FLAVIN BINDING TO THE HIGH AFFINITY RIBOFLAVIN TRANSPORTER RibU

Ria H. Duurkens, Menno B. Tol, Eric R. Geertsma, Hjalmar P. Permentier and Dirk Jan Slotboom

Department of Biochemistry
University of Groningen
Groningen Biomolecular Science and Biotechnology Institute
Nijenborgh 4
9747 AG Groningen
The Netherlands

Running title: The riboflavin transporter RibU

The first biochemical and spectroscopic characterization of a purified membrane transporter for riboflavin (vitamin B₂) is presented. The riboflavin transporter RibU from the bacterium Lactococcus lactis was overexpressed, solubilized and purified. The purified transporter was bright yellow when the cells had been cultured in rich medium. We used a detergent-compatible MALDI-TOF mass spectrometry method (Cadene and Chait, 2000) to show that the source of the yellow color was riboflavin which had been co-purified with the transporter. The method appears generally applicable for substrate identification of purified membrane proteins. Substrate-free RibU was produced by expressing the protein in cells cultured in chemically defined medium. Riboflavin, FMN and roseoflavin bound to RibU with high affinity and 1:1 stoichiometry (Kd for riboflavin 0.6 nM), but FAD did not bind to the transporter. The absorption spectrum of riboflavin changed dramatically when the substrate bound to RibU. Well-resolved bands appeared at 441, 464 and 486 nm, indicating a hydrophobic binding pocket. The fluorescence of riboflavin was almost completely quenched upon binding to RibU and also the tryptophan fluorescence of the transporter was quenched when flavins bound. The results indicate that riboflavin is stacked with one or more tryptophan residues in the binding pocket of RibU. Mutagenesis experiments showed that W68 was involved directly in the riboflavin binding. The structural properties of the binding site and mechanistic consequences of the exceptionally high affinity of RibU for its substrate are discussed in relation to soluble riboflavin binding proteins of known structure.

Riboflavin (vitamin B₂) is a water-soluble vitamin that is converted by flavokinases and FAD-synthases to the cofactors FMN and FAD. These cofactors are indispensable for all living organisms and they are involved in a wide range of reactions (1). Vertebrates have lost the ability to synthesize riboflavin and need to take up the vitamin from their gut (2). The proteins involved in epithelial uptake of riboflavin and membrane transport into other cell-types in the body have not been identified yet.

Most prokaryotes, fungi and plants can synthesize riboflavin using pathways that have been well-studied (3). In addition, some prokaryotes and fungi can also take up riboflavin from the environment. The molecular identities of two very diverse types of riboflavin transporters have recently been established in yeast and bacteria (4-6). A classical genetic approach revealed that riboflavin is transported across the
plasma membrane of the yeast *Saccharomyces cerevisiae* by Mch5p, a homologue of mammalian monocarboxylate transporters (6). *In vivo* transport studies suggested that the protein facilitates diffusion and does not use metabolic energy for transport of riboflavin. In the bacteria *Lactococcus lactis* and *Bacillus subtilis* two homologous riboflavin transporters, YpaA and RibU, respectively, were found (4;5). RibU is a 22.8 kD membrane protein with 5 predicted membrane-spanning segments. RibU is not homologous to any previously characterized flavin binding protein and belongs to a novel family of transport proteins with members found in Bacteria and Archaea. *In vivo* transport studies showed that RibU, like Mch5p in yeast, is likely to mediate facilitated diffusion (4). Here we have overexpressed and purified RibU, and we have spectroscopically and biochemically characterized the binding of flavins to the transporter.

**EXPERIMENTAL PROCEDURES**

*Materials.* Riboflavin, FMN and FAD were obtained from Sigma, roseoflavin from Toronto Research Chemicals. The concentrations of the flavins in solution were determined photometrically (7). For roseoflavin an extinction coefficient of 31 mM⁻¹.cm⁻¹ at 505 nm was used (8). Dodecyl-β-D-maltoside was obtained from Anatrace. [³H]-riboflavin (24 Ci/mmol) was purchased from Moravek Biochemicals. All other chemicals were of analytical grade and obtained from commercial sources.

*Strains, plasmids and growth conditions.* *Lactococcus lactis* strains NZ9000 (9) and NZ9000-*Δ*ribA (10) were used for cloning and expression. The ribU gene was engineered with the coding sequence for a C-terminal 10 Histidine tag by PCR. An *Nco*I site was introduced to coincide with the start codon and an *Xba*I site was engineered immediately after the stop codon. These restriction sites were used to clone the ribU-His gene into expression plasmid pNZ8048 (9) behind the *nisinA* promoter. The DNA sequence was checked (ServiceXS, The Netherlands). Three mutants (W68Y, W79Y and W97Y) were made using the PCR overlap extension method and cloned into a pNZ8048 derived vector by ligase independent cloning (E.R. Geertsma, manuscript submitted). The sequences coding for an N-terminal His-tag and TEV protease cleavage site were introduced. All mutations (TGG to TAT) were confirmed by DNA sequencing (Service XS, The Netherlands).

*L. lactis* strains were grown at 30°C in either GLX or M17 (Difco) medium supplemented with 1.0% (w/v) glucose and 5 μg/ml chloramphenicol. GLX contained 2% (w/v) gistex LS (Strik BV, Eemnes, NL) and 65 mM potassium phosphate (KP) pH 7. Cells were grown in 1 liter bottles to an A₆₀₀ of 0.7, followed by induction of the expression with 0.1% (v/v) culture supernatant of the Nisin A producing strain NZ9700 (9). The cells were harvested 1.5 hr after induction. For the production of riboflavin-free RibU-His, 6 liter of cells were grown in M17 medium to an A₆₀₀ of 0.7. Subsequently, cells were harvested, washed with sterile 50 mM KP, pH 7 and transferred to a pH and temperature controlled fermenter (pH 6.8, 30°C) containing 10 L of Chemically Defined Medium supplemented with 1.0% (w/v) glucose and 5 μg/ml chloramphenicol (11) without riboflavin. After 15 minutes expression was induced with Nisin A as described above. Cells were harvested after 1.5 hours of induction and were frozen and stored at -80°C.

**Purification of RibU-His.** Membrane vesicles were prepared according to standard procedures (12) and stored at -80°C. For solubilization, membrane vesicles were resuspended to a protein concentration of 1.5 mg/ml in buffer A (10% glycerol, 300 mM NaCl, 50 mM Tris/HCl pH 8) supplemented with 15 mM imidazol/HCl, pH 8, and 0.5% dodecyl-β-D-maltoside (DDM) and incubated on ice for 30 min with occasional mixing by inverting. Unsolubilized material was spun down (20 min at 440,000xg and 4°C) and the supernatant was incubated with Ni-Sepharose chromatography medium equilibrated in buffer A containing 15 mM imidazol, pH 8, (1 ml medium/15mg membrane protein, GE Heathcare) for 1 hour at 4°C with gentle agitation. The resin was poured into a disposable column (Biorad) and washed with 20 column volumes of buffer A supplemented with 60 mM imidazol, pH 8, and 0.05% DDM. Proteins were eluted in buffer A supplemented with 500 mM imidazol, pH 8, and 0.05% DDM in 5 fractions of 0.5 column volume each. 5 mM Na-EDTA, pH 7.5, was added to the
fractions and the peak fraction (0.5 ml) was loaded onto a 23 ml Superdex 200 gel filtration column (GE Healthcare) equilibrated with gel filtration buffer (20 mM Tris pH 7.5, 150 mM NaCl and 0.05% DDM). Fractions containing RibU-His were collected and used immediately.

**Mass spectrometry.** Mass spectra were recorded on a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems). For spectra of the purified protein the linear mode was used and samples were prepared according to Cadene and Chait (13). For riboflavin detection the reflectron mode was used.

**Protein concentration determination and UV/VIS absorption spectroscopy.** The protein content of membrane vesicles was estimated using the BCA (Pierce) or Bradford (14) assay with bovine serum albumin as the standard. The concentration of purified RibU-His was determined by measuring absorption at 280 nm using an extinction coefficient of 1.14 ml.mg⁻¹.cm⁻¹. For calculation of the extinction coefficient quantitative amino acid analysis was performed (Eurosequence, The Netherlands). UV/VIS absorption spectra were recorded on a Varian Cary 100 Bio UV-visible spectrophotometer.

**Isothermal Titration Calorimetry (ITC).** ITC experiments were performed using a MCS calorimeter (MicroCal) at 25 °C. Membranes (1.5 mg/ml in 50 mM Tris/HCl, pH 7.5) or purified RibU-His (4.25 μM in gel filtration buffer) were added to the MCS cell. Riboflavin (140 μM) was dissolved in 50 mM Tris/HCl pH 7.5 (for vesicles) or in gel filtration buffer (for purified protein) and titrated into the cell. Data was analyzed using the MicroCal software provided (15).

**Fluorescence titration.** Measurements were performed on a Spex Fluorolog 322 Fluorescence spectrophotometer (Jobin Yvon) at 25 °C in a 1 ml stirred cuvet. For fluorescence titration experiments 0.1-1.8 μM purified RibU-His was used and solutions of riboflavin, roseoflavin, FMN or FAD were added in 1-2 μl steps. For riboflavin, FMN and FAD the excitation and emission wavelengths were 435 nm and 523 nm, respectively. Titrations in the absence of protein were performed as reference. Tryptophan fluorescence was measured using an excitation wavelength of 280 nm.

**Flow dialysis.** Flow dialysis was performed essentially as described by Veldhuis et al. (16) at 25 °C in a 0.38 ml cell. Membranes (0.4 mg/ml in 50 mM Tris/HCl pH 7.5, 150 mM NaCl) or purified RibU-His (1-2 μM in gel filtration buffer) were added to the dialysis cell. Tritiated riboflavin (stock solution 120 μM) was titrated into the cell. Titrations in the absence of protein were performed as reference.

**Data analysis.** Fluorescence titration and flow dialysis data were analyzed essentially as described by Veldhuis et al. (16) with the following modifications. The measured signals (fluorescence or scintillation counts) were subtracted from the signals of the reference titrations and the differences were plotted against the final concentration of added substrate. Curve fitting (OriginLab) was performed to find the best values for p, n₀ and K_d using the following model:

\[
[ES] = \frac{p \left( n + s + K_d \right)}{2} \left( \sqrt{\left( n + s + K_d \right)^2 - 4 * n * s} \right)
\]

in which n is the concentration of binding sites in the cell; s is the final substrate concentration added; K_d is the dissociation constant; p is an instrument response factor; [ES] is the concentration of RibU-substrate complex. Dilution of the concentration of binding sites by the titration of substrate was taken into account:

\[
n = n_0 \left( \frac{s_0 - s}{s_0} \right)
\]

in which n₀ is the concentration of binding sites at the start of the titration and s₀ is the concentration of substrate in the stock solution. In the analysis of the flow dialysis data corrections were made to account for the amount of substrate dialyzed from the cell (16).

**RESULTS**

**Expression and purification of His-tagged RibU.** The riboflavin transporter RibU from *Lactococcus lactis* was tagged with a C-terminal 10 Histidine-tag and expressed in *L. lactis* NZ9000. Strikingly, membranes isolated from the cells expressing the Histidine-tagged transporter (RibU-His) were bright yellow, whereas typically membrane vesicles from *L. lactis* are white. RibU-His was solubilized from the membranes with the
detergent dodecyl-β-D-maltoside (DDM) and purified using Ni-Sepharose and gel filtration chromatography. SDS-PAGE showed that the protein was more than 99% pure (Fig 1A) and the yield was approximately 2 mg per liter culture. The integrity of the purified His-tagged transporter (24.4 kD) was confirmed by mass spectrometry (Fig 1B).

Identification of the bound substrate. Just like the membrane vesicles isolated from the RibU-His expressing cells, the purified protein was also yellow. The absorption spectrum in the visible region of the purified protein had two peaks with maxima at 361 nm and 464 nm, respectively, and the latter peak had well defined shoulders at 441 nm and 486 nm (not shown, but compare Fig 3A). The spectrum differed from the spectrum of riboflavin (dissolved in the same buffer), either because the spectral properties of riboflavin had changed when the molecule bound to the transporter, or because there was a different yellow molecule bound to RibU-His. We used MALDI-TOF mass spectrometry to reveal the source of the yellow color. MALDI-TOF MS can be used to analyze membrane proteins directly in detergent solution without the need for extensive removal of impurities such as detergents, salt and buffer (13). Figure 2A and 2B show mass spectra of riboflavin (MW 376 Da in the oxidized form) and purified, yellow RibU-His, respectively, in the m/z range of singly charged riboflavin. The two spectra look very similar and have peaks at m/z values of 377, 378 and 379 corresponding to three riboflavin species: oxidized and protonated riboflavin ([376+1H]+), the protonated riboflavin radical ([376+2H]•) and protonated reduced riboflavin ([376+3H]+), as previously reported by Ohashi and Itoh (17). The peak at m/z value of 379 also contained a contribution of the α-cyano-4-hydroxy-cinnamic acid matrix, a spectrum of which is shown in Figure 2C. The spectra conclusively demonstrate that riboflavin is bound to the purified RibU-His. Two other small molecules were found in the spectrum of RibU-His: Tris (m/z 122.1) and a sodium ion adduct of DDM (m/z 533.2) (spectra not shown).

Production of substrate-free RibU-His. Because the co-purification of riboflavin and RibU-His complicates kinetic analyses of the transporter, a method to obtain riboflavin-free RibU was developed. Dialysis to remove riboflavin from the protein proved extremely inefficient and time consuming, and therefore a protocol was worked out to produce substrate-free RibU at the expression stage. We used a mutant of L. lactis NZ9000 with a disrupted ribA gene, which codes for 3,4-dihydroxy-2-butanoic acid synthase (10), an essential enzyme in the riboflavin biosynthesis pathway (3). Because riboflavin is required for growth of Lactococcus lactis, the ribA mutant is completely dependent on the uptake of riboflavin from the environment. The ribA mutant was grown to the mid exponential phase in rich medium (M17) containing riboflavin. The recombinant His-tagged RibU was not expressed at this stage, but the chromosomal copy of RibU was present for the uptake of riboflavin from the medium. Cells were then centrifuged, washed with buffer and transferred to Chemically Defined Medium (CDM) from which riboflavin had been omitted. The cultivation was continued in CDM and at the same time the expression of His-tagged RibU was induced with Nisin A. The cells continued to grow until all residual riboflavin had been depleted. Membrane vesicles prepared from these cells expressed His-tagged RibU at similar levels as membrane vesicles prepared from cells grown in rich medium (not shown), but they were colorless indicating that riboflavin-free RibU-His had been produced. The absence of bound riboflavin was confirmed by absorption spectroscopy of the purified protein (Fig 3A).

Absorption and fluorescence spectroscopy. UV/VIS absorption spectra of riboflavin were recorded in the presence of increasing amounts of purified riboflavin-free RibU-His (Fig 3A). Binding of riboflavin to RibU-His resulted in a hypsochromic shift of the absorption peak at 372 nm to 361 nm and a bathochromic shift of the maximum at 444 nm to 464 nm. Both shifts were accompanied by a reduction in the intensity. In addition, well-defined shoulders appeared at 486 and 441 nm. As expected, the spectrum of riboflavin when bound to RibU-His looked very similar to the spectrum of yellow RibU-His purified from cells grown in rich medium (spectrum not shown), again confirming that riboflavin had been co-purified with the transporter.

A similar titration was performed with roseoflavin, an inhibitor of riboflavin transport in Lactococcus lactis (4). The absorption maximum
at 505 nm shifted bathochromically to 518 nm and the shift was accompanied by an increased intensity (Fig 3B).

Fig 4A shows fluorescence emission spectra of riboflavin (0.2 μM) in the presence of increasing concentrations of RibU-His. At the emission wavelength of 523 nm 94% of the riboflavin fluorescence was quenched when the molecule was bound to RibU-His. The small emission peak at 512 nm that remained at high protein:riboflavin ratio’s was not caused by riboflavin fluorescence as it was also seen in the buffer without protein and riboflavin (not shown). The tryptophan fluorescence emission spectrum of RibU-His had a maximum at 342 nm (Fig 4B) and the fluorescence was quenched by the binding of both riboflavin and roseoflavin (Fig 4B for riboflavin). Saturating concentrations of riboflavin and roseoflavin quenched 82% and 72% of the tryptophan fluorescence, respectively, and resulted in a red-shift of the emission peak of 2-3 nm.

Binding parameters. Three independent methods were used to determine the dissociation constant and the binding stoichiometry of RibU-His and riboflavin: isothermal titration calorimetry (ITC) (15), fluorescence titration (7) and flow dialysis (16). For the calculation of the binding stoichiometry an accurate value of the protein concentration was required. We used A280 measurements and an \( \varepsilon_{280} \) of 1.14 ml.mg\(^{-1}\).cm\(^{-1}\), which was determined by quantitative amino acid analysis. Fig 5 shows the results of the ITC of purified RibU-His, from which an apparent dissociation constant of 1.8 +/- 0.7 nM was calculated and a binding stoichiometry of 0.85 molecules of riboflavin per protein molecule. Given the possibility that a fraction of the purified protein may be inactive and possible inaccuracy in the determination of the protein concentration (+/- 20% for quantitative amino acid analysis), we conclude that there is one riboflavin binding site per protein molecule. Flow dialysis using tritiated riboflavin (not shown) yielded very similar values (Table 1). Because both techniques require relatively large concentrations of protein (> 1 μM) and the affinity of RibU-His for riboflavin is very high, the determination of the dissociation constants was not very accurate. Fluorescence titration, which is a more sensitive method, allowed the used of protein concentrations as low as 100 nM which yielded a more accurate value for the dissociation constant of 0.6 nM (Fig 6 and Table 1). Roseoflavin and FMN also bound with high affinity to the transporter, but FAD did not (Table 1). This is consistent with previous in vivo transport experiments, in which it was found that roseoflavin and FMN inhibit uptake of radio-labeled riboflavin by L. lactis cells and that both compounds are likely to be transported into the cells by RibU (4).

ITC was also used to determine the binding parameters in membrane vesicles expressing riboflavin-free RibU-His. The \( K_d \) values in membrane vesicles were similar to the ones determined in detergent solubilized RibU-His (Table 1). In the membrane vesicles 5.3 nmol of binding sites for riboflavin were found per mg membrane protein. Since there is one binding site for riboflavin per RibU molecule (see above), the expression level of active RibU in the membrane vesicles was more than 10 % of total membrane protein. This is consistent with Coomassie stained SDS Polyacrylamide gels of membrane vesicles expressing RibU, which showed huge overexpression levels of RibU.

Mutagenesis of tryptophan residues. RibU contains three tryptophan residues, W68, W79 and W97. Each tryptophan was mutated to tyrosine and the riboflavin dissociation constants were determined by fluorescence titration (cf. Fig 6). Also the quenching of the remaining tryptophan residues by riboflavin binding was measured in the mutants. The riboflavin dissociation constants of the W79Y and W97Y mutants (\( K_d \) 0.8 nM each) were very similar to the wild-type value (Table 1), indicating that W79 and W97 are not involved in substrate binding. In contrast, the riboflavin dissociation constant of the W68Y mutant had changed dramatically (\( K_d \) 81 nM, Table 1).

In contrast to the wild-type protein (Fig. 4B), the tryptophan fluorescence of the W97Y mutant was quenched to completion by riboflavin binding (Fig 4D). From this we conclude that the residual tryptophan fluorescence (~18%) of the wild-type protein saturated with riboflavin can be attributed to W97. The result also demonstrates that the fluorescence of tryptophans 68 and 79 was completely quenched upon riboflavin binding. Consistently, the fluorescence of the W68Y mutant was quenched to a much lesser extent by riboflavin binding than the wild-type protein (Fig 4C). But the intensity of tryptophan fluorescence...
and the quenching behavior of mutant W79Y were very similar to the wild-type protein (data not shown) indicating that tryptophan 79 only marginally contributes to the total tryptophan fluorescence of RibU.

**DISCUSSION**

Binding of riboflavin to the transporter RibU results in marked changes in the absorption spectrum of the flavin (Fig 3). The spectral characteristics of flavins bound to proteins contain information on the properties of the binding pocket. The spectrum of riboflavin bound to RibU has very well-resolved absorption bands at 441, 464 and 486 nm, indicating a non-polar environment of the flavin (18;19). Also the red-shift of the absorption maximum from 444 nm to 464 nm and the decreased intensity of the peak at 361 nm are signs of a hydrophobic binding pocket (20;21). The spectral changes are reminiscent of the changes that occur when riboflavin binds to hen egg riboflavin binding protein (RfBP), a soluble storage protein for the vitamin in egg white (20-22). The crystal structure of hen egg RfBP has been published (23), although the coordinates are not present in the Protein Data Bank (www.pdb.org). In the crystal structure the binding pocket of riboflavin is hydrophobic with the isoalloxazine moiety of riboflavin stacked between the parallel aromatic rings of the side chains of a tryptophan and a tyrosine residue. Binding of riboflavin to hen egg RfBP results in an almost complete quenching of the riboflavin fluorescence, which is attributed to the π-stacking of the isoalloxazine ring and the aromatic residues. In addition, the tryptophan fluorescence of RfBP is quenched by approximately 85% when riboflavin or riboflavin analogues are bound (20;21), which has been attributed to the presence of 5 out the 6 tryptophans in RfBP in the vicinity (<4.5 Å) of riboflavin (23). π-Stacking of riboflavin with aromatic residues and concomitant quenching of the riboflavin fluorescence has also been seen in other riboflavin binding proteins (e.g. in 6,7-dimethyl-8-ribityllumazine synthase and dodecin (24;25)). Binding of riboflavin to RibU also resulted in quenching of both riboflavin and tryptophan fluorescence to similar extents as observed for RfBP (Fig 4). Moreover, the riboflavin dissociation constants of RfBP and RibU are very similar (1.3 nM and 0.6 nM, respectively). We therefore speculate that the riboflavin binding sites in RibU and RfBP may be similar, and that both binding pockets are very hydrophobic as indicated by the features of the absorption spectra. It must be noted that no sequence similarity exists between RfBP and RibU.

The emission maximum of the tryptophan fluorescence of the apo-protein (342 nm) indicates that the tryptophans are in a polar environment when riboflavin is not bound. RibU has three tryptophan residues, W68, W97 and W79, located at the C-terminal ends of putative membrane spanning segments 2 and 4 and at the N-terminal end of segment 3, respectively. None of these is completely conserved in the family of RibU-like proteins (4), but W68 and W97 are located in regions of well-conserved aromatic residues, and we hypothesized that their fluorescence may be quenched by riboflavin. Mutagenesis of each of the tryptophan residues to tyrosine revealed that W79 and W97 are not involved in riboflavin binding, but the properties of the W68Y mutant changed dramatically compared to the wild-type protein. The dissociation constant changed over 100 fold to 81 nM. It is likely that that tryptophan 68 is stacked with isoalloxazine ring in the binding pocket of RibU, because the tryptophan fluorescence of W68 was quenched to completion by binding of the substrate.

The affinity of RibU-His for riboflavin is very high (K_d 0.6 nM), which ranks it among secondary transporters with the highest known affinity for their substrate. For comparison, lactose permease LacY has a substrate affinity which is 6 orders of magnitude lower (26). High affinity binding to RibU is probably necessary to scavenge riboflavin from the environment in which it is present at low concentration (nM range) (2). The affinity of RibU for its substrate is comparable to the riboflavin binding affinity (K_d 5-20 nM) measured in membrane vesicles of *Bacillus subtilis* (27). Very likely the binding of riboflavin to *B. subtilis* membranes is mediated by YpaA, a homologue of RibU (5). The high binding affinity of RibU and riboflavin poses a challenge for the release of riboflavin in the cytoplasm and necessitates the internal concentration of free riboflavin to be kept low. This is very likely achieved by metabolic trapping of riboflavin by flavin kinases/FAD synthases that convert the
vitamin to FMN and FAD (27). Technically, the high affinity brings about difficulties for in vitro transport assays in isolated membrane vesicles or in liposomes reconstituted with the protein. Since the transporter catalyzes facilitated diffusion (4), no accumulation of substrate in the vesicle lumen will occur in the absence of metabolic trapping and therefore transport is easily overshadowed by binding. Similar problems have been observed for example with the high affinity nickel transporter HoxN (28).

We have used MALDI-TOF mass spectrometry to establish that the substrate riboflavin is co-purified with RibU-His. MALDI-TOF mass spectrometry is used routinely to analyze the integrity of purified membrane proteins (Fig 1B and (13)). The advantage of MALDI over other ionization techniques in the analysis of membrane proteins is that impurities (in particular the detergent needed to keep the membrane protein solubilized) do not have to be removed completely (13), which makes rapid and routine measurements possible. To our knowledge the work presented here is the first example in which the technique has been used successfully to identify the substrate bound to a membrane transport protein. Identification of the substrates of membrane transport proteins is becoming increasingly important as genomics projects have revealed large numbers of putative transporters with no known substrates (http://www.membranetransport.org/). MALDI-TOF mass spectrometry provides a generic technique to identify substrates bound to purified membrane proteins. The requirement that the substrate is co-purified with the transporter may be easily met for high-affinity binding systems such as RibU, but proteins that bind their substrates with lower affinity may loose the substrate during the purification. Nonetheless, theoretical calculations have shown that substantial amounts of substrates may be co-purified with proteins regardless of their binding affinity, provided that the protein concentrations are kept high throughout the purification (10-20 fold higher than the dissociation constant) (29). Large amounts of membrane proteins of high concentration are routinely purified for crystallization purposes and therefore MALDI-TOF mass spectrometry may provide a general tool for substrate identification in structural genomics projects in which membrane proteins of unknown function are being crystallized.

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1Abbreviations: MALDI-TOF, matrix assisted laser desorption ionization-time of flight; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; DDM, n-Dodecyl-β-D-maltoside; ITC, isothermal titration calorimetry; CDM, chemically defined medium; RfBP, riboflavin binding protein.
Figure Legends

Figure 1. Purification of RibU-His. (A) SDS-Polyacrylamide gel stained with Coomassie Brilliant Blue; 8-10 μg of protein was loaded in each lane. Lane 1, membrane vesicles expressing RibU-His. The arrow indicates the position of RibU-His. Lane 2 and 3, Supernatant and pellet after solubilization and centrifugation, respectively. Lane 4, Flow-through of the Ni-Sepharose column. Lane 5, Molecular weight markers (from top to bottom: 200, 116, 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kD). Lane 6 and 7, purified protein after Ni-Sepharose and gel filtration chromatography, respectively. (B) MALDI-TOF mass spectrum of purified RibU-His (24.4 kD). Purified RibU-His (approximately 20 μM) was diluted 10-fold in water and 1 μl was spotted on a MALDI target as described by Cadene and Chait (2000). Protein species with single, double, triple and quadruple protonation are indicated.

Figure 2. Identification of the bound substrate to purified RibU-His by mass spectrometry. MALDI-TOF mass spectra of (A) riboflavin (10 pmol) mixed with α-cyano-4-hydroxy-cinnamic acid matrix, (B) purified RibU-His 10-fold diluted in water mixed with α-cyano-4-hydroxy-cinnamic acid matrix (~5 pmol was spotted on the MALDI target) and (C) α-cyano-4-hydroxy-cinnamic acid matrix.

Figure 3. Spectrophotometric titrations of riboflavin (A) and roseoflavin (B) with substrate-free RibU-His. (A) Absorption spectra of 9 μM of riboflavin in the absence of protein (trace 1) and in the presence of 2.5 μM, 5 μM, 7.5 μM and 10 μM RibU-His (traces 2-5). Trace 6: Absorption spectrum of purified RibU-His devoid of substrate (~22 μM). (B) Absorption spectra of 3.4 μM roseoflavin in the absence of protein (trace 1) and in the presence of 0.9 μM, 1.8 μM, 2.7 μM and 3.6 μM RibU-His (traces 2-5). All spectra were recorded in gel filtration buffer.

Figure 4. Fluorescence spectra. (A) Emission spectrum of riboflavin (0.2 μM in gel filtration buffer) in the absence of protein (trace 1) and in the presence of 0.08 μM, 0.16 μM, 0.24 μM, 0.32 μM and 0.4 μM RibU-His (traces 2-6). (B) Tryptophan fluorescence spectrum of RibU-His (3.1 μM) in the absence of riboflavin (trace 1) and in the presence of 0.5 μM, 1.0 μM, 1.5 μM, 2.0 μM, 2.4 μM, 2.9 μM, 3.4 μM, 3.9 μM, 4.3 μM, 4.8 μM and 5.3 μM riboflavin (traces 2-12). (C and D) Tryptophan fluorescence spectra of the mutants W68Y (1.1 μM) and W97Y (0.9 μM) respectively, in the absence of riboflavin (trace 1) and in the presence of 0.13 μM, 0.27 μM, 0.40 μM, 0.53 μM, 0.66 μM, 0.92 μM, 1.19 μM, 1.45 μM 1.71 μM, 1.97 μM, 2.22 μM, 2.48 μM, 2.74 μM, 2.99 μM and 3.24 μM riboflavin (traces 2-16). All spectra were recorded in gel filtration buffer.

Figure 5. Isothermal titration calorimetry of RibU-His (4.25 μM) with riboflavin. The upper panel shows the heat added to the cell over time with 19 successive additions of 0.2 μM riboflavin each. The excess heat added per addition was integrated from the upper panel and plotted in the lower panel as function of the ratio of the concentrations of riboflavin and RibU-His in the cell. From the lower plot, the K_d and binding stoichiometry were calculated. Binding of riboflavin to RibU-His was exothermic (-14.5 kcal per mole of riboflavin).

Figure 6. Fluorescence titration of RibU-His (165 nM) with riboflavin. Inset: Fluorescence of riboflavin when titrated to the protein solution (open squares) or to buffer (open circles). The fluorescence difference between the two curves was calculated and plotted against the riboflavin concentration (main graph). From this plot the K_d and binding stoichiometry were calculated as described in the experimental procedures.
Table 1. Dissociation constants and binding stoichiometries of RibU-His and flavins.

| Substrate  | Method<sup>a</sup> | Sample<sup>b</sup>     | $K_d$ (nM) | Binding Stoichiometry (Riboflavin:RibU-His) |
|------------|---------------------|------------------------|------------|--------------------------------------------|
| Riboflavin | ITC                 | RibU-His               | 1.8 +/- 0.7 | 0.85 +/- 0.005                             |
| Riboflavin | ITC                 | Membranes              | 5.0 +/- 1.0 |                                            |
| Riboflavin | FT                  | RibU-His               | 0.6 +/- 0.2 | 0.86 +/- 0.004                             |
| Roseoflavin| FT-Trp              | RibU-His               | 2.5 +/- 1.2 | 0.89 +/- 0.009                             |
| FMN        | FT                  | RibU-His               | 36 +/- 6    | 0.83 +/- 0.008                             |
| FAD        | FT                  | RibU-His               | No Binding  |                                            |
| Riboflavin | Flow dialysis       | RibU-His               | 1.7 +/- 3.2 | 0.83 +/- 0.016                             |
| Riboflavin | FT                  | RibU-His W68Y          | 81 +/- 4.5  |                                            |
| Riboflavin | FT                  | RibU-His W79Y          | 0.8 +/- 0.2 |                                            |
| Riboflavin | FT                  | RibU-His W97Y          | 0.8 +/- 0.2 |                                            |

<sup>a</sup>ITC, isothermal titration calorimetry; FT, fluorescence titration (excitation 435 nm, emission 523 nm); FT-Trp, fluorescence titration (excitation 280 nm, emission 340 nm); <sup>b</sup>RibU-His, purified substrate-free protein; Membranes, membrane vesicles expressing substrate-free RibU-His.
Figure 1

A

B

% intensity

100

80

60

40

20

0

5000
10000
15000
20000
25000

m/z

12196 [M+2H]⁺

6103 [M+4H]⁺

8134 [M+3H]⁺

24390 [M+1H]⁺
Figure 2

A

B

C

% Intensity

% Intensity

% Intensity

370 375 380 385 390

m/z

378.12 [M+2H]^*
377.11 [M+1H]^*
379.06 [M+3H]^*

379.04 [M+3H]^*
378.09 [M+2H]^*
377.09 [M+1H]^*

379.02
Figure 3

A

Absorption

300 350 400 450 500 550
Wavelength (nm)

B

Absorption

440 460 480 500 520 540 560
Wavelength (nm)
Figure 4

[A] 

[B] 

[C] 

[D]
Figure 5
Figure 6
Flavin binding to the high affinity riboflavin transporter RibU
Ria H. Duurkens, Menno B. Tol, Eric R. Geertsma, Hjalmar P. Permentier and Dirk Jan Slotboom

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