed coordinately. The data illustrated in Figure 1 show that 50% loss of riboflavin-transport and binding activity occurred at 170 and 280 nM concentrations in the growth medium, respectively. Although not shown in Figure 1, uptake and binding in the parent strain, B subtilis 168M, were not significantly affected by the riboflavin concentration supplied during growth. It should be noted that the parent strain does not require riboflavin for growth, and uptake and binding by this strain are extremely low.

The data in Figure 2 demonstrate riboflavin counterflow in membrane vesicles prepared from B subtilis H52. In this type of "entrance counterflow" experiment, vesicles were suspended in 10 μM cold riboflavin and subsequently diluted into media containing 0.4 μM [2-^{14}C]-riboflavin. The concentration of radioactive riboflavin within the vesicles reached a level of 30 pmoles/mg of membrane protein, equivalent to a 1:1 distribution of riboflavin if an internal volume of 3 μl/mg of membrane protein is assumed [20]. This is what would be predicted for a facilitated-diffusion system, where affinities for entrance and exit for a specific substrate are equal and no energy is required for movement. The efficiency of this movement is due in part to the frequency with which the carrier returns from the outer to the inner surface of the membrane in the loaded vs the unloaded form [21–23]. Figure 3 shows that vesicles equilibrated with 10 μM radioactive riboflavin and then diluted 100-fold into media containing 10 μM unlabeled riboflavin rapidly exchange the intravesicular pool of riboflavin, with a $t_{1/2}$ for loss of label of approximately 3 sec. The results in Figures 2 and 3 are consistent with a carrier-mediated uptake for riboflavin in B subtilis H52.

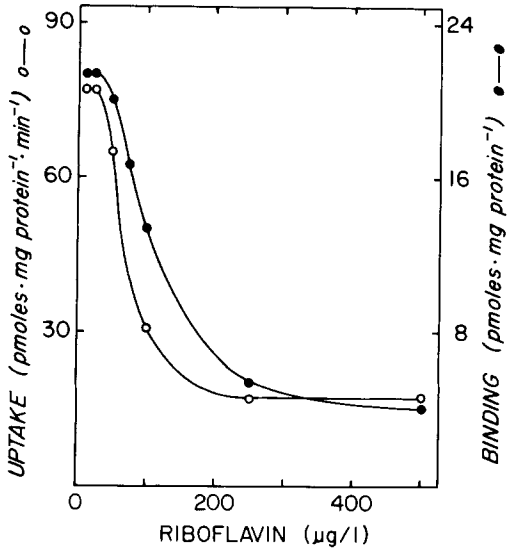

Fig. 1. Effect of riboflavin added to the growth medium upon the uptake (○—○) and binding (●—●) of riboflavin. The assay mixtures contained 0.4 μm [2-^{14}C]-riboflavin, and the assays for uptake and binding were as previously described [15].

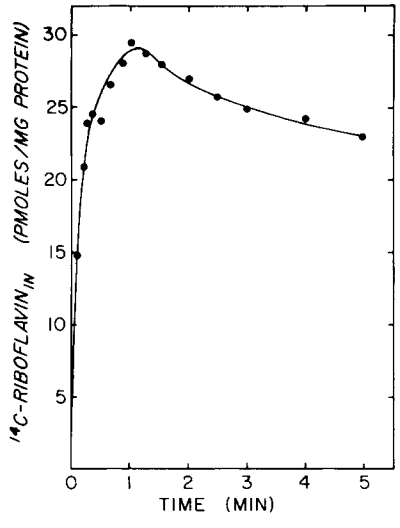

Fig. 2. Riboflavin counterflow in membrane vesicles. B subtilis H52 membrane vesicles were concentrated to 22 mg of protein/ml in 0.1 M potassium phosphate, pH 6.6, and equilibrated with 10 μM riboflavin for 3 h at room temperature. Then aliquots of 4 μl were diluted into 400 μl of 0.1 M phosphate, pH 6.6, containing 0.4 μM[2-^{14}C]-riboflavin, and counterflow was assayed by filtration of the vesicles on Millipore HAMK 0.45 μ filters [15, 21]. The background binding of riboflavin to the vesicles was subtracted before calculating the level of entry of riboflavin.

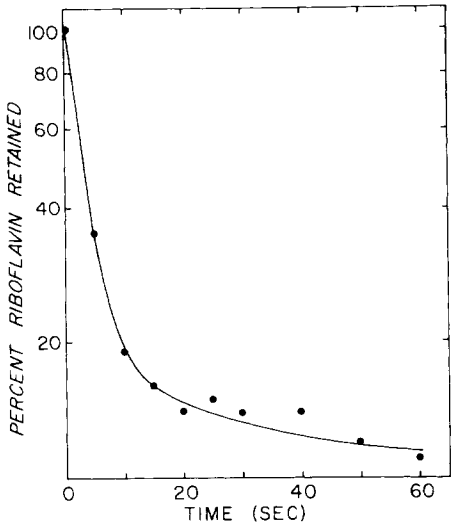

Fig. 3. Riboflavin exchange in membrane vesicles. B subtilis H52 vesicles were concentrated to 23 mg of protein/ml in 0.1 M potassium phosphate, pH 7.0, and equilibrated with 10 μM [2-^{14}C]-riboflavin for 3 h at room temperature. Then 4 μl of the suspension was diluted into 400 μl of potassium phosphate, pH 7.0, containing 10 μM unlabeled riboflavin. The vesicles were separated from the suspension as described in Figure 2.

It was of interest to attempt to purify the membrane-bound component responsible for riboflavin-binding in these vesicles. Table I shows the purification obtained by the procedure detailed in Materials and Methods. The degree of purification in the final step varies from 10- to 20-fold in various preparations. The most highly purified fraction obtained shows 3 major Coomassie blue-staining bands on sodium dodecyl sulfate slab gel electrophoresis and 4–5 bands that stain less intensely (data not shown). All bands have apparent molecular weights between 20,000 and 70,000, based on marker proteins on the gels. The band associated with riboflavin-binding activity has not yet been purified.

The properties of the partially purified binding component are similar to those observed with intact vesicles, including the effects of pH and temperature and the affinity for riboflavin. The probable identity of the purified binding component and that expressed in the vesicles is affirmed by the strikingly similar specificity for riboflavin analogues documented in Table II. The riboflavin analogues tested in Table II also inhibit riboflavin transport in whole cells, as previously shown [15].

It has been previously shown that riboflavin uptake in B subtilis H52 was intimately coupled to the action of the flavokinase of this organism [15]. In addition, it is known that the flavokinase of this organism has the unusual property of catalyzing phosphorylation only of reduced flavins [16]. It was of interest, therefore, to see what effect reduction of riboflavin might have on riboflavin binding to vesicles. It may be seen in Table III that riboflavin binds to a slightly greater extent in intact vesicles when it is reduced rather than oxidized. When vesicles are made permeable by treatment with toluene, however, reduced riboflavin binds to a greater extent. We have observed that detergent-solubilized extracts of

TABLE I. Purification Procedure for Riboflavin-Binding Component

Step	Total protein (mg)	Total activity (units)	Specific binding activity (pmoles/mg protein)
Vesicles	464	41,760	90
Cholate supernatant	76	5,092	67
Triton X-100 pellet	250	17,500	70
Triton X-100 supernatant	120	15,840	132
DEAE-cellulose eluate	38	9,310	245
ISCO electrophoretic concentrate	20	7,700	385
Hydroxylapatite effluent	3	2,976	992

TABLE II. Inhibition of Riboflavin-Binding by Riboflavin Analogues*

Analogue	Inhibition in vesicles (%)	Inhibition in purified preparations (%)
5-Deazariboflavin	75	60
7α-Methylriboflavin	98	75
2-Thioriboflavin	42	47
Lumiflavin	< 10	< 10

*Binding of riboflavin by vesicles and the purified binding component was measured by equilibrium dialysis in the presence of 0.4 μM [2-^{14}C]-riboflavin and 4 μM analogues.

the vesicles exhibit increased binding of reduced vs oxidized riboflavin (data not shown). Perhaps the cytoplasmic face of the membrane vesicle has more sites available to bind reduced riboflavin.

DISCUSSION

Previous work in our laboratory had shown that riboflavin uptake in B subtilis was sensitive to metabolic poisons and to temperature changes, and that this process followed saturation kinetics with a K_m value in the range of 5–20 nM [15]. Further evidence has been presented here that transport of riboflavin is carrier-mediated and that a binding component is an integral part of the transport system. Riboflavin-binding activity has proved to be repressed coordinately with transport activity in whole cells, as are the thiamine and folate transport systems of L casei [2, 3] and the branched-chain amino acid transport system of E coli [24]. In addition, a binding component for riboflavin in the bacterial membrane has been extracted and partially purified, which shows binding properties similar to those observed with intact membrane vesicles in terms of affinity for riboflavin and 5-deazariboflavin [15], broad pH range for activity [15], and specificity for analogues of riboflavin (Table II). The component requires detergent for solubilization and behaves like a hydrophobic protein.

TABLE III. Binding of Reduced versus Oxidized Riboflavin by Vesicles*

Additions	Riboflavin bound (pmoles/mg protein)
Oxidized riboflavin	66.2
Oxidized riboflavin + toluene[a]	75.7
Reduced[b] riboflavin	90.1
Reduced[b] riboflavin + toluene[a]	268.5

*Riboflavin binding was measured by equilibrium dialysis.
[a]Vesicles treated with toluene (1%, v/v) were incubated for 30 min at 37°C prior to beginning dialysis against riboflavin.
[b]All components were pre-equilibrated with argon, and a flow of argon was maintained over the solutions during additions. Riboflavin was reduced by the addition of solid sodium dithionite to give 2 mM final concentration. Dialysis was carried out under anaerobic conditions, and the riboflavin remained reduced throughout, as judged by lack of fluorescence.

The counterflow and exchange of riboflavin by membrane vesicles are also properties indicative of carrier-mediation [22, 25]. In addition, these experiments show more definitively that riboflavin actually enters the vesicles, since binding of riboflavin to the membranes is corrected for and further net changes in isotope content of the vesicles are observed. The counterflow experiment further indicated that re-equilibration of the internal riboflavin with the external solution on dilution of the vesicles to a lower external riboflavin concentration was very slow, whereas the initial change resulting in "overshoot," like exchange, was fast. This may imply that the rate of transport is accelerated when the carrier is bound to substrate in each direction, much like trans-stimulation [23], or that the affinity of the unbound carrier for riboflavin may be lower, or that exit of riboflavin may occur by a different route.

In the bacterial membrane vesicles it has not yet been possible to demonstrate convincingly any energy requirement for riboflavin uptake, as is immediately evident in studies with whole cells, where riboflavin is rapidly converted upon entry to the phosphorylated derivatives, FMN and FAD [15]. Although this conversion in itself could explain the energy dependence of riboflavin uptake in whole cells, if it is required for intracellular retention of the vitamin, full understanding of the process is complicated by the peculiar requirements of the enzymes involved in the synthesis of FMN and FAD, namely that the flavin substrates must be in the reduced form (at least with these enzymes isolated in the cell-free form [16]). Thus, energy in the form of reducing equivalents must also be supplied within the cells, probably as NADH or NADPH, to generate reduced flavin. It is conceivable that the reduction step could occur in the membrane as part of the transport process, to be followed immediately by flavokinase and FAD synthetase action. To this end, we have attempted, with negative outcome, to determine if NADH or NADPH could stimulate transport and accumulation of riboflavin by membrane vesicles. Direct testing of reduced riboflavin as substrate has given results similar to those with oxidized riboflavin, in that binding occurs but no net uptake by vesicles is evident. Interestingly, the reduced flavin was bound to a greater extent than oxidized flavin, especially by vesicles rendered permeable by exposure to toluene. If riboflavin is reduced during transport, binding of

reduced riboflavin by the interior face of the vesicles might be expected, but not necessarily to a greater extent. The increased binding might reflect exposure of other sites with affinity for reduced riboflavin that are not necessarily related to transport. Further work on the specificity of these binding sites is clearly indicated.

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