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The antibiotics roseoflavin and 8-demethyl-8-amino-riboflavin from *Streptomyces davawensis* are metabolized by human flavokinase and human FAD synthetase

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ABSTRACT

The non-pathogenic Gram-positive soil bacterium *Streptomyces davawensis* synthesizes the riboflavin (vitamin B_2) analogs roseoflavin (RoF) and 8-demethyl-8-amino-riboflavin (AF). Both compounds are antibiotics. Notably, a number of other riboflavin analogs are currently under investigation with regard to the development of novel antiinfectives. As a first step towards understanding the metabolism of riboflavin analogs in humans, the key enzymes flavokinase (EC 2.7.1.26) and FAD synthetase (EC 2.7.7.2) were studied. Human flavokinase efficiently converted RoF and AF to roseoflavin mononucleotide (RoFMN) and 8-demethyl-8-amino-riboflavin mononucleotide (AFMN), respectively. Human FAD synthetase accepted RoFMN but not AFMN as a substrate. Consequently, roseoflavin adenine dinucleotide (RoFAD) was synthesized by the latter enzyme but not 8-demethyl-8-amino-riboflavin adenine dinucleotide (AFAD). The cofactor analogs RoFMN, AFMN and RoFAD have different physicochemical properties as compared to FMN and FAD. Thus, the cofactor analogs have the potential to render flavoenzymes inactive, which may negatively affect human metabolism. RoF, but not AF, was found to inhibit human flavokinase. In summary, we suggest that AF has a lower toxic potential and may be better suited as a lead structure to develop antimicrobial compounds.

1. Introduction

Riboflavin (vitamin B_2) analogs have the outstanding potential to serve as basic structures for the development of novel antiinfectives [1] and it is possible that in the future they will be able to meet the urgent need for new molecules to fight multiresistant microorganisms [2]. Vitamin analogs are interesting antimicrobials for two reasons. First, many microorganisms have efficient vitamin transporters, which catalyze the rapid uptake of vitamins and also vitamin analogs [3]. Consequently, the delivery of the vitamin analog to the target molecules is very efficient. Second, many vitamin analogs have multiple cellular targets and thus the chance of developing a resistance is much less likely.

In all organisms, riboflavin serves as the direct precursor for the flavoenzyme cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [4]. FMN is synthesized from riboflavin and ATP by flavokinase (EC 2.7.1.26). FAD is synthesized from FMN and ATP by FAD synthetase (EC 2.7.7.2) [5]. Human flavokinase (Fmn1p; encoded by the gene *RFK*) is an 18.5 kDa protein, whose crystal structure has been determined [6]. At least two isoforms of human FAD synthetase exist, which are the gene products of *FLAD1* transcript variant 1 (65 kDa) and transcript variant 2 (54 kDa). Isoform 1 probably represents the mitochondrial enzyme [7–9].

Streptomyces davawensis produces the riboflavin analogs roseoflavin (RoF) [10,11] and 8-demethyl-8-amino-riboflavin (AF) (Fig. 1). Both compounds show antimicrobial activity against Gram-positive bacteria such as *Bacillus subtilis* but also against Gram-negative bacteria if uptake systems for flavins/flavin analogs are present [10,12]. In *B. subtilis*, the intracellular synthesis of the flavin cofactor analogs roseoflavin mononucleotide/8-demethyl-8-amino-riboflavin mononucleotide (RoFMN/AFMN) and roseoflavin adenine dinucleotide/8-demethyl-8-amino-riboflavin adenine dinucleotide (RoFAD/AFAD) (Fig. 1) most likely explains why RoF is an antibiotic [13] (although the enzymatic synthesis of AFMN and AFAD has not been validated experimentally). First, at least some flavoenzymes are thought to be less active or completely inactive in the presence of RoFMN/AFMN and RoFAD/AFAD, cofactor analogs which have different physicochemical properties as

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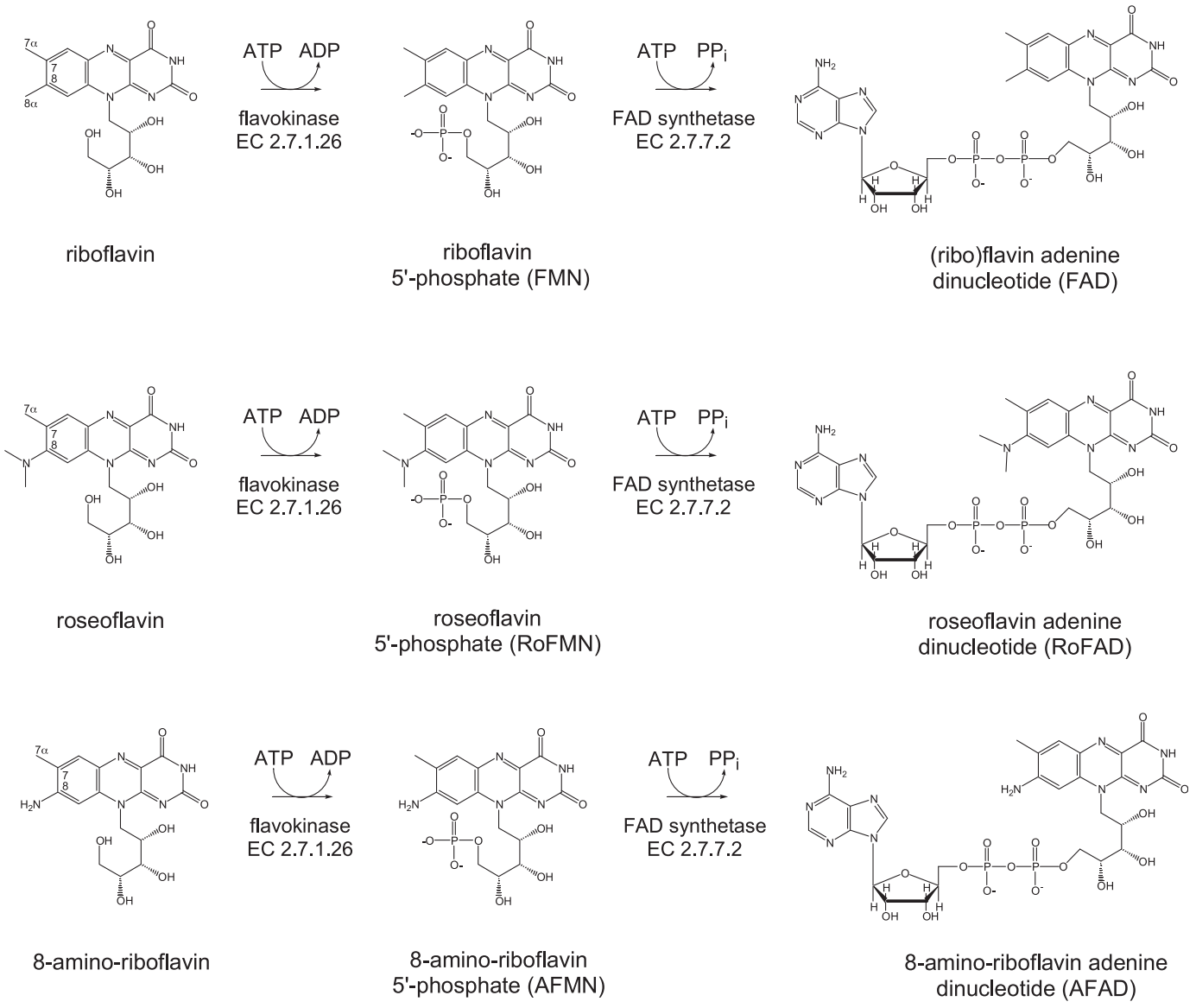

Fig. 1. The conversion of riboflavin (top) into FMN/FAD, of roseoflavin (middle) into roseoflavin-5′-phosphate (roseoflavin mononucleotide; RoFMN) and roseoflavin adenine dinucleotide (RoFAD) and of (8-demethyl)-8-amino-riboflavin (bottom) into 8-amino-riboflavin-5′-phosphate (8-amino-riboflavin mononucleotide; AFMN) and (8-demethyl)-8-amino-riboflavin adenine dinucleotide (AFAD). The enzymatic synthesis of AFAD by human FAD synthetase was not observed.

compared to FMN/FAD. Second, a non-protein cellular target for roseoflavin has been identified in the model organism *B. subtilis*. According to these results roseoflavin (or very likely RoFMN) blocks FMN-riboswitches rendering cells riboflavin auxotrophic [14,15]. In a very recent study it was investigated how roseoflavin affected FMN-riboswitch mediated gene-expression, growth and infectivity of the human bacterial pathogen *Listeria monocytogenes* [16]. The results showed that roseoflavin had a profound inhibiting effect on the growth of *L. monocytogenes* at very low concentrations. In parallel to developing flavin analogs as antiinfectives it is important to study their metabolism in humans. Therefore, we set out to exemplarily test the activity of human flavokinase and human FAD synthetase on RoF, AF, RoFMN and AFMN.

2. Materials and methods

2.1. Materials

All chemicals were from Sigma–Aldrich (Munich, Germany), if not otherwise specified. The flavin 8-demethyl-8-amino-riboflavin was a gift from Peter Macheroux (Technical University of Graz, Austria). Restriction endonucleases and other cloning reagents were purchased from Fermentas (St Leon Rot, Germany). Bacto peptone and bacto yeast extract were from Difco (Becton Dickinson, Heidelberg, Germany).

2.2. Microbial strains, plasmids and growth conditions

Escherichia coli BL21 and Rosetta (DE3) [17] were used as hosts for gene cloning and expression experiments and were aerobically grown at 37 °C on Lysogeny Broth (LB) [17]. When required 34 μg ml^{-1} chloramphenicol and/or 100 μg ml^{-1} ampicillin were added. Oversynthesis of the recombinant proteins was stimulated by 1 mM IPTG. *Pichia pastoris* X33 (Invitrogen) was used as a host for expression of the gene for human FAD synthetase (transcript variant 2). The recombinant *P. pastoris* was cultured on YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with 100 μg ml^{-1} of the antibiotic zeocin. *P. pastoris* cultures were grown at 30 °C for 4 days for the constitutive expression of human FAD synthetase.

2.3. Construction of plasmids and purification of recombinant proteins

The plasmid which was used for the synthesis of His_6-tagged recombinant human flavokinase in *E. coli* strain BL21 [17] was constructed earlier [6]. The gene for human FAD synthetase (transcript variant 2) was amplified by PCR from pH6EX3-$_{UTR}$h-FADS2 [7] employing the forward and reverse modifying primers 5′-AGC CGA ATT CGC CAT GAC TTC TAG GGC CTC TGA ACT TTC TC-3′ and 5′-GAC CCT CGA GCC TGT GCG GGA GTT CCG-3′. The restriction endonuclease sites *Eco*RI and *Xho*I are underlined. The *Eco*RI/*Xho*I treated PCR product was ligated to the *Eco*RI/*Xho*I digested expression vector pGAPZA (zeocin resistance) (Invitrogen, Darmstadt, Germany). The resulting plasmid was used to transform *P. pastoris* X33. A corresponding transformant strain overproduced human FAD synthetase (isoform 2) carrying the additional C-terminal amino acids GSSRGGRQLGPEQKLISEEDLN-SAVD and the additional C-terminal amino acids HHHHHH (His_6-tag). Both His_6-tagged proteins were purified from cell free extracts by column chromatography using Ni^{2+}-nitrilotriacetic-agarose (GE Healthcare, Munich, Germany) and a standard protocol. Fractions containing the purified enzyme were pooled and desalted using a Sephadex G-25 column (GE Healthcare). For deglycosylation, protein (1 mg) was treated with 0.1 U endoglycosidase H (β-N-acetylglucosaminidase H; EC 3.2.1.96) (Sigma) for 12 h at 37 °C.

2.4. Enzyme assays and HPLC analysis of flavins

Flavokinase activity was measured in a final volume of 2 ml of 50 mM potassium phosphate (pH 7.5) containing 25–350 μM flavin, 1 mM ATP, 6 mM NaF, 12 mM $MgCl_2$ and 24 mM sodium dithionite ($Na_2S_2O_4$). The mixture was preincubated at 37 °C for 5 min and the reaction was started by addition of the enzyme. After appropriate time intervals aliquots were removed and applied directly to an HPLC column (ReproSil-Pur C18 AQ; 4.0 mm × 250 mm; Dr. Maisch GmbH, Ammerbich-Entringen, Germany). The following solvent system was used at a flow rate of 0.8 ml min^{-1}: 30% (vol/vol) methanol, 100 mM formic acid and 100 mM ammonium formate (pH 3.7). Detection of flavins was carried out with a diode array detector (Agilent Technologies, Waldbronn, Germany). Flavokinase activity is expressed as micromoles of phosphorylated flavin formed from the corresponding flavin and ATP. FAD synthetase activity was measured as described above using 25–180 μM of the 5′-monophosphorylated flavin as a substrate. Roseoflavin mononucleotide (RoFMN), roseoflavin adenine dinucleotide (RoFAD) and 8-demethyl-8-amino-riboflavin mononucleotide (AFMN) were not commercially available and were enzymatically synthesized using human flavokinase and human FAD synthetase, respectively. The compounds were purified by preparative HPLC and the identities of the compounds were verified by HPLC coupled to a mass spectrometer (Agilent Technologies, Waldbronn, Germany) (Fig. 2). All apparent K_m and V_{max} values were determined from Lineweaver–Burk plots.

2.5. Other assays

Protein was determined by the method of Bradford using BSA as a standard. Proteins were separated on 7–20% polyacrylamide by SDS-PAGE performed according to Laemmli.

2.6. Measurement of flavokinase/FAD-synthetase activity in human hepatocytes

Human hepatocytes (HepG2 cells) were cultured in RPMI 1640 medium (Sigma Aldrich) with L-glutamine (2 mM) supplemented with 10% fetal bovine serum (PAA Laboratories), at 37 °C in a 5% CO_2 environment. The cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.5% Nonidet P-40; 50 mM NaF; 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. Cell-free extracts were used directly in the flavokinase/FAD-synthetase assay described above.

2.7. Protein structures

The figures showing three-dimensional protein structures for human flavokinase were generated using PyMol (Portland, USA).

3. Results

3.1. Overproduction and purification of recombinant human flavokinase and human FAD synthetase in E. coli and P. pastoris

Human flavokinase and FAD synthetase (transcript variant 2) were separately overproduced as His_6-tagged recombinant enzymes in *Escherichia coli*. Human flavokinase (19.4 kDa, including His_6-tag) could easily be purified (>90%) as an active soluble enzyme by affinity chromatography from a cell-free extract of a corresponding recombinant *E. coli* strain (Fig. 3, lane 2). The purification of active His_6-tagged human FAD synthetase from *E. coli*, however, was more challenging. The latter enzyme (overproduced in *E. coli*) apparently was not soluble under the applied conditions and purification of sufficient amounts of active enzyme was not possible (data not shown). Therefore, His_6-tagged human FAD synthetase was overproduced employing an alternative expression system, the methylotrophic yeast *P. pastoris*. Purification of His_6-tagged human FAD synthetase from a recombinant overproducing *P. pastoris* strain was successful and yielded soluble and pure (>90%) enzyme (Fig. 3, lane 3). It was already reported previously that overproduction/purification of soluble human FAD synthetase was difficult [7,8]. We now provide an alternative strategy to synthesize this enzyme using *P. pastoris*. Slower growth of *P. pastoris* (as compared to *E. coli*) may have promoted correct folding in turn leading to enhanced solubility of the recombinant protein. Potential glycosylation sites (N-X-S/T) are absent in human FAD synthetase. Furthermore, a band-shift of the enzyme upon treatment with endoglycosidase H was not observed (data not shown) and we therefore conclude that glycosylation by the host *P. pastoris* apparently was not responsible for enhanced solubility.

3.2. The effect of sodium dithionite on the activity of human flavokinase and human FAD synthetase and kinetic constants for the natural substrates riboflavin and FMN

Some bacterial bifunctional flavokinases/FAD synthetases previously were found to be more active in the presence of reducing agents [13,18,19]. In fact, the *B. subtilis* bifunctional flavokinase/FAD synthetase RibC was reported to be specific for reduced flavins (dihydroriboflavin/$FMNH_2$), although, it could not completely be ruled out that the tertiary structure of the enzyme itself was affected by the reducing agent [18]. For human flavokinase we found that the addition of sodium dithionite (24 mM) enhanced the activity (V_{max}) by a factor of 1.8 (Table 1 and Fig. 4A and B). The apparent K_m value for riboflavin was 117 μM in the absence, and 36 μM in the presence of sodium dithionite (V_{max} 1667 U mg^{-1} total protein). The k_{cat}/K_m ratio was 2.6×10^{-3} in the absence, and 1.4×10^{-2} in the presence of sodium dithionite. These data indicate that the reduced form of riboflavin (dihydroriboflavin), which upon reduction assumes a folded configuration, may have a higher affinity towards the active site of flavokinase, as compared to planar oxidized riboflavin.

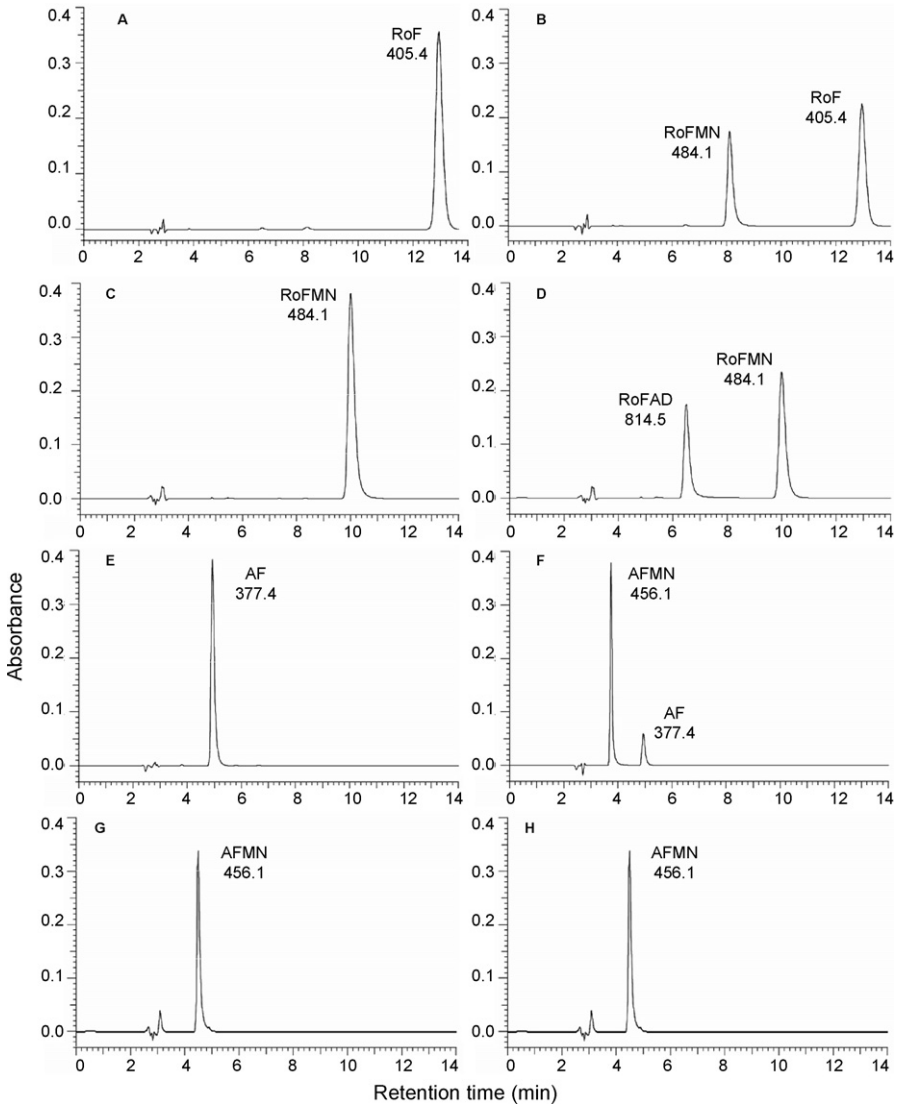

Fig. 2. Enzymatic synthesis of phosphorylated/adenylylated flavins. Reaction mixtures containing different flavin substrates and human flavokinase or human FAD synthetase were incubated at 37 °C. At indicated times samples were taken and analysed by HPLC/mass spectrometry (masses are shown in the diagram). A (0 min) and B (10 min) Roseoflavin monophosphate (RoFMN) formed from RoF and ATP upon addition of human flavokinase. C (0 min) and D (10 min) RoFAD formed from RoFMN and ATP upon addition of human FAD synthetase. E (0 min) and F (15 min) AFMN formed from AF and ATP upon addition of human flavokinase. G (0 min) and H (120 min) No formation of AFAD from AFMN and ATP upon addition of human FAD synthetase.

Similar results (with respect to the addition of a reducing agent) were obtained for human FAD synthetase, which apparently was more active (factor of 4.9) in the presence of sodium dithionite (Table 2). Notably, using the latter assay conditions, we determined an activity V_{max} of 88 U mg^{-1} for this enzyme which was much higher then what has been reported previously (6.8 mU mg^{-1}) [7]. The apparent K_m value for the adenylylation reaction was 109 μM FMN in the absence, and 68 μM FMN in the presence of sodium dithionite. The latter values were also different to what has previously been reported (K_m for FMN of 1.5 μM) [7]. The presence or absence of sodium dithionite cannot be responsible for these large discrepancies. Earlier, *E. coli* was used as a host for overproduction of largely insoluble human FAD synthetase [7] and we attribute our conflicting data to this. In comparison with FAD synthetase human flavokinase *in vitro* apparently is more active (about 19-fold) and thus is able to provide sufficient amounts of FMN for the following FAD synthetase reaction. Notably, FAD contributes the majority (about 90%) of total flavins in most tissues [6].

3.3. Human flavokinase accepts RoF and AF as substrates

As a first step towards analysing the metabolism of flavin analogs in humans, RoF and AF (Fig. 1) were tested as substrates for human flavokinase (Table 1) (see also Fig. 2A and B; E and F). Both flavin analogs were good substrates with turnover numbers (k_{cat}) being three times as high when compared to k_{cat}-values of (dihydro)riboflavin. The K_m-values for the phosphorylation of the flavin analogs thereby were significantly higher (RoF, 160 μM; AF, 885 μM) when compared to the K_m-value for the reaction with (dihydro)riboflavin (36 μM). First, we conclude from the kinetic data that there is apparently enough space in the active site of the enzyme to accommodate the dimethylamino group of RoF. Inspection of the three-dimensional structure of human flavokinase [6] indeed suggests that RoF could fit into the active site without strongly disturbing the overall structure of the enzyme (Fig. 5A). The same should in principle be true for AF (Fig. 5B). However, for the latter substrate a significantly higher K_m-value was found suggesting that substrate binding is less efficient. AF

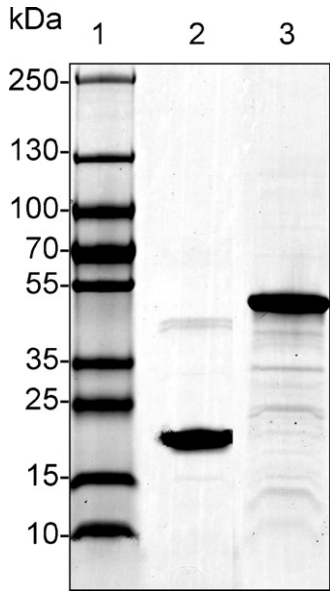

Fig. 3. His_6-tagged human flavokinase (lane 2) was purified to near homogeneity from a cell-free extract of a recombinant *Escherichia coli* strain using immobilized metal affinity chromatography. Accordingly, His_6-tagged (soluble) human FAD synthetase (lane 3) was purified from a cell-free extract of a recombinant *Pichia pastoris* strain. Protein samples were analysed by SDS-PAGE/Coomassie Brilliant Blue R-250 staining. Lane 1, molecular mass marker (in kDa).

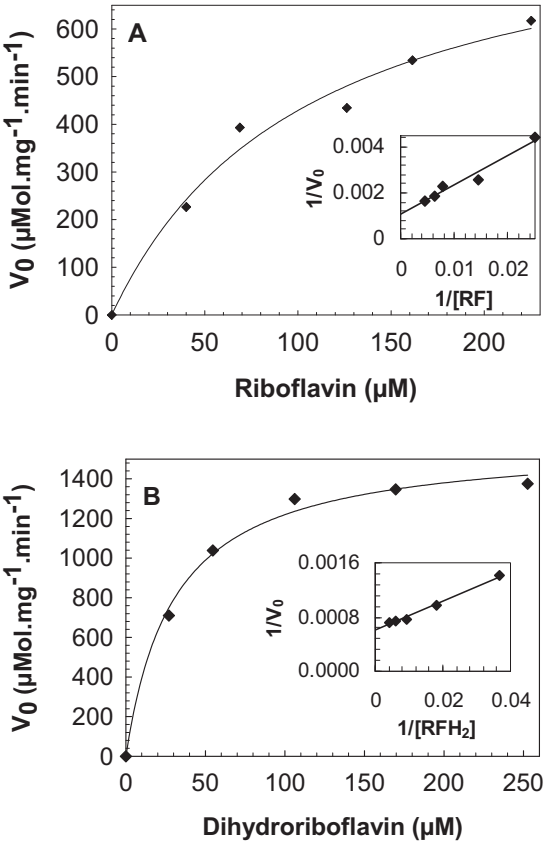

Fig. 4. Determination of steady-state kinetic parameters for the phosphorylation of riboflavin (RF) by recombinant human flavokinase in the presence (A) and absence (B) of the reducing agent sodium dithionite. Rates of individual reactions were obtained by incubating varying amounts of RF (0–250 μM) with 2.3 μM human flavokinase and 1.0 mM ATP as described in the materials and methods section. The initial rates (V_0) were plotted against the RF concentrations to obtain the data. The insert graphs show the Lineweaver–Burk plots of the data.

contains a hydrophilic amino group at C8 and a hydrophobic methyl group at C7 (C7α) (Fig. 1). In contrast, the isoalloxazine ring of the natural substrate riboflavin contains two hydrophobic methyl groups (C8α and C7α) (“dimethylbenzene portion”). The structure of human flavokinase revealed that this hydrophobic dimethylbenzene portion of riboflavin resides at the bottom of a pocket surrounded by the hydrophobic residues I53, V69, F116, L122 and I126 [6]. The hydrophilic C8 amino group of AF does not allow hydrophobic interactions (Fig. 5B) and thus could be the reason for the higher K_m-value of AF. RoF (K_m = 160 μM) clearly is a better substrate as compared to AF (K_m = 885 μM). An explanation for this may be that the more hydrophobic dimethylamino group at C8 of RoF allows interaction with the hydrophobic environment I53, V69, F116, L122 and I126 of human flavokinase (Fig. 5A). In contrast to riboflavin, the flavin analogs RoF and AF may become protonated at the C8 amino group during the enzymatic reaction. Protonation in turn may cause an electrostatic repulsion enhancing release of the reaction products. The latter could explain why the apparent k_{cat}-values of RoF and AF are significantly higher as compared to the k_{cat}-value of the natural substrate riboflavin.

If flavin analogs in the future should be used to treat infectious diseases they will be present in the cell in addition to the natural flavins RF, FMN and FAD. In order to study the influence of different flavin species on the activity of human flavokinase we tested the enzyme in the presence of both RF and RoF at the same time. The addition of RoF apparently reduced the activity of human flavokinase, an inhibition constant of 109 μM (±29) was determined (Fig. 6A). Interestingly, AF did not affect human flavokinase activity even at a maximum concentration of AF (200 μm) just below its solubility limit (Fig. 6B).

3.4. Human FAD synthetase accepts RoFMN but not AFMN as a substrate

The following experiments were carried out in order to study the metabolism of RoFMN and AFMN, which, according to the results reported above, were synthesized *in vitro* by human flavokinase. The latter enzyme was used to produce the cofactor analogs RoFMN and AFMN, which were not commercially available (Fig. 2A and B; E and F). RoFMN was a good substrate (K_m 116 μM; V_{max} 77 U mg^{-1}, k_{cat} 0.08) for human FAD synthetase when compared to the natural substrate FMN (K_m 68 μM; V_{max} 88 U mg^{-1}, k_{cat} 0.07) (Table 2) (see also Fig. 2C and D). The structure of human FAD synthetase is not yet available, however, the structure of the corresponding enzyme from yeast has been solved [20]. This work revealed that the dimethylbenzene portion

Table 1
Kinetic constants for human flavokinase with different flavin substrates.

Substrate	K_m (μM)	V_{max}[a] (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Riboflavin (without $Na_2S_2O_4$)	117	909	0.3	2.6×10^{-3}
Riboflavin (24 mM $Na_2S_2O_4$)	36	1667	0.5	1.4×10^{-2}
Roseoflavin (24 mM $Na_2S_2O_4$)	160	5000	1.6	1.0×10^{-2}
8-Aminoriboflavin (24 mM $Na_2S_2O_4$)	885	5000	1.6	1.8×10^{-3}

[a] Specific activities (U mg^{-1}) are in μMol min^{-1} mg^{-1} protein.

Table 2
Kinetic constants for human FAD synthetase with different flavin substrates.

Substrate	K_m (μM)	V_{max}[a] (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
FMN (without $Na_2S_2O_4$)	109	18	0.02	8.3×10^{-4}
FMN (24 mM $Na_2S_2O_4$)	68	88	0.08	1.2×10^{-3}
RoFMN (24 mM $Na_2S_2O_4$)	116	77	0.07	6.0×10^{-4}
AFMN (24 mM $Na_2S_2O_4$)	–	0	–	–

[a] Specific activities (U mg^{-1}) are in μMol min^{-1} mg^{-1} protein.

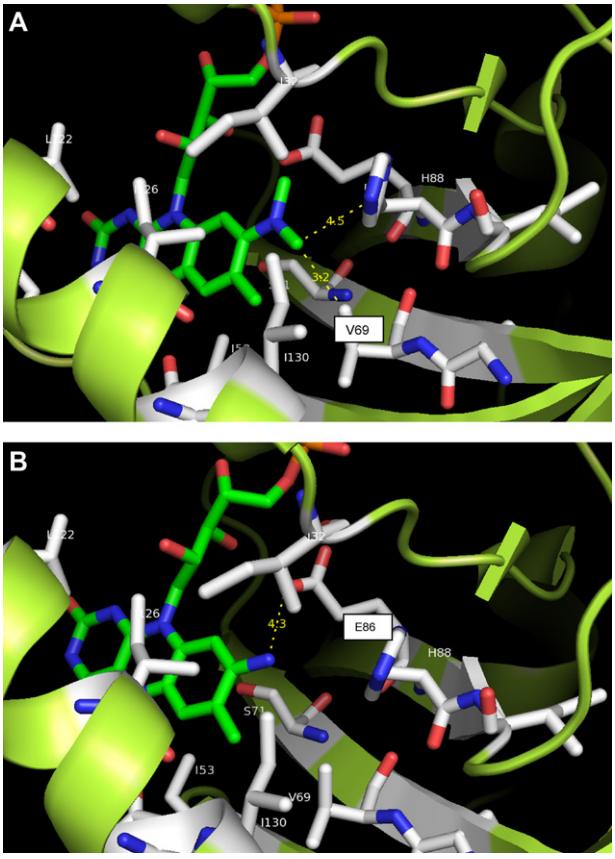

Fig. 5. (A) The FMN-binding site of human flavokinase is shown whereby FMN was *in silico* replaced by roseoflavin-5′-phosphate (RoFMN). RoFMN was fitted using PyMol, the distances are in Å. One of the methyl groups of RoFMN apparently is close to the hydrophobic amino acid residue V69. It is important to note that the crystal which originally was used for solving the structure contained FMN (not RoFMN) [6]. (B) The FMN-binding site of human flavokinase is shown whereby FMN was *in silico* replaced by (8-demethyl)-8-amino-riboflavin 5′-phosphate (AFMN). AFMN was fitted using PyMol, the distances are in Å. The hydrophilic amino group at C8 of AFMN may not be able to interact with the hydrophobic pocket of the enzyme (I53, V69, F116, L122 and I126) which naturally accommodates the dimethylbenzene portion of FMN. The hydrophilic amino group at C8 of AFMN also may be repulsed by E86. It is important to note that the crystal which originally was used for solving the structure contained FMN (not AFMN) [6].

of FMN fills a hydrophobic pocket upon binding to the enzyme. Assuming a similar structure for the FMN binding site in the human enzyme, and, in light of our kinetic data, we conclude that RoFMN with its relatively hydrophobic dimethylamino group at C8 effectively binds to the protein. This seems not to be the case for AFMN (for reasons described for apparent reduced binding of AF to human flavokinase) since no activity was measured employing this substrate under the standard assay conditions (Fig. 2G and H). Human FAD synthetase was also tested in the presence of both flavin substrates, FMN and RoFMN (Fig. 7). The presence of RoFMN, however, did not negatively affect the adenylylation of FMN carried out by human FAD synthetase.

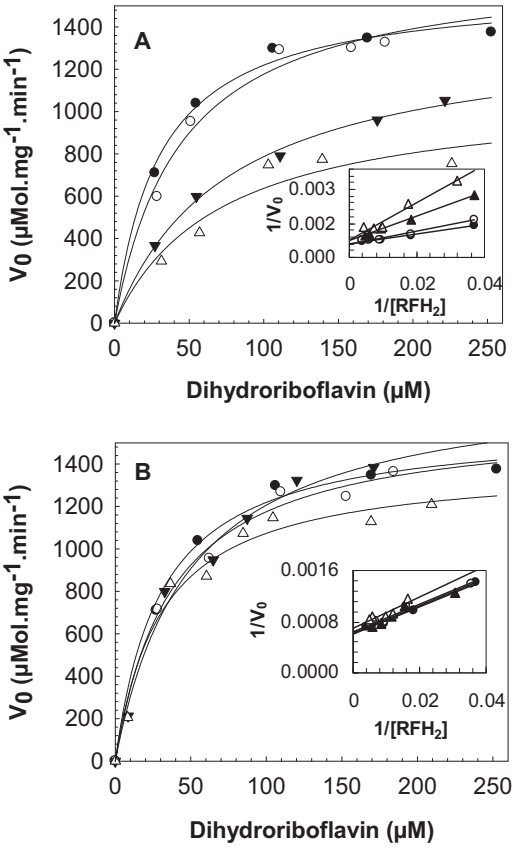

Fig. 6. Inhibition of human flavokinase by the toxic riboflavin analogs roseoflavin (A) and 8-demethyl-8-aminoriboflavin (B). The assay and the detection of flavins was carried out as described in Fig. 2 and Fig. 4A. The upper curve (solid circles) shows the saturation of human flavokinase with increasing concentrations of riboflavin. Upon addition of 50 μM roseoflavin the initial activity of the enzyme was slightly reduced (open circles). Addition of 100 μM (solid triangles) and 250 μM roseoflavin (open triangles) more strongly reduced enzyme activity. The insert graphs show the Lineweaver–Burk plot of the data for determining the inhibition constant K_i. (B) Human flavokinase was tested as in (A) in the presence of 8-demethyl-8-aminoriboflavin (AF), however, no significant inhibition of the enzyme was observed (0 μM AF, solid circles; 50 μM AF, open circles; 100 μM AF, solid triangles; 250 μM AF, open triangles).

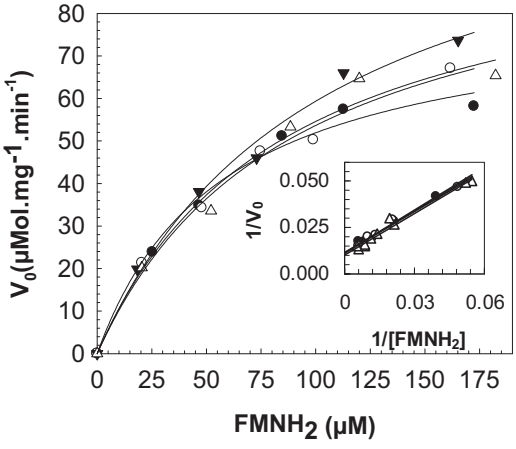

Fig. 7. Human FAD synthetase is not affected by roseoflavin-5′-phosphate (RoFMN). The assay was carried out as described in Fig. 4A. The data show saturation of FAD synthetase activity with increasing concentrations of the substrate flavin mononucleotide FMN (solid circles). Upon addition of RoFMN (25 μM, open circles; 50 μM, solid triangles; 100 μM, open triangles) the initial activity of the enzyme apparently was not affected.

3.5. A hepatocyte cell-free extract catalyzes the formation of FMN, RoFMN and AFMN

Cell-free extracts from freshly grown human hepatocytes were tested with respect to flavokinase and FAD-synthetase activity using the substrates RF, AF and RoF and the corresponding 5′-phosphates FMN, RoFMN and AFMN (100 μM each), respectively. The flavokinase reaction, i.e. the 5′-phosphorylation of RF to FMN (0.4 μMol min^{-1} mg^{-1} total protein), of RoF to RoFMN (0.7 μMol min^{-1} mg^{-1} total protein) and of AF to AFMN (1.1 μMol min^{-1} mg^{-1} total protein) could be measured. The latter data suggest that synthesis of the flavin cofactor analogs RoFMN and AFMN also occurs *in vivo*. Furthermore, RoF and AF seem to even be better substrates as compared to RF, a finding, which supports our data generated using recombinant human flavokinase. FAD-synthetase activity was not detected in hepatocyte cell-free extracts. This was not surprising since our data using recombinant enzymes revealed that FAD-synthetase activity was much lower (at least 20-fold) as compared to flavokinase activity.

4. Discussion

In mammals, dietary riboflavin is actively adsorbed by intestinal cells. Subsequently, the vitamin is imported into the peripheral cells *via* specific plasma membrane transporter(s) [21,22]. For bacterial riboflavin transporters RoF was found to be a good substrate [3], possibly human riboflavin transporters accept flavin analogs as substrates as well. According to our present *in vitro* studies using human flavokinase and FAD synthetase the flavin analogs RoF and AF are efficiently converted to the cofactor analogs RoFMN, AFMN and RoFAD. The relatively high K_m-value for the phosphorylation of AF (885 μM) and the fact that AFMN was not adenylylated at all suggest that AF has a lower toxic potential as compared to roseoflavin. We conclude that, since most flavoenzymes within the cell use FAD as a cofactor, AF, having a good antibacterial potential, is probably a better lead structure for the development of novel antiinfectives based on flavin analogs. We cannot rule out, however, that flavin analogs and/or their degradation products negatively interfere with human metabolism. The synthesis of RoFMN and AFMN by human hepatocyte cell-free extracts supports the data which were generated *in vitro* using recombinant enzymes and strongly suggests that cofactor analogs are also generated *in vivo*. We only can speculate on the molecular activity of the potentially toxic flavin cofactor analogs RoFMN, AFMN and RoFAD in human cells. For D-amino acid oxidase (EC 1.4.3.3) from *Sus scrofa* it was shown that RoFAD is an inactive cofactor [13]. Riboflavin analogs with electron-donating substituents at position 8 were described to be inert to several biological reductants and consequently were thought not to function as redox-active components (roseoflavin $E_0' = -222$ mV; riboflavin $E_0' = -208$ mV). It was discussed that these analogs may be good steric replacements for riboflavin but not catalytic substitutes [23]. Accordingly, RoFMN, AFMN and RoFAD may combine with FMN- or FAD-dependent flavoenzymes, reduce their activity and negatively affect human metabolism [13]. Furthermore, it was reported that protonation of flavin analogs which carry an amino group at C8 have a strongly altered redox potential (e.g. for protonated RoF an E_0' of +190 mV was published [23]). It remains to be elucidated whether the different physicochemical properties of RoFMN, AFMN and RoFAD are indeed relevant for human physiology.

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