Switching the Mechanism of NADH Photooxidation by Supramolecular Interactions

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1. General Information

**UV-vis absorption spectroscopy** was performed on a JASCO V-670 UV-vis-NIR Spectrophotometer using gas-tight quartz glass cuvettes (d = 10.0 mm, Hellma).

**Emission spectroscopy** was performed on a JASCO FP-8500 Fluorescence Spectrometer with gas-tight quartz glass cuvettes (d = 10.0 mm, Hellma).

**¹H-NMR** (400 MHz) spectra were recorded on a Bruker Avance 400 MHz at room temperature.

**¹³C-NMR** (126 MHz) spectra were recorded on a Bruker Avance 500 MHz at room temperature. The shift values are given in ppm and are referenced to the corresponding solvent residual peaks (4.79 ppm (singlet, ¹H-spectra) for D₂O, 3.31 ppm (quintet, ¹H-spectra) or 49.00 ppm (septet, ¹³C-spectra) for d₄-MeOD as well as 2.50 ppm (quintet, ¹H-spectra) for d₆-DMSO).

**O₂ detection** was performed using a FireStingO₂ optical oxygen meter (Pyroscience, Germany) using oxygen sensitive optical sensor spots (OXSP5, with optical isolation). The spot was glued (transparent silicone glue, SPGLUE) to the inner glass wall of a screw-capped cuvette (d = 10.0 mm, Hellma). The O₂ concentration was measured in μmol/l. Two-point calibration of the liquid phase was performed using a de-oxygenated aqueous solution (aqueous sodium dithionite solution) and air equilibrated deionized water. More details can be found in the respective literature.[1]

**¹⁰O₂ emission measurements**: All samples were prepared in 1 cm quartz cuvette with 3000 μl volume with optical density between 0.055 to 0.065. MeCN was supplied by Roth. All UV-vis absorption measurements were done in a JASCO V-780. FLS 980 of Edinburgh Instruments with NIR-PMT detector was used for emission measurements with integration time/ dwell time of 1 s per step with slit width of excitation: 20 nm and slit width of emission: 20 nm. The excitation wavelength was set to \( \lambda = 450 \, \text{nm} \). The temperature of the NIR-PMT detector was maintained to -80 °C using liquid nitrogen cooling pump. The emission spectrophotometer already has the lamp intensity correction file integrated in the program.

**Irradiation setup**: For all catalysis runs analyzed via UV-vis, emission or ¹H-NMR spectroscopy the irradiation setup consisted of a custom-made reactor equipped with four ventilators to exclude heating of the samples and one blue light emitting LED-stick (\( \lambda_{\text{max}} = 465 \, \text{nm} \), 45-50 mW/cm²) in the center of the reactor on which the GC-vials or cuvettes were placed. The only difference when measuring the O₂
consumption during photocatalysis was that the irradiation was not performed from the bottom of the glass vial/cuvette but rather from one side of the cuvette equipped with the O2 sensor spots.

**Nanosecond transient absorption spectroscopy** was performed using a 10 Hz NdYAG laser (Surelite Continuum) in combination with an optical parametric oscillator (Surelite OPO) as the pump source. The energy of the pump at the sample position was maintained at 0.2 mJ. The white light probe was generated using a Xenon arc lamp. Transient absorption kinetics from individual probe wavelengths were recorded using a commercially available detection system from Pascher Instruments AB with a time resolution of 10 ns.

**Femtosecond transient absorption spectroscopy**: The setup for femtosecond transient absorption spectroscopy has been described elsewhere.[2,3] In brief, 800 nm beam is split into two, one of which is used to generate the pump beam of 470 nm. A residual of the other beam is taken and focused on a rotating CaF\(_2\) plate which generates a white light continuum. Both the pump and the probe are then spatially and temporally overlapped at the sample position at the magic angle. The probe beam is then directed to a spectrophotometer integrated with a diode array which records the spectra.

**Chemicals and Materials**: 1,10-Phenanthroline (phen) was obtained from abcr (Germany) and 4,4\(^{-}\)tert-butyl-2,2\(^{-}\)-bipyridine (tbbpy) as well as 9,10-anthracenediyi-bis(methylene)dimalonic acid (ABDA) were obtained from Sigma. All chemicals were used without further purification. Also NADH (disodium salt, grade I approx. 100 %, Roche), NADPH (tetrasodium salt, grade I ≥ 98%, Roche), NAD\(^{+}\) (free acid, grade I approx. 100 %, Roche), NADP\(^{+}\) (Gebr. Biotechnik) ADP (disodium salt, Roche), L-malate (disodium salt, Merck) and Pyruvate (sodium salt, Carl Roth) were used as received. Similarly, sodium benzoate (Merck), NaN\(_3\) (Carl Roth) and L-histidine (Merck) were purchased from commercial suppliers and used as received. NADP-dependent malic enzyme (oxaloacetate decarboxylating malte dehydrogenase) from chicken liver (EC 1.1.1.40) was obtained from Sigma and used as described in chapter 2. Catalase from *Corynebacterium glutamicum* was received from Roche. All solvents for synthetic purposes were of technical grade and redistilled prior to use. Size exclusion chromatography was performed on a Sephadex LH-20 column using MeOH as mobile phase. Additionally, [(tbbpy)\(_2\)]RuCl\(_2\)[4] and dppz[5] were synthesized according to published methods.
2. Experimental details

If not otherwise stated, all preparation steps and photochemical reactions were performed under ambient conditions. The molecular structure and corresponding abbreviations of the three different Ru complexes used in this study are depicted in the scheme below.

![Scheme of Ru complexes](image)

**Photooxidation of NAD(P)H, analyzed with UV-vis and emission spectroscopy:**

For a typical NAD(P)H photooxidation run, the following reaction parameters were chosen: \( V = 1.5 \text{ ml} \), \( c(\text{Ru-complex}) = 2 \mu\text{M} \) (1 eq.) and \( c(\text{NAD(P)H}) = 1 \text{ mM} \) (500 eq.). First, a glass vial (VWR Screw Vial, 4 ml, 45 x 14.15 mm) was charged with the corresponding amount of Ru complex via a MeOH stock solution using Hamilton micro syringes (for 2 \( \mu\text{M} \) solutions of \( V = 1.5 \text{ ml} \), 0.00267 mg \([\text{tbbpy}]_2\text{Ru(phen)}\)Cl\(_2\) (Ruphen, \( M = 888.99 \text{ g/mol} \)), 0.00293 mg \([\text{Ru(tbbpy)}_3]\)Cl\(_2\) (Rutbbpy, \( M = 977.18 \text{ g/mol} \)) or 0.00297 mg \([\text{tbbpy}]_2\text{Ru(dppz)}\)Cl\(_2\) (Rudppz, \( M = 991.08 \text{ g/mol} \)) were added). After evaporation of MeOH using pressured air, a 1 mM aqueous solutions of NADH (1.065 mg per 1.5 ml, \( M = 709.4 \text{ g/mol} \)) or NADPH (1.25 mg per 1.5 ml, \( M = 833.4 \text{ g/mol} \)) was prepared using deionized water. After adding 1.5 ml of the 1 mM nicotinamide solutions and a small stirring bar to the corresponding glass vials they were closed with a screw cap and allowed to incubate for 5 min in the dark (for \([\text{Ru(tbbpy)}_3]\)Cl\(_2\) the complex had to be first dissolved in 20 \( \mu\text{l} \) MeOH or DMSO prior to the addition of the aqueous nicotinamide solution as the pure solid was not well soluble in pure water itself; however no particle formation upon addition of water to this 20 \( \mu\text{l} \) complex solution was observed). After taking a first aliquot (see next paragraph) for the 0 min measurement, the glass vial was irradiated as described above (chapter 1).

Aliquots for the UV-vis and emission measurements were drawn at fixed time intervals as follows: From the 1.5 ml reaction solutions, 100 \( \mu\text{l} \) were added to 2400 \( \mu\text{l} \) of deionized water, i.e. the reaction mixture was diluted by a factor of 25 for UV-vis and emission spectroscopy. Analysis of the obtained UV-vis and emission spectroscopy data was performed as follows: Conversion of NAD(P)H was calculated by the relative decrease of the UV-vis absorbance band at 340 nm and the emission band at 460 nm using a value of 0 for both bands for 100% conversion as the (main) oxidation product, NAD(P)\(^+\), was non-absorbing and non-emitting at these wavelengths using an excitation wavelength for the emission measurements of 340 nm.

For the NAD(P)H photooxidations performed in D\(_2\)O instead of deionized H\(_2\)O, the protocol was completely identical as stated above apart from the preparation of the 1 mM NAD(P)H stock solution in D\(_2\)O instead of H\(_2\)O.

For the NAD(P)H photooxidations performed in presence of 15 mM OH radical quencher (sodium benzoate, \( M = 144.11 \text{ g/mol} \), \( m = 3.24 \text{ mg} \)) or 15 mM \(^1\text{O}_2\) quenchers NaN\(_3\) (\( M = 65.01 \text{ g/mol} \), \( m = 1.46 \text{ mg} \)) or histidine (\( M = 155.16 \text{ g/mol} \), \( m = 3.49 \text{ mg} \)) the protocol from above was followed as well. However, whereas sodium benzoate and histidine were added as solids to the glass vials already...
charged with the corresponding Ru complex, for NaN$_3$ a stock solution was prepared which was then used to prepare a stock solution containing NaN$_3$ and NAD(P)H. This combined stock solution was then used for dissolving the corresponding Ru complex in the glass vial.

The “photooxidation” experiment in absence of oxygen was performed as follows: In a glovebox (Ar atmosphere) a 1 ml solution containing 1 mM NADH and 2 µM Rutbbpy was prepared using freshly degassed (1.5 min per 1 ml H$_2$O) deionized water (a glass vial (VWR Screw Vial, 4 ml, 45 x 14.15 mm) was used as reaction vessel). Inside the glovebox, the reaction mixture was irradiated with one blue light emitting LED-stick ($\lambda_{\text{max}} = 465$ nm, 45-50 mW/cm$^2$) from the bottom of the glass vial. After fixed time intervals, 100 µL samples were taken out from the reaction mixture and diluted by a factor of 25 inside the glovebox by adding the sample to 2.4 ml of degassed water before they were analyzed using UV-vis spectroscopy.

Photooxidation of NADH in presence of ADP

As an increased NADH autoxidation rate was observed in presence of ADP in pure deionized water, the photooxidation process was performed in a 0.1 M phosphate buffer (pH = 7.5; therefore 140.6 mg Na$_2$HPO$_4$ (M = 141.96 g/mol) and 61.2 mg NaH$_2$PO$_4$ (M = 119.98 g/mol) were dissolved in 15.0 ml of deionized water). Using this buffer solution, no effect of ADP on the stability of NADH was observed (data not shown).

Typically, 700 µL of a solution containing 2 µM of Ru complex (either Ruphen or Rudppz), 1 mM NADH and 10 mM ADP were prepared in 4 ml glass vials (see above). Therefore, the glass vials were first charged with the corresponding Ru complex via a MeOH stock solution and Hamilton micro syringes. After evaporation of MeOH using pressured air, 350 µL of a 2 mM phosphate buffered NADH stock solution and 350 µL of a 20 mM phosphate buffered ADP stock solution (both stored on ice prior to use) were added to the glass vial. If the photooxidation was meant to be measured in the absence of ADP, 350 µL of buffer were added instead of the ADP stock solution. After vigorous shaking, a “0 min” measurement was performed by diluting 70 µL of the solution with 2.40 ml of deionized water in a cuvette and measuring absorption and emission spectra ($\lambda_{\text{exc}} = 340$ nm). The glass vials were irradiated with one blue light emitting LED-stick ($\lambda_{\text{max}} = 465$ nm, 45-50 mW/cm$^2$) from the bottom of the glass vial in a custom-made air-cooling apparatus. After every 10 min of irradiation 70 µl samples were drawn, diluted and evaluated as described above to kinetically trace the photooxidation process. All reactions were performed in duplicates.

Photooxidation of NAD(P)H, analyzed with $^1$H-NMR spectroscopy:

For a typical NAD(P)H photooxidation run, the following reaction parameters were chosen: $V = 2.0$ ml, c(Ru-complex) = 2 µM (1 eq.) and c(NAD(P)H) = 4 mM (2000 eq.). First, a glass vial (VWR Screw Vial, 4 ml, 45 x 14.15 mm) was charged with the corresponding amount of Ru complex via a MeOH stock solution using Hamilton micro syringes. After evaporation of MeOH using pressured air, NAD(P)H was added as a solid. Then, 2.00 ml D$_2$O were added and the reaction mixture was allowed to incubate for 5 min in the dark. After taking a first 600 µl aliquot for $^1$H-NMR analysis using a standard NMR tube, the glass vial was irradiated as described above. After fixed time intervals, a second and a third 600 µL sample was analyzed via $^1$H-NMR spectroscopy. In order to allow for $^1$H-NMR spectroscopic comparison with pure NAD$^+$ as reference compound, 80 µL of a PBS buffer (500 µL D$_2$O containing 80 mg NaCl, 2 mg KCl, 14.2 mg Na$_2$HPO$_4$ and 2.7 mg KH$_2$PO$_4$) were added to the 600 µL samples to compensate for peak shifts between the photooxidation products of NADH and the pure NAD$^+$ observed in pure D$_2$O (possibly due to different pH values or ion concentrations of the prepared NADH
and NAD⁺ solutions; note that NADH is used as disodium salt whereas NAD⁺ is used as the free acid). After addition of the PBS buffer the peaks of the main photooxidation product of NADH and of the pure NAD⁺ were almost identically shifted clearly showing the high selectivity of the established photooxidation process.

**1H-NMR spectroscopic determination of binding constants between Rudppz and NADH or ADP, respectively:**

Whereas the samples for 1H-NMR measurements containing Rudppz and ADP could be prepared under ambient conditions, the mixtures of Rudppz and NADH had to prepared under exclusion of oxygen in an argon filled glovebox to avoid photooxidation. Typically, 4 mM Rudppz was added to the respective NMR-tube via a methanolic stock solution. After evaporation of the solvent under reduced pressure, the NMR tube was subsequently charged with 45 µL d6-DMSO to guarantee well dissolved complex. Afterwards, 555 µL D2O containing an appropriate amount of ADP or NADH was added to the NMR tube. After mixing, 1H-NMR spectra were recorded at room temperature. Binding constants were determined using a publicly accessible online program.⁶,⁷

**Photooxidation of NAD(P)H, analyzed with the FireStingO2 optical oxygen meter:**

In an appropriate glass vial, 5 ml of a solution containing 500 µM NADH (100 eq.) and 5 µM [(tbbpy)2Ru(dppz)]Cl2 (0.0248 mg, 1 eq. via a MeOH stock solution using a Hamilton microsyringe) were prepared. Afterwards, the cuvette serving as a reaction vessel for detecting the photoinduced O2-consumption (see chapter 1), was filled completely to the top and then sealed using a screw cap to minimize oxygen diffusion from the surrounding atmosphere into the reaction mixture. Then, the program recording the oxygen content was started (one data point is acquired every second) and after an equilibration period and reaching a constant O2 value of roughly 250 µM, the LED-stick was switched on leading to the consumption of oxygen in the solution. The same protocol was followed if 1 mM instead of 500 µM NADH were used.

**Photooxidation of histidine:**

For a typical histidine photooxidation run, the following reaction parameters were chosen: V = 2.5 ml, c(Ru-complex) = 10 µM (1 eq.) and c(histidine) = 5 mM (500 eq.; M(histidine) = 155.16 g/mol). First, a glass vial (VWR Screw Vial, 4 ml, 45 x 14.15 mm) was charged with the corresponding amount of Ru complex via a MeOH stock solution using Hamilton micro syringes. After evaporation of MeOH using pressured air, 2.5 ml of a 5 mM histidine containing aqueous stock solution was added to the complex. After allowing the reaction mixture to incubate for 5 min in the dark, the solutions were transferred into sealable cuvettes. After taking “0 min measurements”, the cuvettes were placed in the reactor and irradiated as described above. After fixed irradiation times (5 min) the cuvettes were first vortexed for 10 sec each, then placed in the UV-vis spectrometer for one measurement, then placed in the emission spectrometer (λexc = 335 nm) and afterwards placed back to the reactor for further irradiation. Data analysis with respect to the different kinetics of photoinduced endoperoxide formation (see scheme below) was performed by plotting the increase of absorbance at 335 nm or the increase in emission intensity at 445 nm against the irradiation time. By simply dissolving 10 µM of the corresponding Ru complexes in 2.5 ml pure H2O, the photostability of the dyes was checked in absence of histidine.
Stability of NADH in presence of H$_2$O$_2$.

In order to evaluate the stability of NADH in the presence of H$_2$O$_2$, 2.5 ml of a 100 µM NADH solution in pure deionized water was prepared and transferred into a sealable cuvette. Afterwards, a 9.7 M aqueous H$_2$O$_2$ solution (30 w%, VWR) was diluted to a concentration of 9.7 mM (100µL of 9.7 M H$_2$O$_2$ dissolved in 100 ml deionized water). From this 9.7 mM H$_2$O$_2$ solution different amounts were transferred into the cuvette containing 100 µM NADH and analyzed using UV-vis spectroscopy. Typically, the addition of 13 µL of this 9.7 mM H$_2$O$_2$ solution increased the H$_2$O$_2$ concentration in the cuvette by 50 µM. In the end even 20 µL of the 9.7 M aqueous H$_2$O$_2$ solution (30 w%) were added, generating a H$_2$O$_2$ concentration in the cuvette of roughly 70 mM. Even under these highly concentrated conditions NADH was completely stable as analyzed from the dilution corrected absorbance at 340 nm which showed no change at all.

Determination of the reaction stoichiometry and formation of H$_2$O$_2$ as O$_2$-reduction product:

In order to determine the amount of oxygen consumed per molecule of NADH as well as to evaluate the amount of H$_2$O$_2$ formed per molecule of NADH, a combination of oxygen determination using the FireStingO2 optical oxygen meter setup (see above), UV-vis as well as emission spectroscopy was used. Therefore, 5 ml of a solution containing 5 µM Rudppz and 500 µM or 1 mM NADH in a 50 mM aqueous phosphate buffer (pH = 7.5) was prepared in the beginning. Then, a 200 µl sample was diluted with 2.30 ml deionized water for the “pre-irradiation” UV-vis and emission measurements. The remaining 4.80 ml of the prepared solution were transferred in the reaction chamber equipped with the FireStingO2 optical oxygen meter. Upon irradiation, the oxygen level dropped to 0 µM after some reaction time. At this point 200 µl of the solution were again diluted by 2.30 ml deionized water for analyzing the remaining amount of NADH after complete oxygen consumption via UV-vis and emission spectroscopy. Afterwards, 50 µl of a solution containing 0.50 mg catalase per 1 ml of 50 mM phosphate buffer were added which induced a significant evolution of oxygen.

Photocatalytic NADH oxidation under argon atmosphere using H$_2$O$_2$ as oxidant

The samples were prepared as follows: First, a glass vial (VWR Screw Vial, 4 ml, 45 x 14.15 mm) was charged with the corresponding amount of Ru complex via a MeOH stock solution using Hamilton micro syringes. After evaporation of MeOH using pressured air, the GC vials were introduced into an argon filled glovebox. Afterwards, 2.7 ml of a 1.04 mM aqueous NADH solution (degassed H$_2$O) were added, followed by 100 µL of a 56 mM aqueous H$_2$O$_2$ solution (degassed H$_2$O). Then, 400 µL of this solution were transferred into 6 glass vials each. After sealing these glass vials with screw caps, five of them were irradiated with one LED-stick (465 nm, 45-50 mW/cm$^2$) from the bottom, whereas one was not irradiated at all, as it was used as 0 min irradiation reference. In all cases, 100 µL of the samples were diluted with 2.4 mL H$_2$O under ambient conditions prior to spectroscopic analysis.

Optimization of reaction conditions for the NADP-dependent malic enzyme catalyzed conversion of L-malate to pyruvate

To optimize the conditions for the malic enzyme (ME), reference experiments were performed. For this reason, different enzyme concentrations as well as L-malate concentrations were used as starting
conditions under two pH-values, pH 7.5 and pH 8.0. This was important because the ME showed substrate inhibition in some species which, in some cases, could be overcome by changing the pH value.

The experiment was done in 500 µl solutions in Eppendorf tubes using 50 µl of 10 mM NADP⁺ (→ 1 mM) and 50 µl of L-malate in different concentrations, 100 mM, 50 mM and 20 mM (→ 10/5/2 mM). After adding 5 µl or 10 µl of a 1:5 dilution of the ME (initial concentration of the purchased enzyme: 25 U) the volume of 500 µl was filled with a 25 mM TrisHCl-buffer containing 4 mM MnCl₂ (ME-buffer). The reaction solutions were incubated at 30 °C and samples of 10 µl were taken in certain timesteps and diluted in 190 µl of 25 mM TrisHCl-buffer containing 4 mM MgCl₂ (LDH-buffer) (1:20 dilution). These samples were kept on ice until use for concentration determination with the lactate dehydrogenase (LDH) assay. The ME in the samples was inactivated by heat for 10 min at 95 °C in a heating block and centrifuged for another 10 min with 16,000 x g.

For the determination of the pyruvate concentration an LDH assay was performed in a 96-well plate measuring always the samples and a standard at the same time. The wells were filled with either 100 µl or 85 µl of the LDH-buffer, 50 µl of 1 mM NADH and either 50 µl (total dilution 1:80) or 65 µl of the sample (total dilution 1:60). Calibration was done based on pyruvate standards in a serial dilution. To ensure a direct comparability a stock of 10 mM pyruvate was diluted 1:20 in HPLC grade water and a dilution series of 1:2 was used. The absorption was measured three times at 340 nm (NADH; E₁) and then 2 µl LDH were added. After 20 min of incubation at room temperature the absorption at 340 nm was measured again three times (E₂). These experiments showed highest concentrations of about 0.8 mM pyruvate at pH 8.0 with 5 mM L-malate using 10 µl/500 µl of the 1:5 dilution of the ME (total dilution 1:250). The amount of pyruvate reached its maximum after 15 min of incubation with ME at 30 °C.

Discontinuous photobiocatalysis

The photooxidation of NADPH by Rudppz was coupled to the enzymatic reaction using NADP⁺ in a discontinuous way. A total volume of 1.2 ml was used in the beginning, starting with 4 µM Rudppz in ME-buffer with 1 mM NADPH and 5 mM L-malate. As reaction vessels, glass vials (VWR Screw Vial, 4 ml, 45 x 14.15 mm) were used. The solution was irradiated with blue light leading to the photooxidation of NADPH. It was vortexed for 10 s every 5 min and UV-Vis and emission spectra were measured every 20 min. One irradiation cycle was completed after 40 min of irradiation.

For all spectroscopic measurements, 40 µl of the samples were diluted in 960 µl of deionized water in a cuvette (dilution 1:25). After 40 min of irradiation 10 µl of a 1:5 dilution of the ME were added, and the glass vials were incubated for 15 min at 30 °C (water bath) in the dark. Then samples were taken for UV-vis and emission measurements (as described above) and the reaction mixtures were irradiated with blue light for another 20 min in which precipitates containing Rudppz, ME and L-malate were formed. After analyzing the solution via UV-vis and emission spectroscopy (see above), the solution was centrifuged for 10 min with 11,000 x g. The supernatant was used for further cycles beginning with the irradiation with blue light and repeating the steps described above.

From the second cycle on, also 10 µl of L-malate (500 mM) were added with the addition of ME to the reaction solution to compensate a possible loss of L-malate from previous precipitation. Likewise, an appropriate volume (i.e. adding 4 µM Rudppz) of a 1.50 mg/5 ml aqueous Rudppz stock solution was added to the supernatant after centrifugation via Eppendorf pipettes to compensate for Rudppz losses during the photoinduced precipitate formation as well.
3. Synthesis and characterization of the Ru complexes (Figures S1-S20)

All reactions and purification steps were performed under ambient conditions.

**Synthesis of [Ru(tbbpy)$_3$]Cl$_2$ (Rutbbpy):**

To 122 mg [(tbbpy)$_2$RuCl$_2$] (0.172 mmol, 1 eq.) placed in a 100 ml round bottom flask, 50.8 mg (0.189 mmol, 1.1 eq.) of 4,4’-tert-butyl-2,2’-bipyridine (tbbpy) were added. After suspending the two compounds in 40 ml of EtOH:H$_2$O = 3:1 (v:v), the reaction mixture was refluxed for 16 h at 85 °C. After removal of all volatiles the crude reaction product was suspended in 30 ml Et$_2$O and stirred for 2 h. After filtration and removal of all volatiles, 156 mg (93 %, 0.160 mmol) of Rutbbpy were obtained as orange solid.

$^1$H-NMR (d$_4$-MeOD, 400 MHz): δ = 8.71 (d, J = 2.1 Hz, 6H), 7.67 (d, J = 6.1 Hz, 6H), 7.54 (dd, J = 6.1, 2.1 Hz, 6H), 1.45 (s, 54H).

$^{13}$C-NMR (CD$_3$CN, 126 MHz): δ = 164.14, 158.44, 151.86, 126.15, 122.68, 36.62, 30.61.

HRMS (MALDI-FT-ICR): calcd. for [M - 2Cl]$^+$ 906.4876; found 906.4875; calcd. for [M - HCl - H - CH$_4$]$^+$ 923.4092; found 923.4102; calcd. for [M - Cl]$^+$ 941.4562; found 941.4577.

**Synthesis of [(tbbpy)$_2$Ru(dppz)]Cl$_2$ (Rudppz):**

To 150 mg [(tbbpy)$_2$RuCl$_2$] (0.212 mmol, 1 eq.) placed in a 100 ml round bottom flask, 65.6 mg (0.232 mmol, 1.1 eq.) of dppz were added. After suspending the two compounds in 40 ml of EtOH:H$_2$O = 3:1 (v:v), the reaction mixture was refluxed for 22 h at 85 °C. After evaporation of most volatiles the resulting suspension was filtered through celite. Then, the remaining solvent of the clear red solution was removed completely. After suspending the crude product in 30 ml of Et$_2$O and stirring for 30 min a filtration was performed. The obtained red powder was then subjected to size-exclusion chromatography using Sephadex as stationary phase and MeOH as mobile phase. Collection of the first main fraction resulted in the isolation of 183 mg (87 %, 0.184 mmol) pure Rudppz after a final removal of all MeOH.

$^1$H-NMR (d$_4$-MeOD, 400 MHz): δ = 9.76 (dd, J = 2.1 Hz, 2H), 8.79 (d, J = 2.0 Hz, 2H), 8.76 (d, J = 2.0 Hz, 2H), 8.50 (dd, J = 6.6, 3.4 Hz, 2H), 8.25 (dd, J = 5.4, 1.2 Hz, 2H), 8.14 (dd, J = 6.6, 3.4 Hz, 1H), 8.00 (dd, J = 8.2, 5.4 Hz, 2H), 7.81 (d, J = 6.0 Hz, 2H), 7.73 (d, J = 6.1 Hz, 2H), 7.62 (dd, J = 6.0, 2.0 Hz, 2H), 7.40 (dd, J = 6.1, 2.0 Hz, 2H), 1.50 (s, 18H), 1.40 (s, 18H).

$^{13}$C-NMR (d$_4$-MeOD, 126 MHz): δ = 164.55, 164.41, 158.56, 158.38, 154.38, 152.43, 152.18, 151.99, 144.22, 141.25, 134.73, 133.51, 132.27, 130.83, 128.61, 126.31, 126.18, 122.87, 122.79, 36.70, 36.60, 30.65, 30.55.

HRMS (MALDI-FT-ICR): calcd. for [M - 2Cl]$^+$ 920.3842; found 920.3836; calcd. for [M - HCl - H - CH$_4$]$^+$ 937.3058; found 937.3046; calcd. for [M - Cl]$^+$ 955.3528; found 955.3520.

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S9
Synthesis of [(tbbpy)$_2$Ru(phen)]Cl$_2$ (Ruphen):

To 122 mg [(tbbpy)$_2$RuCl$_2$] (0.172 mmol, 1 eq.) placed in a 100 ml round bottom flask, 37.5 mg (0.189 mmol, 1.1 eq.) of 1,10-phenanthroline were added. After suspending the two compounds in 40 ml of EtOH:H$_2$O = 3:1 (v:v), the reaction mixture was refluxed for 16 h at 85 °C. After removal of all volatiles the crude reaction product was suspended in 30 ml Et$_2$O and stirred for 2 h. After filtration and removal of all volatiles, 139 mg (91 %, 0.157 mmol) of Ruphen were obtained as red solid.

$^1$H-NMR (d$_4$-MeOD, 400 MHz): $\delta = 8.77$ (d, J = 1.9 Hz, 2H), 8.73 (d, J = 1.9 Hz, 2H), 8.70 (dd, J = 8.3, 1.1 Hz, 2H), 8.31 (s, 2H), 8.17 (dd, J = 5.3, 1.1 Hz, 2H), 7.85 (dd, J = 8.3, 5.3 Hz, 2H), 7.80 (d, J = 6.0 Hz, 2H), 7.60 (dd, J = 6.0, 1.9 Hz, 2H), 7.49 (d, J = 6.0 Hz, 2H), 7.34 (dd, J = 6.0, 1.9 Hz, 2H), 1.48 (s, 18H), 1.39 (s, 18H).

$^{13}$C-NMR (d$_4$-MeOD, 126 MHz): $\delta = 164.39$, 164.25, 158.61, 158.41, 153.30, 152.21, 152.04, 149.01, 137.93, 132.46, 129.42, 127.41, 126.20, 126.04, 122.77, 122.70, 36.67, 36.57, 30.64, 30.55.

HRMS (MALDI-FT-ICR): calcd. for [M - 2Cl]$^+$ 818.3622; found 818.3604; calcd. for [M - HCl - H - CH$_3$]$^+$ 835.2839; found 835.2828; calcd. for [M - Cl]$^+$ 853.3309; found 853.3293.
Figure S1: $^1$H-NMR spectrum of Rutbbpy in d$_4$-MeOD.

Figure S2: $^{13}$C-NMR spectrum of Rutbbpy in d$_4$-MeOD.
Figure S3: $^1$H-NMR spectrum of Rudpz in d$_4$-MeOD.

Figure S4: $^{13}$C-NMR spectrum of Rudpz in d$_4$-MeOD.
Figure S5: $^1$H-NMR spectrum of Ruphen in $d_4$-MeOD.

Figure S6: $^{13}$C-NMR spectrum of Ruphen in $d_4$-MeOD.
Figure S7: Obtained mass spectrum of Rutbbpy.

Figure S8: Expanded mass spectrum of Rutbbpy showing the [M-2Cl]^+ peak.
Figure S9: Simulated mass spectrum of the \([\text{M-2Cl}]^+\) peak of Rutbbpy.

Figure S10: Expanded mass spectrum of Rutbbpy showing the \([\text{M-HCl-HCH}_4]^-\) peak.
Figure S11: Simulated mass spectrum of the [M-HCl-H-CH₄]^+ peak of Rutbppy.

Figure S12: Expanded mass spectrum of Rutbppy showing the [M-Cl]^+ peak.
Figure S13: Simulated mass spectrum of the [M-Cl]⁺ peak of Rutbbpy.

Figure S14: Obtained mass spectrum of Rudppz.
Figure S15: Expanded mass spectrum of Rudppz showing the [M-2Cl]^+ peak.

Figure S16: Simulated mass spectrum of the [M-2Cl]^+ peak of Rudppz.
Figure S17: Obtained mass spectrum of Ruphen.

Figure S18: Expanded mass spectrum of Ruphen showing the [M-2Cl]⁺ peak.
Figure S19: Simulated mass spectrum of the [M-2Cl]^+ peak of Ruphen.

Figure S20: UV-vis absorption (solid lines) and emission spectra (dotted lines, $\lambda_{\text{exc}} = 335\,\text{nm}$) of Rutbppy (diagram 1), Rudppz (diagram 2) and Ruphen (diagram 3) in H$_2$O ($c = 10\,\mu\text{M}$) under air.
4. Figures S21-S51

Figure S21: Photostability of Rutbbpy (diagram 1), Rudppz (diagram 2) and Ruphen (diagram 3) in pure deionized H$_2$O under ambient conditions. Diagram 4 shows the decrease in MLCT absorbance due to the photodegradation of the Ru complexes with increasing irradiation time, revealing Rudppz as the most stable one, followed by Rutbbpy and Ruphen (i.e. Rudppz > Rutbbpy > Ruphen (1 %, 14 % and 18 % loss of $^1$MLCT band absorbance after 2 h of irradiation)).

Figure S22: UV-vis (A-C) and emission spectroscopic (D-F) analysis of the NADH photooxidation using Rutbbpy (A,D), Ruphen (B,E) and Rudppz (C,F). The reactions were performed at room temperature and under air, using 1.5 mL H$_2$O containing 20 µL DMSO each, c(NADH) = 1 mM, c(Ru-complex) = 2 µM. A spectrum was collected every 20 min, the black spectra indicate the measurements prior to irradiation. Based on the residual emission intensity after 120 min, an NADH to NAD$^+$ conversion of >99 % for Rutbbpy, >95% for Ruphen and >99 % for Rudppz could be determined.
Figure S23: UV-vis spectrum (black) and emission spectrum (gray) of NAD⁺ (40 µM) in deionized water. As the molecule does not absorb at 340 nm no emission signal other than that from the pure solvent is observed.

Figure S24: ¹H-NMR spectroscopic comparison of the NADH photooxidation process (4 mM NADH and 2 µM Rudppz dissolved in D₂O). Bottom spectrum: reaction mixture before irradiation; spectrum in the center: reaction mixture after 1 h of irradiation; top spectrum: pure NAD⁺ as reference. The peak at 3.65 ppm can be attributed to EtOH as part of the utilized NADH.
Figure S25: $^1$H-NMR spectroscopic changes of a mixture containing 2 µM Rutbbpy and 4 mM NADPH in D$_2$O under air. Bottom spectrum: mixture before irradiation; spectrum in the center: mixture after 1 h of irradiation; top spectrum: commercially purchased NADP$^+$ (4 mM) as reference compound.

Figure S26: Photosensitized $^1$O$_2$ emission spectra in pure MeCN (diagram 1) or MeCN:H$_2$O (4:1, v:v, diagram 2) using Ruphen, Rutbbpy, Rudppz as well as [Ru(bpy)$_3$]Cl$_2$ as reference. Excitation of the complexes was performed with $\lambda_{exc} = 450$ nm. The low $^1$O$_2$ production by Rudppz in panel 2 can be attributed to its well-known light-switch effect.[10] Also, $^1$O$_2$ yields of structurally very similar compounds as Rudppz have previously been determined to be very low in pure aqueous PBS buffer (PBS = phosphate buffered saline)[11] due to the formation of a $^3$MLCT excited state localized on the central phenazine sphere which possesses a lifetime of only 250 ps in pure water.[12]
Figure S27: Diagram 1: UV-vis spectroscopic changes of a reaction mixture containing 1 mM NADH and 2 µM Rutbbpy in H₂O. The solution was prepared as described in chapter 2 of the SI and stored in the dark at room temperature. Diagram 2: UV-vis spectroscopic changes of a reaction mixture containing 1 mM NADH and 2 µM Rutbbpy in degassed H₂O under argon upon irradiation with blue light (λ<sub>max</sub> = 465 nm, 45-50 mW/cm²). Diagram 3: UV-vis spectroscopic changes of a solution containing 1 mM NADH in H₂O upon irradiation with blue light (λ = 465 nm, 45-50 mW/cm²). Diagram 4: UV-vis spectroscopic changes of a reaction mixture containing 1 mM NAD⁺ and 2 µM Rutbbpy in H₂O upon irradiation with blue light (λ<sub>max</sub> = 465 nm, 45-50 mW/cm²). Samples were drawn and analyzed as described in chapter 2 of the SI (i.e. 100 µL aliquots were diluted with 2.4 ml deionized H₂O prior to spectroscopic investigation).

Figure S28: ¹H-NMR spectroscopic changes of a solution containing 4 mM of NAD⁺ and 80 µM Rutbbpy in D₂O (peak at 4.75 ppm can be assigned to residual non deuterated water). Bottom spectrum: ¹H-NMR spectroscopic signature of NAD⁺ before irradiation; top spectrum: ¹H-NMR spectroscopic signature of NAD⁺ after 3 h of irradiation.
Figure S29: Effect of $^1$O$_2$ quenchers on the photooxidation of NADH (1 mM) by Ruphen (2 µM) in H$_2$O under ambient conditions.

Figure S30: Emission spectra of Rutbbpy (c = 10µM, diagram 1, left) and Ruphen (c = 10 µM, diagram 2, right) upon excitation into the maximum of the MLCT band at 460 nm (Rutbbpy) and 456 nm (Ruphen), respectively. In all cases, deionized water was used as solvent. The obtained emission intensity was corrected for the absorbance at the respective MLCT maxima to consider minor concentration differences between the samples (therefore, the emission intensities were divided by the absorbances at 460 nm or 456 nm of the respective samples).
Figure S31: Photocatalytic NADH (c = 1 mM) oxidation using different amounts of Ruphen. The reaction was performed in D₂O under ambient conditions using one LED-stick (see chapter 2 of the SI). Diagram 1: UV-vis spectroscopic changes using 1.0 µM of Ruphen (a spectrum was recorded every 10 minutes); diagram 2: UV-vis spectroscopic changes using 0.4 µM of Ruphen (a spectrum was recorded every 15 minutes); diagram 3: UV-vis spectroscopic changes using 0.2 µM of Ruphen (a spectrum was recorded every 30 minutes); diagram 4: Comparison of the TON values using different amounts of Ruphen.

Figure S32: For all measurements aqueous solutions containing 5 mM histidine and 10 µM of the respective complex were used; these solutions were irradiated with one LED-stick (λ_{max} = 465 nm, 45-50 mW/cm²) and analyzed every 5 minutes. Diagram 1: Temporal evolution of the emission intensity at 446 nm comparing the three different Ru complexes. Diagram 2: UV-vis spectroscopic changes using Ruphen; diagrams 3 and 4: Emission spectroscopic changes using Ruphen (3) and Rudppz (4), respectively (λ_{exc} = 335 nm).
Figure S33: UV-vis and emission spectroscopic changes during the photooxidation of histidine (5 mM) using 10 µM Rutbbpy (diagrams 1 and 2) or 10 µM Rudppz (diagram 3); diagram 4: Kinetic analysis of the UV-vis spectroscopic changes using Ruphen, Rutbbpy and Rudppz followed by the absorbance increase at 335 nm. Preparation and irradiation of the solutions was performed as described in chapter 2 of the SI.

Figure S34: Femtosecond transient absorption spectra of A) Rudppz under Ar; B) Rudppz under air; C) Rudppz and NADH under Ar; D) Rudppz and NADH under air with 470 nm excitation.
Figure S35: Nanosecond transient absorption spectra of A) Rudppz and NADH under air and B) Rudppz and NADH under Ar with 470 nm excitation. In B) the signal at 390 nm corresponds to the spectral signature from radicals of NADH. The signal at initial time of the experiment i.e at the lowest probe wavelength corresponds to a scenario where Rudppz is in excess. These Rudppz can successfully react with NADH to form its corresponding radicals. As time passes, the hydrogenated Rudppz is the predominant species preventing further oxidation of NADH. Hence no spectral signature corresponding to the radicals of NADH is observed at higher probe wavelengths. This occurs due to the inability of the detector to detect all probe wavelengths at the same instant.

Figure S36: UV-vis absorption spectra of a solution containing 1 mM NADH, 2 mM H₂O₂ and either 2 µM Rudppz (diagram 1) or 2 µM Ruphen (diagram 2) under argon atmosphere, respectively, after diluting the solutions by a factor of 25 using pure water. Only for Rudppz NADH oxidation was observed after 50 min of irradiation. The absorbance decrease at 340 nm is ca. 17 % thus yielding a TON for NAD⁺ formation of 85 after 50 min.
Figure S37: UV-vis spectra of NADH (c = 100 µM) in H₂O upon addition of increasing amounts of H₂O₂ under air. The stability of NADH is indicated by the constant absorbance at 340 nm, whereas increasing H₂O₂ amounts induce an absorbance increase between 250 and 325 nm. The dilution of the initial NADH solution by adding increasing amounts of H₂O₂ has been mathematically taken into consideration.

Figure S38: ¹H-NMR spectra (aromatic region) of Rudppz at different concentrations (bottom: 2 mM, center: 4 mM, top: 6mM) in a mixture of D₂O:d₆-DMSO = 555:45 (v:v). The arrows connect signals originating from the same H-atoms; black arrows indicate a shift to smaller ppm-values upon an increase of concentration, blue arrows indicate the opposite shift. The signal assignment was based on literature reports,[16,17] a H,H-COSY spectrum (see Figure S39) and a comparison to Ruphen in the same solvent mixture (Figures S45) showing no peak above 8.7 ppm.
Figure S39: H,H-COSY spectrum of Rudppz (6 mM) in D$_2$O:d$_6$-DMSO = 555:45 µL; peaks that belong to the same spin systems are marked with the same symbols. The two tbppy systems are marked with orange and green filled circles, respectively; the phenanthroline sphere of the dppz ligand is marked with black filled circles whereas signals belonging to the benzene like terminal moiety of the dppz ligand are marked with black squares. Signal assignments are in accordance with the molecular structure of Rudppz shown in Figure S38.

Figure S40: $^1$H-NMR spectra of NADH (4 mM) in absence (bottom spectrum) and in presence (top spectrum) of Rudppz (4 mM). As solvent a mixture of D$_2$O:d$_6$-DMSO = 555:45 (v:v) was used.
Figure S41: $^1$H-NMR spectra of NAD$^+$ (4 mM) in absence (bottom spectrum) and in presence (top spectrum) of Rudppz (4 mM). As solvent a mixture of D$_2$O:d$_6$-DMSO = 555:45 (v:v) was used.

Figure S42: $^1$H-NMR spectra of ADP (4 mM) in absence (bottom spectrum) and in presence (top spectrum) of Rudppz (4 mM). As solvent a mixture of D$_2$O:d$_6$-DMSO = 555:45 (v:v) was used.
Figure S43: Aromatic region of the $^1$H-NMR spectra of 4 mM Rudppz, mixed with 0 mM (0.0 eq.), 2 mM (0.5 eq.), 4 mM (1.0 eq.), 6 mM (1.5 eq.), 8 mM (2.0 eq.), and 12 mM (3.0 eq.) ADP in D$_2$O:d$_6$-DMSO = 555:45 (v:v).

Figure S44: Aliphatic region of the $^1$H-NMR spectra of 4 mM Rudppz, mixed with 0 mM (0.0 eq.), 2 mM (0.5 eq.), 4 mM (1.0 eq.), 6 mM (1.5 eq.), 8 mM (2.0 eq.), and 12 mM (3.0 eq.) ADP in D$_2$O:d$_6$-DMSO = 555:45 (v:v).
Figure S45: $^1$H-NMR-spectroscopic changes (between 7.1 and 9.7 ppm) of a solution containing 4 mM Ruphen in a mixture of D$_2$O:d$_6$-DMSO = 555:45 (v:v; bottom spectrum) upon addition of 4mM NADH (top spectrum). The signals originating from NADH are marked with filled black circles.

Figure S46: $^1$H-NMR spectra of NADH (4 mM) in absence (bottom spectrum) and in presence (top spectrum) of Ruphen (4 mM). As solvent a mixture of D$_2$O:d$_6$-DMSO = 555:45 (v:v) was used.
Figure S47: Aromatic region of the $^1$H-NMR spectra of 4 mM Rudppz, mixed with 0 mM (0.0 eq.), 1 mM (0.25 eq.), 2 mM (0.5 eq.), 4 mM (1.0 eq.), 6 mM (1.5 eq.), or 8 mM (2.0 eq.) NADH (from bottom to top) in D$_2$O:d$_6$-DMSO = 555:45 (v:v) under an atmosphere of argon.

Figure S48: Aliphatic region of the $^1$H-NMR spectra of 4 mM Rudppz, mixed with 0 mM (0.0 eq.), 1 mM (0.25 eq.), 2 mM (0.5 eq.), 4 mM (1.0 eq.), 6 mM (1.5 eq.), or 8 mM (2.0 eq.) NADH (from bottom to top) in D$_2$O:d$_6$-DMSO = 555:45 (v:v) under an atmosphere of argon.
Figure S49: Effect of ADP addition on the photocatalytic NADH oxidation using Rudppz (diagram 1) or Ruphen (diagram 2). The kinetic plots were obtained by tracing the emission maximum of NADH at 460 nm over time. For experimental details, see chapter 2 of the SI.

Figure S50: Diagram 1: Course of O$_2$-concentration (starting point at 265 µM) during a typical NADH photooxidation experiment using a PBS buffered aqueous solution containing 1 mM NADH and 5 µM Rudppz under ambient conditions. Arrow a indicates the start of blue light irradiation of the solution which ends after 14 min, indicated by arrow b (0 µM O$_2$ in the reaction mixture). Addition of catalase is marked by arrow c and the oxygen level rises to 109 µM (assuming the typical stoichiometries of the reaction it should theoretically rise to 132 µM; see Figure S35 for a possible explanation). Diagram 2: UV-vis (solid lines) and corresponding emission spectra (dotted lines) of the reaction mixture before irradiation (black curves, a) and after complete consumption of oxygen (gray curves, b). Based on the amount of consumed oxygen (diagram 1, 265 µM) a decrease of 27 % in the initial 1 mM NADH absorbance and emission was anticipated. The experimentally detected values were 31 % for the UV-vis measurement and 27 % for the emission measurement (decrease of the emission maximum by 27 %).
Figure S51: Main panel: Emission spectra of Rudppz upon irradiation with \( \lambda_{\text{exc}} = 443 \) nm in the malic enzyme buffer in absence (black curve) and presence (red curve) of the malic dehydrogenase. The peak at ca. 545 nm can be attributed to light scattered by the solvent. Inset: emission behavior of Rudppz in presence of ME depending on whether oxygen is present in the sample or not.
5. References:

[1] F. L. Huber, S. Amthor, B. Schwarz, B. Mizaikoff, C. Streb, S. Rau, *Sustain. Energy Fuels* **2018**, *2*, 1974–1978.

[2] R. Siebert, D. Akimov, M. Schmitt, A. Winter, U. S. Schubert, B. Dietzek, J. Popp, *ChemPhysChem* **2009**, *10*, 910–919.

[3] M. Karnahl, C. Kuhnt, F. Ma, A. Yartsev, M. Schmitt, B. Dietzek, S. Rau, J. Popp, *ChemPhysChem* **2011**, *12*, 2101–2109.

[4] S. Rau, B. Schäfer, A. Grüßing, S. Schebesta, K. Lamm, J. Vieth, H. Görls, D. Walther, M. Rudolph, U. W. Grummt, et al., *Inorganica Chim. Acta* **2004**, *357*, 4496–4503.

[5] C. Wang, L. Lystrom, H. Yin, M. Hetu, S. Kilina, S. A. McFarland, W. Sun, *Dalts. Trans.* **2016**, *45*, 16366–16378.

[6] https://supramolecular.org 2021.

[7] D. Brynn Hibbert, P. Thordarson, *Chem. Commun.* **2016**, *52*, 12792–12805.

[8] R. Y. Hsu, T. A. Pry, *Biochemistry* **1980**, *19*, 962–968.

[9] E. Detarsio, C. E. Alvarez, M. Saigo, C. S. Andreo, M. F. Drincovich, *J. Biol. Chem.* **2007**, *282*, 6053–6060.

[10] A. E. Friedman, J.-C. Chambron, J.-P. Sauvage, N. J. Turro, J. K. Barton, *J. Am. Chem. Soc.* **1990**, *112*, 4960–4962.

[11] C. Mari, V. Pierroz, R. Rubbiani, M. Patra, J. Hess, B. Spingler, L. Oehninger, J. Schur, I. Ott, L. Salassa, et al., *Chem. Eur. J.* **2014**, *20*, 14421–14436.

[12] E. J. C. Olson, D. Hu, A. Hörmann, A. M. Jonkman, M. R. Arkin, E. D. A. Stemp, J. K. Barton, P. F. Barbara, *J. Am. Chem. Soc.* **1997**, *119*, 11458–11467.

[13] B. Czochralska, L. Lindqvist, *Chem. Phys. Lett.* **1983**, *101*, 297–299.

[14] S. Fukuzumi, O. Inada, T. Sueonobu, *J. Am. Chem. Soc.* **2003**, *125*, 4808–4816.

[15] J. Gębicki, A. Marcinek, J. Zielonka, *Acc. Chem. Res.* **2004**, *37*, 379–386.

[16] M. G. Pfeffer, C. Pehlen, R. Staehle, D. Sorsche, C. Streb, S. Rau, *Dalts. Trans.* **2014**, *43*, 13307–13315.

[17] A. K. Mengele, S. Kaufhold, C. Streb, S. Rau, *Dalts. Trans.* **2016**, *45*, 6612–6618.