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TURNING RIBOFLAVIN-BINDING PROTEIN INTO A SELF-SUFFICIENT MONOOXYGENASE BY COFACTOR REDESIGN

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1. General

Reagents were purchased from Sigma-Aldrich, Acros or Alfa Aesar and were used as provided unless otherwise stated. Mass spectra were recorded on an AEI-MS-902 mass spectrometer. High resolution mass spectra were determined on a FTMS Orbitrap FischerScientific mass spectrometer by ESI measurements in positive mode. NMR spectra were obtained on Varian AMX400 spectrometer (^1H : 400 MHz; ^{13}C 100.59 MHz). Chemical shifts (δ) are given in parts per million (ppm) relative to the residual solvent peak, while the coupling constants (J) are given in Hz. Gas chromatography was carried out on a Hewlett-Packard 6890 Series using a flame ionization detector. For all the analyses, the injector temperature was 225 °C and the FID temperature was 250 °C. HPLC was performed on a Shimadzu LC-10ADVP equipped with a Shimadzu SPD-M10AVP diode array detector. UV-Vis spectra were obtained using a Hewlett-Packard HP 8543 FT spectrophotometer in a 1.0 cm quartz cuvette.

Racemic sulfoxides were prepared by treatment of the starting sulfides with H_2O_2 in methanol at room temperature (yields higher than 80%). All other reagents and solvents were of the highest quality grade available and were obtained from Sigma-Aldrich-Fluka and Acros Organics.

The absolute configurations of the final sulfoxides were determined by comparison of retention time on GC or HPLC with the published data.¹

For docking studies on the flavins in RfBP, the crystallographic coordinates of holo RfBP were obtained from Prof. H.L. Monaco (University of Verona, Italy). The structural models of the flavins were built using ChemBioDraw Ultra 12.0 and Chem3D Pro 12.0 was used for initial energy minimization. The Molegro Virtual Docker (MVD 2010.4.0.0, Molegro, Aarhus, Denmark) was used for docking simulations. For this, the

structural RfBP model and all flavins were first subjected to energy minimization in MVD. The flavins were docked into RfBP within a radius of 18 Å of the riboflavin binding site. The crystallographically bound riboflavin was used for template-assisted docking. For the docking calculations default settings were used except for the grid resolution which was set to highest resolution (0.2). The poses with lowest MolDock energy scores were used in this study. For visualizing the docked flavins in RfBP the PyMOL software was used (www.pymol.org).

2. Experimental procedures

2.1. Synthesis of 5-ethylriboflavin (2).

To a flask charged with 100 mL of degassed ethanol/water (5:4), riboflavin (1.12 g, 3.0 mmol) and 10% Pd/C (200 mg) were added. Hydrogen atmosphere was applied and after 5 minutes, freshly distilled acetaldehyde (5.6 mL, 100 mmol) was added. The system was stirred at room temperature for one week and after this time the reaction mixture was filtered over celite in a double Schlenk flask. The residue was washed several times with degassed ethanol. Solvents were then removed under reduced pressure in order to obtain the product as a pale yellow solid (0.75 g, 62% yield). The product was kept in a reduced state by storage under a nitrogen atmosphere adding a small amount of Na₂S₂O₄. ¹H-NMR (400 MHz, *d*₆-DMSO): 0.97 (br d, 3H), 2.05 (br s, 6H), 3.08 (br s, 1H), 3.31-3.69 (br m, 8H), 4.10 (br s, 1H), 4.54 (br s, 1H), 4.93 (br s, 1H), 5.10 (br s, 1H), 6.26 (br s, 1H), 6.73 (br s, 1H), 10.32 (br s, 1H), 10.59 (br s, 1H). HRMS (ESI⁺): calcd. for C₁₉H₂₅N₄O₆ 405.1779, found 405.1780.²

2.2. General procedure for the preparation of the flavin catalysts 3-6.

A three-step procedure, following the described methodology by Bäckvall *et al.*,³ was performed in order to prepare the final trialkylated *N*¹-, *N*³- and *N*⁵-flavins. First, alloxane and a diamine were coupled via imine formation in order to yield the desired alloxazines, which were *N*¹- and *N*³-dialkylated by treatment with the corresponding alkyl iodides in presence of base and DMF. Finally, the dialkylated flavin was subjected to reductive alkylation using palladium on carbon with dihydrogen and acetaldehyde as described by Bäckvall *et al.*,⁴ in order to introduce an ethyl group at the *N*⁵ of the flavin.

Alloxazine. o-Phenylenediamine (3.38 g, 31 mmol) was dissolved in 50 mL of acetic acid. A mixture of alloxane (5.00 g, 31 mmol) and boric acid (2.13 g, 34.5 mmol) in 200

mL of hot acetic acid was added to this solution and the reaction was stirred for 2 hours at room temperature. The formed precipitate was removed by filtration and washed with acetic acid, diethyl ether and dried under reduced pressure in order to obtain alloxazine as a yellow solid (6.36 g, yield 95%). ¹H-NMR (500 MHz, *d*₆-DMSO): 7.69-7.87 (m, 1H), 7.94 (d, ³*J*_{HH} = 2.8, 2H), 8.17 (d, ³*J*_{HH} = 8.4, 1H), 11.76 (s, 1H), 11.94 (s, 1H); ¹³C-NMR (126 MHz, *d*₆-DMSO): 127.6 (CH), 129.1 (CH), 130.8 (CH), 132.3 (C), 134.0 (CH), 139.9 (C), 143.3 (C), 147.5 (C=O), 150.8 (C=O), 161.1 (C). EI-MS (*m/z*): 214.0487 (100).³

7,8-Dimethylalloxazine. The same procedure was followed, starting from 3,4-dimethyl-1,2-phenyldiamine (0.92 g, 7.0 mmol), in order to obtain 1.46 g of a yellow solid (86% yield). ¹H-NMR (400 MHz, *d*₆-DMSO): 1.88 (s, 6H), 7.68 (s, 1H), 7.89 (s, 1H), 11.63 (s, 1H), 11.80 (s, 1H); ¹³C-NMR (100 MHz, *d*₆-DMSO): 21.2 (CH₃), 21.7 (CH₃), 127.6 (CH), 129.1 (CH), 130.8 (CH), 132.3 (C), 134.0 (CH), 139.9 (C), 143.3 (C), 147.5 (C=O), 150.8 (C=O), 161.1 (C). EI-MS (*m/z*): 242.08617 (100).³

1,3,7,8-Tetramethylalloxazine. 7,8-Dimethylalloxazine (1.25 g, 5.15 mmol) and K₂CO₃ (3.54 g, 25.7 mmol) were added to 20 mL of dry DMF. Methyl iodide (710 μL, 11.3 mmol) was added and the reaction mixture was stirred for 4 hours at room temperature. 50 mL of water was added to the system and the formed precipitate was filtered, washed several times with water and dried under reduced pressure. No further purification was required in order to obtain the final compound as a yellow solid (0.91 g, 68% yield). ¹H-NMR (400 MHz, *d*₆-DMSO): 2.68 (s, 3H), 2.71 (s, 3H), 3.77 (s, 3H), 3.98 (s, 3H), 7.97 (s, 1H), 8.24 (s, 1H); ¹³C-NMR (126 MHz, CDCl₃): 21.2 (CH₃), 21.7 (CH₃), 30.7 (CH₃), 31.5 (CH₃), 127.0 (CH), 128.7 (C), 129.5 (CH), 139.1 (C), 140.2 (C), 142.5 (C), 145.2 (C), 145.9 (C), 150.9 (C=O), 160.3 (C=O). HRMS (ESI⁺): calcd. For C₁₄H₁₅N₄O₂ 271.1190, found 271.1877.⁵

7,8-Dimethyl-1,3-diethylalloxazine. The same procedure was employed, using ethyl iodide as alkylating reagent. The product was obtained as a yellow solid with 89% yield (1.36 g). $^1\text{H-NMR}$ (400 MHz, CDCl_3): 1.36 (t, $^3J_{\text{HH}} = 7.0$, 3H), 1.41 (t, $^3J_{\text{HH}} = 7.0$, 3H), 2.52 (s, 3H), 2.55 (s, 3H), 4.26 (q, $^3J_{\text{HH}} = 7.1$, 2H), 4.52 (q, $^3J_{\text{HH}} = 7.1$, 2H), 7.81 (s, 1H), 8.07 (s, 1H); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): 13.3 (CH_3), 13.4 (CH_3), 20.6 (CH_3), 21.1 (CH_3), 37.9 (CH_2), 38.1 (CH_2), 127.1 (CH), 129.0 (C), 129.6 (CH), 139.4 (C), 140.1 (C), 142.8 (C), 144.8 (C), 145.7 (C=O), 150.1 (C=O), 160.0 (C); HRMS (ESI^+): calcd. for $\text{C}_{16}\text{H}_{19}\text{N}_4\text{O}_2$ 299.1503, found 299.1501.

7,8-Dimethyl-1,3-dipropylalloxazine. This compound was obtained as a yellow solid (1.26 g, 75% yield) by employing propyl iodide. $^1\text{H-NMR}$ (400 MHz, CDCl_3): 1.03-1.08 (m, 6H), 1.80-1.84 (m, 4H), 2.52 (s, 3H), 2.54 (s, 3H), 4.16 (t, $^3J_{\text{HH}} = 7.5$, 2H), 4.41 (t, $^3J_{\text{HH}} = 7.4$, 2H), 7.80 (s, 1H), 8.06 (s, 1H); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): 11.6 (CH_3), 11.7 (CH_3), 20.6 (CH_3), 21.1 (CH_3), 21.2 (CH_2), 21.4 (CH_2), 44.2 (CH_2), 44.3 (CH_2), 127.1 (CH), 128.9 (C), 129.6 (CH), 139.4 (C), 140.0 (C), 142.7 (C), 145.0 (C), 145.7 (C=O), 150.6 (C=O), 160.2 (C); HRMS (ESI^+): calcd. for $\text{C}_{18}\text{H}_{23}\text{N}_4\text{O}_2$ 327.1816, found 327.1814.

1,3-Diethylalloxazine. Alloxazine (1.00 g, 4.6 mmol) and K_2CO_3 (3.3 g, 23.7 mmol) were added to 30 mL of dry DMF. Ethyl iodide (0.85 mL, 11.5 mmol) was added dropwise and the reaction mixture was stirred for 3 hours at room temperature. 60 mL of water was added to the system and the formed precipitate was filtered, washed several times with water and dried under reduced pressure. No further purification was required in order to obtain the final compound as a yellow solid (1.1 g, 87% yield). $^1\text{H-NMR}$ (400 MHz, CDCl_3): 1.29 (t, $^3J_{\text{HH}} = 8.0$, 3H), 1.35 (t, $^3J_{\text{HH}} = 7.5$, 3H), 4.22 (q, $^3J_{\text{HH}} = 7.8$, 2H), 4.45 (q, $^3J_{\text{HH}} = 7.6$, 2H), 7.71 (m, 1H), 7.79 (m, 1H), 7.96 (d, $^3J_{\text{HH}} = 8.5$, 1H), 8.32 (d, $^3J_{\text{HH}} = 8.4$, 1H); $^{13}\text{C-NMR}$ (126 MHz, CDCl_3): 13.2 (CH_3), 13.4 (CH_3),

38.1 (CH₂), 38.3 (CH₂), 128.0 (CH), 129.3 (CH), 130.1 (C), 131.0 (CH), 134.0 (CH), 140.1 (C), 143.7 (C), 145.1 (C), 150.8 (C=O), 161.1 (C=O). HRMS (ESI⁺): calcd. For C₁₄H₁₄N₄O₂ 271.1190, found 271.1197.

7,8-Dimethyl-1,3-dimethyl-5-ethyl-5,10-dihydroalloxazine (3). 1,3,7,8-

Tetramethylalloxazine (200 mg, 0.83 mmol) and 10% Pd/C (80 mg) were dissolved in 50 mL of a degassed mixture ethanol/water (5:4) and a hydrogen atmosphere was applied. After stirring the system for 5 minutes, freshly distilled acetaldehyde (1.2 mL, 21 mmol) and concentrated hydrochloric acid (1.2 mmol) were added and the suspension was stirred at room temperature for 48 hours. The crude reaction was then filtered over celite in a double Schlenk flask, washing the residue with degassed ethanol. Solvents were then removed in vacuo in order to obtain a yellow air sensitive powder (190 mg, 84% yield). The product was kept in a reduced state by storage under a nitrogen atmosphere adding a small amount of Na₂S₂O₄. ¹H-NMR (400 MHz, CDCl₃): 1.03 (t, ³J_{HH} = 7.4, 3H), 2.01 (s, 3H), 2.06 (s, 3H), 3.30 (s, 3H), 3.37 (q, ³J_{HH} = 7.0, 2H), 3.41 (s, 3H), 6.54 (s, 1H), 6.59 (s, 1H); ¹³C-NMR (100 MHz, CDCl₃): 10.9 (CH₃), 18.9 (CH₃), 28.2 (CH₃), 28.9 (CH₃), 48.6 (CH₂), 100.2 (C), 115.2 (CH), 116.1 (CH), 125.8 (CH), 127.9 (CH), 131.2 (C), 132.7 (C), 133.2 (C), 147.4 (C), 151.1 (C=O), 160.0 (C=O). HRMS (ESI⁺): calcd. For C₁₆H₁₉N₄O₂ 299.1508, found 299.1495.⁵

7,8-Dimethyl-1,3-diethyl-5-ethyl-5,10-dihydroalloxazine (4), was prepared in a similar way, starting from 7,8-dimethyl-1,3-diethylalloxazine (200 mg, 0.67 mmol) in order to obtain the final product kept in a reduced state by adding Na₂S₂O₄ as an orange solid (190 mg, 86% yield). ¹H-NMR (400 MHz, CDCl₃): 1.03 (t, ³J_{HH} = 7.0, 3H), 1.14 (t, ³J_{HH} = 7.0, 3H), 1.24 (t, ³J_{HH} = 7.4, 3H), 2.02 (s, 3H), 2.05 (s, 3H), 3.32 (q, ³J_{HH} = 7.1, 3H), 3.93 (q, ³J_{HH} = 7.1, 4H), 6.36 (s, 1H), 6.57 (s, 1H); ¹³C-NMR (100 MHz, CDCl₃): 10.4 (CH₃), 12.2 (CH₃), 12.8 (CH₃), 18.1 (CH₃), 18.2 (CH₃), 35.1 (CH₂), 36.1

(CH₂), 49.8 (CH₂), 98.8 (C), 114.8 (CH), 114.9 (CH), 122.8 (CH), 122.9 (CH), 130.7 (C), 131.8 (C), 131.9 (C), 142.3 (C), 152.8 (C=O), 158.1 (C=O). HRMS (ESI⁺): calcd. For C₁₈H₂₃N₄O₂ 327.1899, found 327.1906.

7,8-Dimethyl-1,3-dipropyl-5-ethyl-5,10-dihydroalloxazine (5) was synthesized in a similar way from 7,8-dimethyl-1,3-dipropylalloxazine (200 mg, 0.61 mmol), achieving 140 mg (64% yield) of brown solid, kept in a reduced state by adding Na₂S₂O₄. ¹H-NMR (400 MHz, CDCl₃): 0.86 (t, ³J_{HH} = 7.4, 3H), 0.91 (t, ³J_{HH} = 7.4, 3H), 1.03 (t, ³J_{HH} = 7.4, 3H), 1.57-1.62 (m, 4H), 2.02 (s, 3H), 2.04 (s, 3H), 3.32 (q, ³J_{HH} = 7.0, 2H), 3.85 (m, 4H), 6.40 (s, 1H), 6.58 (s, 1H); ¹³C-NMR (100 MHz, CDCl₃): 10.9 (CH₃), 11.2 (2CH₃), 18.3 (CH₃), 18.5 (CH₃), 20.2 (CH₃), 20.8 (CH₂), 41.3 (CH₂), 41.8 (CH₂), 50.5 (CH₂), 99.4 (C), 115.9 (CH), 116.0 (CH), 123.8 (CH), 123.9 (CH), 131.7 (C), 132.7 (C), 133.1 (C), 146.4 (C), 150.1 (C=O), 158.1 (C=O). HRMS (ESI⁺): calcd. For C₂₀H₂₇N₄O₂ 355.2134, found 355.2119.

1,3,5-Triethyl-5,10-dihydroalloxazine (6), was prepared in a similar fashion starting from flavin 1,3-diethylalloxazine (200 mg, 0.74 mmol). The product, 160 mg of an orange/red solid, was kept in a reduced state by adding Na₂S₂O₄ (73% yield). ¹H-NMR (400 MHz, CDCl₃): 1.06 (t, ³J_{HH} = 7.0, 3H), 1.13 (t, ³J_{HH} = 7.0, 3H), 1.19 (t, ³J_{HH} = 7.1, 3H), 3.34 (q, ³J_{HH} = 7.1, 2H), 3.88-4.03 (m, 4H), 6.63-6.75 (m, 3H), 6.80-6.85 (m, 1H); ¹³C-NMR (100 MHz, CDCl₃): 10.5 (CH₃), 12.3 (CH₃), 12.6 (CH₃), 34.8 (CH₂), 36.3 (CH₂), 48.6 (CH₂), 98.5 (C), 113.1 (CH), 121.5 (CH), 122.7 (CH), 123.7 (CH), 134.8 (C), 135.1 (C), 144.3 (C), 150.7 (C=O), 158.6 (C=O). HRMS (ESI⁺): calcd. For C₁₆H₁₉N₄O₂ 299.1508, found 299.1493.

2.3. Isolation and purification of riboflavin binding protein (RfBP) from *Gallus gallus* eggwhite.

The eggwhite from 10 eggs was separated from the yolk (final volume $\approx$ 400 mL), stored on ice and diluted with 400 mL of NaAc buffer 50 mM pH 4.5. 5 mg of riboflavin was added and the resulting mixture was stirred gently for 10 minutes, brought to pH 4.5 by addition of acetic acid and filtered. The remained of the precipitate was removed by centrifugation (30 min, 9500 rpm, 4°C), after which the supernatant was stored on ice and brought onto a DEAE-Sepharose column which was equilibrated with NaAc buffer 50 mM pH 4.5. The column was washed with the buffer until the A_{280} was below 0.1. Elution was performed with a high salt solution (NaCl 1.0 M), using a gradient, releasing the RfBP from the column. The fraction with A_{450} , indicative for holo riboflavin binding protein, was collected (100 mL). Pulverized ammonium sulfate was added to the protein solution which was kept at 0°C until 55% saturation was reached (32.6 g). A white precipitate was removed by centrifugation (10 min, 10000 rpm, 4°C). The yellow supernatant was collected and pulverized ammonium sulfate was added until 80% saturation (additional 16.1 g). The yellow precipitate was collected by centrifugation of the solution (10 min, 10000 rpm 4°C). The precipitate was dissolved in 10 mL NaAc buffer 0.1 M pH 5.6. This protein solution was desalted by employing a desalting column (BioRad Econopac 10), and equilibrated with NaAc 0.1 M pH 5.6. Elution of the yellow fraction was performed with the same buffer in order to obtain the purified RfBP (3.0 mL, 250 μ M).

2.4. Purification of *Gallus gallus* apo-RfBP

Ammonium sulfate (1.6 g) was added at 0 °C up to 85% saturation to the riboflavin binding protein solution (3.0 mL). The suspension was centrifuged (15 min, 9000 rpm, 4°C) and the precipitate was dissolved in 5.0 mL of NaAc buffer 50 mM pH 3.8. Salts

were removed using a desalting column, equilibrated, and eluted with NaAc buffer 50 mM pH 3.8. The yellow fraction recovered was subjected to cation-exchange chromatography using an S-Sepharose column, equilibrated with NaAc buffer 50 mM pH 3.8. The column was washed using the same buffer in order to elute the free riboflavin. After disappearance of the yellow colour, apo-RfBP was eluted using a phosphate buffer 50 mM NaCl 1.0 M pH 7.5 and concentrated in 50 mM phosphate buffer pH 7.5 using a an Amicon Ultra 15 filter (Millipore) with a cut-off of 10 KDa, in order to obtain 6.0 mL of colourless solution (100 μ M).

2.5. Isolation and purification of apo-RfBP from *Coturnix japonica*

For the isolation and purification of the apo-riboflavin binding protein from Japanese quail, the same procedure as described in sections 2.3 and 2.4 was followed, starting from 30 quail eggs. From this amount of starting material, 1.5 mL of apo-RfBP (230 μ M) was obtained.

2.6. Binding of flavin catalysts 2-6 to RfBP

To 1.0 mL of 100 μ M apo-RfBP in phosphate buffer 0.1 M pH 7.5, 10 μ mol of the flavin catalyst was added. To the resulting solution, excess $\text{Na}_2\text{S}_2\text{O}_4$ was added to keep the catalyst in a reduced state. The solution was washed 5 to 7 times using an Amicon Ultra 15 filter by repeated diluting to 10 mL with phosphate buffer 50 mM pH 7.5 and subsequent concentrating to 1.0 mL. After the final washing step, the solution retained colour due to the presence of the flavin catalyst, while the flowthrough showed no presence of unbound flavin. The solution containing the RfBP with the incorporated flavin catalyst was frozen with liquid nitrogen and stored at -80°C .

2.7. Fluorescence-based binding assay with apo-RfBP and flavin derivative 2.

The fluorescence binding assay was done by titration of apo-RfBP to fluorescent flavin **2** at 25°C using a fluorescence spectrophotometer Hitachi F-2000. The excitation wavelength was set at 369 nm. The slits of excitation/emission were set at 2 nm and 5 nm receptivity, with a potential of 900V on the photomultiplier. Measurements were done in a 50 mM phosphate buffer pH 7.5, with 6.0 µM concentration of the flavin. In a titration experiment 1.0 mL of the buffer containing 6.0 µM flavin **2** was titrated with apo-RfBP to molar ratios up to 2.

2.8. Optimisation of the reaction conditions.

In order to minimise the quantity of racemic sulfoxide formed in the catalysed processes by the non-enzymatic oxidation in presence of hydrogen peroxide, different reaction parameters, such as substrate (2.0, 5.0 or 10.0 mM) and oxidant concentration (1.1 or 3.3 equivalents), reaction medium (pH 7.5 or 9.0, use of milliQ water), temperature (4, 17 or 30°C) or additives (EDTA), were studied. The best condition for achieving the lowest conversion for this reaction were obtained by employing 5.0 mM of substrate, 6.6 mM hydrogen peroxide and 0.2 mM EDTA at pH 7.5. These conditions have been employed in all reactions, unless stated otherwise.

2.9. General procedure for the enzymatic sulfoxidations employing *Gallus gallus* RfBP-flavin catalysts 2-6.

Unless otherwise stated, prochiral sulfides (5.0 mM) were dissolved in a 50 mM phosphate buffer pH 7.5 (1.0 mL) containing an aqueous H₂O₂ solution (6.6 mM), EDTA (0.2 mM) and the corresponding RfBP-flavin catalyst **1-5** (50 µM). Reactions were shaken at 4°C for 24 hours. Once finished, the crude mixtures were extracted with EtOAc, dried on Na₂SO₄ and analyzed by GC and/or HPLC in order to determine the

conversions and the enantiomeric excesses of the final sulfoxides (Table S.1). For those sulfides in which enzymatic activity was found, a small amount (less than 5%) of sulfone was observed. Blank reactions in the absence of RfBP-flavin catalyst were performed, as shown in Table S.2. In all cases the formation of the corresponding racemic sulfoxides was observed. Blank reactions were also carried out using native RfBP with the natural riboflavin still bound, with conversions similar to those in absence of this protein (Table S.2).

Table S1. Some other results obtained in the enzymatic sulfoxidations catalyzed by RfBP-Flavin catalysts **2-6**.

Entry	Sulfide	RfBP-Flavin catalyst	<i>c</i> (%) ^a	<i>ee</i> (%) ^b
1	BuSEt	2	48	9 (<i>R</i>)
2	BnSMe	3	47	15 (<i>S</i>)
3	CySMe	3	50	13 (<i>S</i>)
4	BnSMe	4	40	10 (<i>S</i>)
5	BuSEt	4	63	12 (<i>R</i>)
6	BnSMe	5	35	6 (<i>R</i>)
7	CySMe	5	41	7 (<i>R</i>)
8	<i>p</i> -TolSMe	6	49	5 (<i>R</i>)
9	CySMe	6	56	10 (<i>R</i>)

^a Measured by GC. ^b Measured by GC or HPLC.

Table S2. Blank reactions: Oxidation of prochiral sulfides while using H₂O₂ as oxidant in the presence and absence of RfBP.

Entry	Sulfide	RfBP	<i>c</i> (%) ^a
1	PhSMe	Yes	13
2	PhSMe	No	14
3	PhSEt	Yes	12
4	PhSEt	No	10
5	<i>p</i> -TolSMe	Yes	13
6	<i>p</i> -TolSMe	No	9
7	BnSMe	Yes	19

8	BnSMe	No	16
9	BnSEt	Yes	16
10	BnSEt	No	14
11	CySMe	Yes	17
12	CySMe	No	20
13	BuSEt	Yes	19
14	BuSEt	No	17

^a Measured by GC.

2.10. General procedure for the enzymatic sulfoxidations employing *Coturnix japonica* RfBP-flavin catalysts **5-6**.

Prochiral sulfides (5.0 mM) were dissolved in a 50 mM phosphate buffer pH 7.5 (1.0 mL) containing an aqueous H₂O₂ solution (6.6 mM), EDTA (0.2 mM) and the corresponding quail RfBP-flavin catalyst **5-6** (50 μM). Reactions were shaken at 4°C for 24 hours. Once finished, the crude mixtures were extracted with EtOAc, dried on Na₂SO₄ and analysed by GC and/or HPLC in order to determine the conversions and the enantiomeric excesses of the final sulfoxides, as shown in Table S3.

Table S3. Sulfoxidations catalyzed by quail riboflavin binding protein-flavin catalysts **5-6**.

Entry	Sulfide	RfBP-Flavin catalyst	<i>c</i> (%) ^a	<i>ee</i> (%) ^b
1	PhSMe	5	32	≤ 3
2	<i>p</i> -TolSMe	5	35	9 (<i>S</i>)
3	BnSMe	5	51	≤ 3
4	PhSMe	6	38	≤ 3
5	BnSMe	6	60	6 (<i>R</i>)

^a Measured by GC. ^b Determined by GC or HPLC.

2.11. General procedure for the enzymatic sulfoxidations employing flavin catalysts 2-6.

Unless otherwise stated, prochiral sulfides (5.0 mM) were dissolved in a 50 mM phosphate buffer pH 7.5 (1.0 mL) containing an aqueous H₂O₂ solution (6.6 mM) and the corresponding RfBP-flavin catalyst **2-6** (50 μM). Reactions were shaken at 4°C for 24 hours. Once finished, the crude mixtures were extracted with EtOAc, dried onto Na₂SO₄ and analysed by GC and/or HPLC in order to determine the conversions of the final racemic sulfoxides, as shown in Table S4.

Table S4. Sulfoxidation of prochiral sulfides catalyzed by *N*5-ethylated flavins **2-6** and H₂O₂.

Entry	Sulfide	Flavin-catalyst	<i>c</i> (%) ^a
1	PhSMe	2	36
2	<i>p</i> -TolSMe	2	42
3	BnSMe	2	43
4	CySMe	2	45
5	BuSEt	2	50
6	PhSMe	3	30
7	PhSEt	3	35
8	<i>p</i> -TolSMe	3	37
9	BnSMe	3	48
9	CySMe	3	42
10	PhSMe	4	33
11	<i>p</i> -TolSMe	4	35
12	BnSMe	4	46
13	CySMe	4	42
14	BuSEt	4	57
15	PhSMe	5	28
16	<i>p</i> -TolSMe	5	33
17	BnSMe	5	39
18	CySMe	5	40
19	PhSMe	6	32
20	<i>p</i> -TolSMe	6	35

21	BnSMe	6	55
22	BnSEt	6	45
23	CySMe	6	50
24	BuSEt	6	55

^a Measured by GC.

2.12. Time dependence of the biocatalytic sulfoxidation of thioanisole employing RfBP-flavin catalyst **3**.

Thioanisole (5.0 mM) was dissolved in a 50 mM phosphate buffer pH 7.5 (6.0 mL) containing an aqueous H₂O₂ solution (6.6 mM), EDTA (0.2 mM) and the corresponding RfBP-flavin catalyst **3** (50 μM). Reactions were shaken at 4°C and aliquots of 1.0 mL were taken after 1, 2, 8, 24, 48 and 72 hours. Samples were extracted with EtOAc, dried on Na₂SO₄ and analyzed by GC to determine the conversions and the enantiomeric excesses of the final sulfoxides. Blank reactions in the absence of RfBP-flavin catalyst were performed. Final results of both biocatalyzed and blank reactions are shown in Figure S1.

Figure S1. Time-dependence of the catalytic sulfoxidation of thioanisole employing RfBP-flavin **3**. Conversion (♦) and enantiomeric excess (▲) of (*S*)-methyl phenyl sulfoxide are shown. Conversion in the absence of the RfBP-flavin (●) is also indicated.

3. GC and HPLC analyses.

The following columns were used for the determination of conversions and enantiomeric excesses of the sulfoxides by GC: A: GT-A (Alltech, 30 m x 0.25 mm x 0.25 μ m), B: Hewlett Packard HP-1 (Agilent, 30m x 0.32 mm x 0.25 μ m) or C: Restek RT-BetaDEXse (30 m x 0.25 mm x 0.25 μ m, 12 psi N₂).

Table S5. Determination of conversion and enantiomeric excess values by GC.

Substrate	Program ^a	Column	t_R (min) sulfides	t_R (min) sulfoxides
PhSMe	100/0/10/160/8	A	3.6	10.0 (<i>R</i>); 11.5 (<i>S</i>)
PhSEt	100/0/10/160/8	A	4.1	11.3 (<i>R</i>); 12.7 (<i>S</i>)
<i>p</i> -TolSMe	100/0/10/160/8	A	4.6	12.3 (<i>R</i>); 13.0 (<i>S</i>)
BnSMe	70/5/10/200/5	B	13.2	17.1
BnSEt	70/5/10/200/5	B	14.5	18.8
BuSEt	50/5/3/200/5	C	14.2	33.1 (<i>S</i>), 35.4 (<i>R</i>)
CySMe	60/5/5/160/5	B	15.7	21.0

^a Program: initial T (°C)/ time (min)/ slope (°C/min)/T (°C)/ time (min).

For the determination of the enantiomeric excesses of the sulfoxides by HPLC, the following columns, both from Daicel, were employed: A: Chiralcel OD (0.46 cm x 25 cm) or B: Chiralcel OB-H (0.46 cm x 25 cm).

Table S6. Determination of enantiomeric excess values by HPLC.

Substrate	Flow rate (mL min ⁻¹)	T (°C)	Column	Eluent ^a	<i>t_R</i> (min)
BnSMe	0.8	25	A	<i>n</i> -hexane-IPA 9:1	18.3 (<i>R</i>); 20.1 (<i>S</i>)
BnSEt	0.8	25	A	<i>n</i> -hexane-IPA 9:1	21.8 (<i>R</i>); 23.5 (<i>S</i>)
CySMe	0.7	25	B	<i>n</i> -hexane-IPA 8:2	7.8 (<i>S</i>); 9.2 (<i>R</i>)

^a All the experiments were performed with isocratic eluent.

4. UV Spectra

UV spectra of riboflavin binding protein bound to flavin catalyst **3** (■) and of unbounded flavin catalyst **3** (▲). The same pattern was observed for the flavin catalysts **4-6**. Measurements were performed in phosphate buffer 50 mM pH 7.5, using 0.40 mM catalyst.

5. Supporting references

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6. NMR Spectra