Structure Elucidation

The Cofactor of the Iron–Sulfur Cluster Free Hydrogenase Hmd: Structure of the Light-Inactivation Product**

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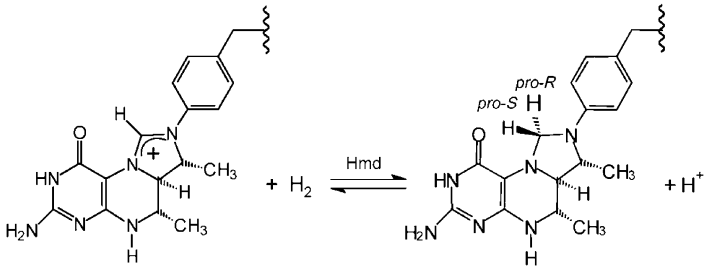
Hydrogenases are enzymes that catalyze reversible reactions that have H_2 as a substrate or product. There are three classes of hydrogenases which appear not to be structurally and phylogenetically related: [NiFe]-hydrogenases, [FeFe]-hydrogenases, and the iron–sulfur cluster free hydrogenase Hmd (H_2-forming methylenetetrahydromethanopterin dehydrogenase; previously referred to as metal-free hydrogenases).[1] [NiFe]-hydrogenases are generally found in bacteria and archaea;[2] they contain a dinuclear [NiFe] center as a prosthetic group which is in long-range electronic interaction with a [4Fe-4S] cluster.[3–5] [FeFe]-hydrogenases are found mainly in anaerobic Gram-positive bacteria with low guanine/cytosine content and in some anaerobically grown Eucarya;[6] they contain a dinuclear [FeFe] center covalently bound to a [4Fe-4S] cluster through a bridging cysteic sulfur ligand.[7,8] The iron–sulfur cluster free hydrogenase Hmd is found only in some methanogenic archaea growing on H_2 and CO_2.[9,10] The enzyme catalyzes the reversible reduction of methenyltetrahydromethanopterin with H_2 to methylenetetrahydromethanopterin (Scheme 1), which is an intermediary step in the reduction of CO_2 to methane.[11,12]

Hmd from *Methanothermobacter marburgensis* was recently shown to harbor a tightly bound iron-containing cofactor, which is required for activity.[13] The cofactor is released only after unfolding of the enzyme in urea, guanidine·HCl, or methanol in the presence of 2-thioethanol. The Hmd apoprotein is inactive, but becomes active upon addition of cofactor. The cofactor was found to be inactivated upon irradiation with UV-A/blue light.[14] Light inactivation is associated with the bleaching of the cofactor's color and the release of one iron atom[14] and most probably two CO molecules.[15] The active cofactor is also temperature-sensitive:[13] it is inactivated at 50 °C, with a half-life of only a few minutes. The half-life increases to approximately 60 minutes in the presence of 10 mM 2-thioethanol. Stabilization is most probably a consequence of the binding of the thiol group of 2-thioethanol to the cofactor's iron center.

Attempts to determine the structure or the molecular mass of the active cofactor have until now failed. It was found, however, that a relatively stable product with mass 542 Da is formed upon light inactivation or heat inactivation (Figure 1) which could be purified by HPLC and characterized. Inactivation was much more rapid than the formation of the product with mass 542 Da, and at least one of the intermediates was seen by HPLC analysis.

The exact mass of the product was determined by FT-ICR MS (ICR = ion-cyclotron resonance) to be 542.1167 ± 0.0003 Da (calculated: 541.1089+1.0078 Da; see the Supporting Information), which is consistent with an elemental composition of $C_{19}H_{23}N_6P_1O_{11}$. Placing the compound in ^2H_2O resulted in the mass increasing by 10 Da when analyzed in the negative mode and by 12 Da when analyzed in the positive mode, which indicates that 11 of the 23 hydrogen atoms were exchangeable with protons of water. When the cofactor was purified from enzyme isolated from cells grown in the presence of $^{15}NH_4Cl$ rather than $^{14}NH_4Cl$, the light-inactivation product with mass 548 Da, thus indicating that the compound with mass 542 Da indeed contained 6 N atoms. The presence of phosphorus was substantiated by the finding of a fragment with mass 79 Da in the ESI mass spectrum (negative mode); this signal corresponds to the molecular mass of PO_3^-. The ^{31}P NMR spectrum obtained with proton decoupling showed only one singulet at $\delta = -6.18$ ppm, which suggested the presence of one P atom. The stoichiometry H_{23}:P_1 was confirmed by comparison of the ^1H and ^{31}P NMRspectra obtained using methylphosphonic acid as an internal standard. A fragment with mass 498 Da was always seen in the mass spectra (data not shown), irrespective of whether the samples were prepared in buffers



Scheme 1. Reaction catalyzed by the iron–sulfur cluster free hydrogenase Hmd. Molecular hydrogen reacts with N^5N^{10}-methenyltetrahydromethanopterin reducing it to N^5N^{10}-methylenetetrahydromethanopterin and a proton, $\Delta G^{\circ\prime} = -5.5$ kJ mol^{-1}. A hydride is stereospecifically transferred from H_2 into the *pro-R* site of methylenetetrahydromethanopterin.

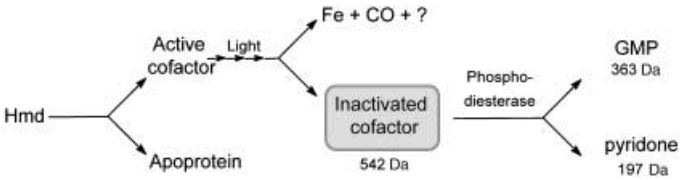


Figure 1. Scheme showing the formation of inactivated cofactor, CO, and other products from active Hmd cofactor by irradiation with light. The active cofactor is released from the holoenzyme after denaturation in the presence of 2-thioethanol. The holoenzyme was found to contain two moles of CO per mole of Fe by FTIR analysis and 2.4 ± 0.2 moles of CO per mole of Fe by chemical analysis.[15] Upon incubation of the inactivated cofactor (542 Da compound) for 60 min at 37 °C with phosphodiesterase I (0.07 U *Crotalus adamanteus* Venom from Amersham Bioscience, Freiburg, plus 40 μmol inactived cofactor in 50 μL 100 mM Gly/NaOH pH 8.9 containing 100 mM NaCl and 14 mM $MgCl_2$) two main products were formed that are referred to as 363 Da and 197 Da.

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containing sodium (23 Da) or lithium (7 Da) ions. The intensity of the signal at 498 Da increased with increasing CID voltage. The mass difference difference of 44 Da (542–498) indicates that the compound with mass 542 Da was partially decarboxylated during the determination of the mass.

A signal at mass 150 Da was found in the ESI mass spectrum of the compound with mass 542 Da (see the Supporting Information). This mass corresponds to the mass of guanine minus one proton. The UV/Vis spectrum of the compound with mass 542 Da resembles in part that of guanosine ($\varepsilon_{252} = 13.7\ \text{mM}^{-1}\,\text{cm}^{-1}$). The shoulder at 300 nm indicates, however, the presence of a second chromophore. Subtraction of the spectrum of guanosine from that of the compound with mass 542 Da gave a difference spectrum with an absorbance maximum at 300 nm ($\varepsilon = 8\ \text{mM}^{-1}\,\text{cm}^{-1}$; Figure 2). The compound with mass 542 Da was found to fluoresce with a maximum excitation at 300 nm and emission at 375 nm (Figure 3). These findings suggested that it was composed of a guanosine moiety and a moiety which has a maximum absorption of light at 300 nm.

The observation of a ^{31}P resonance at $\delta = -6.18$ ppm indicated that the product with mass 542 Da could be a phosphodiester. This turned out to be correct. Incubation of the compound with phosphodiesterase at pH 8.9 (optimum pH value of the enzyme) resulted in the formation of two main products: one with a mass of 363 Da and one with a mass of 197 Da (Figure 1) that could be separated by analytical HPLC on a Synergi 4μ Polar RP 80A column (250 mm × 4.6 mm) using a linear H_2O/CH_3OH gradient. The masses add up to 560 Da and indicate that the two products were formed by hydrolysis (18 Da) of the compound with mass 542 Da. The product with mass 363 Da had the same mass, retention time ($R_t = 6$ min), and UV/Vis spectrum as GMP. The absorption spectrum of the product with a mass of 197 Da ($R_t = 13$ min) was very similar to that shown in the inset of Figure 2 (data not shown).

The UV/Vis spectrum of the compound with mass 542 Da changed when the sample was incubated at pH 8.9 in the presence of phosphodiesterase: the shoulder at 300 nm almost completely disappeared and the spectrum became almost identical to that of GMP (not shown). This finding indicates that after hydrolysis of the compound with mass 542 Da to form GMP and the product with mass 197 Da, the absorbance of the latter species at 300 nm was much lower than when it was still covalently bound.

^1H NMR and ^{13}C NMR spectra of the compound with mass 542 Da containing nitrogen atoms with the natural isotopic distribution and labeled with ^{15}N were recorded in ^2H_2O and [D_6]DMSO to obtain more information on the structure of the inactivated cofactor, especially on the structure of the moiety with mass 197 Da. The 1D ^1H NMR spectrum obtained in ^2H_2O revealed the presence of 15 protons associated with the compound of mass 542 Da, 3 of which slowly exchanged with ^2H_2O at pH 7; in DMSO 22 protons associated with the compound of mass 542 Da were seen. The 1D ^{13}C NMR spectrum recorded with proton decoupling in ^2H_2O revealed the presence of 14 carbon atoms. Five additional carbon atoms were detected by $^{13}C,^1H$ HMBC (C,H-long range correlation) studies.[16] HSQC[17] ($^1J_{C,H}$ correlation), HMBC, and HNN[18] experiments performed on the ^{15}N-labeled compound in DMSO revealed the presence of all the nitrogen atoms (1–3, 7, 9, and a; Scheme 2). All the results together are consistent with the structure shown in Scheme 2, as explained in the following.

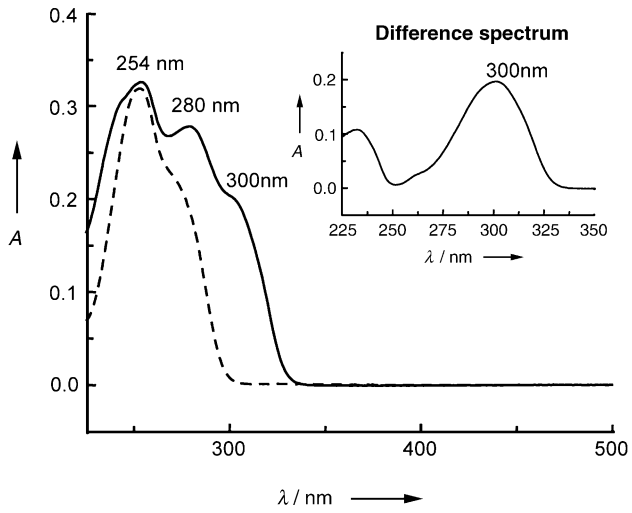


Figure 2. UV/Vis spectrum of the inactivated cofactor and of GMP. The inset shows the difference spectrum: ——: inactivated cofactor; -----: GMP. The concentrations of the inactivated cofactor and of GMP were 80 μM in 100 mM sodium phosphate at pH 7.0. The path length *d* was 0.3 cm. The spectra were recorded on a Specord UV/Vis S10 Diode Array (Zeiss, Jena). $\varepsilon_{254} = 14\ \text{mM}^{-1}\,\text{cm}^{-1}$; $\varepsilon_{280} = 12\ \text{mM}^{-1}\,\text{cm}^{-1}$; $\varepsilon_{300} = 9\ \text{mM}^{-1}\,\text{cm}^{-1}$. The cofactor concentration was determined from its phosphate content for the calculation of the extinction coefficients.

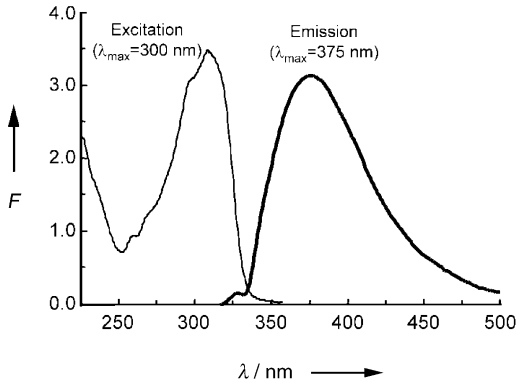
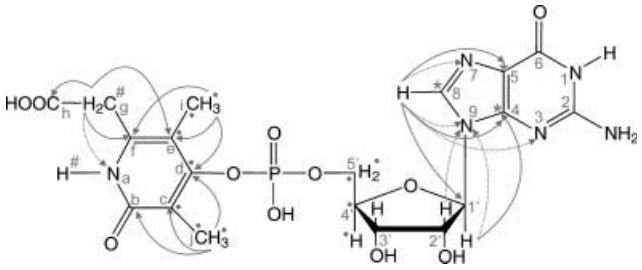


Figure 3. Fluorescence excitation and emission spectra of the inactivated cofactor. The concentration of the compound was 125 μM in 100 mM sodium phosphate at pH 7.0. The buffer was used as a reference. The spectra were recorded with a Cary Eclipse Spectrometer (Varian, Darmstadt).

The 11 hydrogen atoms in the structure that are exchangeable with protons of water are the protons at positions 1, 2, and 8 of the guanine ring, hydroxy groups at positions 2′ and 3′ of the ribose, the hydroxy group of the phosphate group, and the g, h, and a protons of the pyridone moiety (Scheme 2). Three of the 11 hydrogen atoms have been shown to exchange only slowly at pH 7 and room temperature; these include the hydrogen atom bound to C(8) of the guanine ring and the two

hydrogen atoms bound to C(g) in the pyridone moiety. The hydrogen atom at C(8) of guanosine is known to exchange slowly and the two hydrogen atoms at C(g) are expected to have similar properties as the two hydrogen atoms at C(2) of malonic acid. The hydrogen atom of the carboxy group C(h) is the only one not seen by NMR spectroscopy; this is because of fast exchange of the carbonic acid protons both in water and in DMSO. The hydrogen atom N(a) was visible in the spectrum recorded in DMSO and displayed a signal intensity of 85% compared to the reference hydrogen atom H(1′); this is probably because of the existence of tautomeric forms in DMSO. We observed three different signals for H(a) and H(g), whose intensities changed simultaneously over time. The most probable explanation of this result is the presence of



Scheme 2. Structure proposed for the inactivated cofactor. It is a (6-carboxymethyl-3,5-dimethyl-2-pyridone-4-yl)-(5′-guanosyl) phosphate. The arrows indicate heteronuclear connectivities measured in the HMBC spectra (in ^2H_2O or DMSO), full lines for ^{13}C–^1H connectivities, and dashed lines for the ^{15}N–^1H ones. Although a complete COSY spin system was observed for the ribofuranose ring, these connectivities are not shown. The stars indicate extremely broadened signals arising from the presence of iron, the hash indicates two protons H(g) and H(a) that show tautomeric exchanges, and the filled circles indicate the signals correlated to the phosphorus atom ($^{13}P^1H$ HMBC correlations or $^nJ_{P,C}$).

tautomeric forms that depend on the water content, which changed over time because of the unavoidable increase in the water content of DMSO samples that are only sealed with parafilm. Only one set of signals each was observed for H(a) and H(g), thus indicating a fast exchange between the previously slowly exchanging tautomeric or hydration forms.

Five of the nineteen carbon resonances in the compound with mass 542 Da (C(4), C(5), C(8), C(g), and C(h)) were very broad in a ^{13}C NMR spectrum recorded in ^2H_2O with proton decoupling during detection. The line-broadening of the signals corresponding to C(8) and C(g) can be explained by the fact that the hydrogen atoms at these carbon positions had exchanged with the ^2H_2O solvent and the ^{13}C-^2H coupling was not decoupled. Indeed, when the same sample was measured directly after dissolution in ^2H_2O the resonance for C(8) and for C(g) were as narrow as the other ^{13}C resonances. In ^1H_2O, where no exchange of hydrogen atoms with deuterium can occur, the signals corresponding to the C(8) and C(4) atoms were observed in the indirect frequency dimension of an HMBC experiment by a $^3J_{C,H}$ coupling with hydrogen atom H(1′). In a similar manner, we observed the resonance for the C(8) atom in an HSQC spectrum and the carbon atoms C(4) and C(5) by HMBC (Scheme 2 and Figure 4) in DMSO, where no exchange of protons for deuterium can occur.

The C(h) resonance was broadened in the direct-detection ^{13}C NMR experiment performed in ^2H_2O, probably because of the same effect discussed for the C(g) resonance above. The presence of this carboxy group was first indicated by the finding of a decarboxylation product in the mass spectrum. The resonance of C(h) was observed in an HMBC spectrum obtained in ^1H_2O or in DMSO through correlation with the hydrogen atoms at C(g). In the case of C(4) and C(5), the explanation for the extremely broadened resonances in the 1D ^{13}C NMR spectrum is less obvious since both carbon atoms are observable in the ^{13}C NMR spectrum of GMP. We found, however, that both signals were extremely broadened when trace amounts of Fe^{II} ions were added to a solution of GMP (1% Fe^{II} relative to GMP). Such low amounts of iron were probably present in our preparations of the compound with mass 542 Da since it was derived from the iron-containing Hmd cofactor. The signal corresponding to C(8) of GMP was also slightly broadened upon addition of trace amounts of Fe^{II} ions. Thus, the broadening of this signal in the compound with mass 542 Da (see above) is probably not only a consequence of exchange with the solvent.

All the signals of the pyridone moiety were assigned based on through-bond interactions (Scheme 2 and Figure 4). The ^{13}C NMR signals attributed to C(4′), C(5′), C(c), C(d), C(e), and C(i) were split through *J* couplings with ^{31}P, while for the $C(i)H_3$ and the $C(j)H_3$ protons a strong and a weak ^{31}P,^1H HMBC interaction was observed, respectively (Scheme 2 and Figure 4). The protons of C(g) showed HMBC correlations with N(a) after ^{15}N-labeling of the cofactor. The N(1) and N(2) atoms of the GMP moiety were assigned through HSQC cross peaks with their directly bound protons. In addition, HMBC correlations were observed from H(8) to N(7) and N(9), while HNN correlations were observed from H(8) to N(3) (Scheme 2). The β-D-ribofuranose was clearly assigned using $^3J_{H,H}$ COSY connectivities, the ^{13}C spectrum, and the NOESY spectrum (see the Supporting Information).

The NMR spectra of the compound with mass 542 Da, the NMR-derived connectivities, and the observation that the hydrolysis product of mass 197 Da has a composition of $C_9H_{11}NO_4$ and five exchangeable protons (see the Supporting Information) indicate that the chromophore bound to GMP through a phosphoester linkage is the pyridone derivative shown in scheme 2. A pyridone structure is also supported by the fluorescence spectrum of the inactivated cofactor (Figure 3). 2-Pyridone derivatives in the tautomeric keto form are known to exhibit room-temperature fluorescence with an excitation maximum near 300 nm and an emission maximum near 370 nm.[19] As far as we know, a similar structure has not been encountered in any biological system.

It currently remains unclear how the compound with mass 542 Da is involved in iron complexation in the active Hmd cofactor. A possible coordination to N(a) and the carboxy group to yield a six-membered ring can be envisaged from the structure of the pyridone moiety. However, since the active cofactor is stable in the presence of high concentrations of EDTA, there must be additional ligands stabilizing the bidentate iron complex, if it is present. Since the compound with mass 542 Da is formed from the active Hmd cofactor via

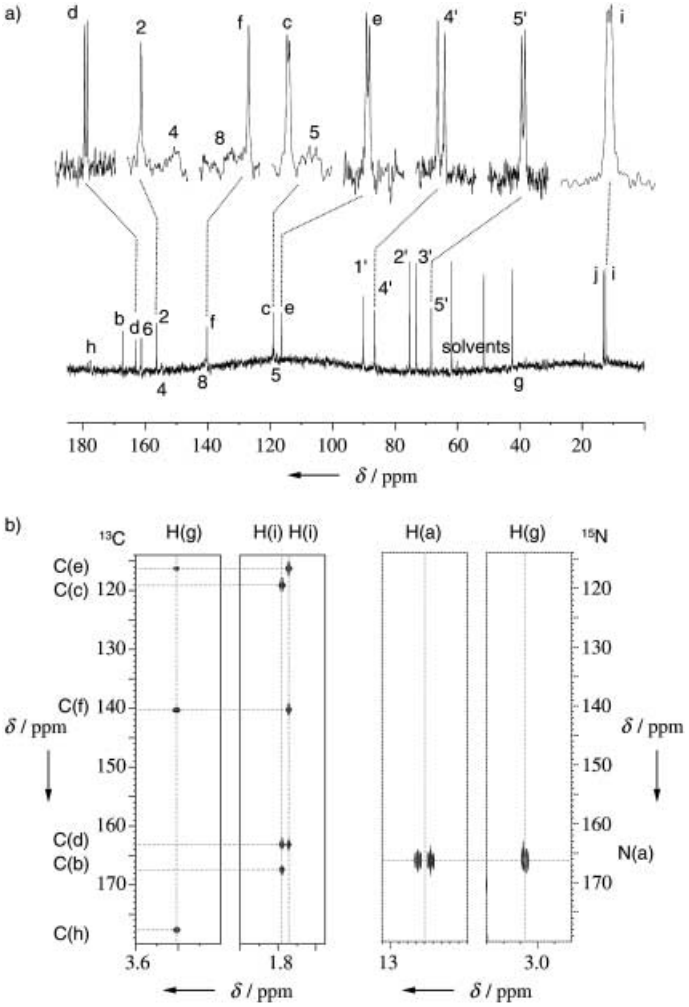


Figure 4. a) 1D ^{13}C decoupled spectrum of the cofactor in $^{2}H_2O$ at 300 K. The lower part shows the full spectrum and the upper part some particular signals obtained using different processing parameters to show the extremely broadened ones. The splittings for the 4′, 5′, c, d, e, and i signals result from $^{n}J_{C,P}$ couplings. The signals 4, 5, 8, g, and h are broadened because of the presence of iron (4, 5, and 8) and/or to chemical exchange (8, h, and g). b) Two dimensional HMBC correlation NMR spectrum in $^{2}H_2O$ at 300 K highlighting the connectivities within the pyridone ring. The two left panels (full lines) show the ^{1}H-^{13}C connectivites of (g) and the methyl groups (i) and (j). The right part (dashed lines) shows the ^{1}H–^{15}N connectivites of N(a).

intermediates it also has to be considered that the pyridone ring is formed only upon inactivation. We also do not want to exclude a complexation of iron to the pyridone moiety through π binding, such as in cyclopentadienyl–Fe^{II}–carbonyl complexes[20] or in butadiene–Fe^{0}–carbonyl complexes,[21,22] since there is evidence for the presence of two CO molecules bound to the iron center in the active Hmd cofactor. It is interesting to note that CO is also a ligand to the iron center in [NiFe]- and [FeFe]-hydrogenases.[2,6,23,24]

Experimental Section

Hmd (subunit molecular mass 38 kDa) was purified from *M. marburgensis* cells (5 × 100 g, wet mass) grown under nickel-limiting conditions and the active cofactor was extracted and purified according to the published procedure.[14] The active cofactor from the enzyme (600 mg, 16 μmol, 300 000 U) was obtained in approximately 25 % yield as determined from its activity after reconstitution with the apoenzyme. The cofactor dissolved in 10 mM ammonium carbonate at pH 9 (125 mL) and 10 mM 2-thioethanol (500 U per mL) was then inactivated at 0 °C by exposure to room light for 5 h. This was done in five 100-mL glass vials each containing 25 mL of solution, with 100 % N_2 as the gas phase. Inactivation was continued until activity of less than 1 U mL^{-1} was detected. Once inactivated, each 25-mL sample was passed through a 0.22-μm filter and applied to a 6 mL Resource Q column equilibrated with H_2O. The inactivated cofactor was eluted by washing the column with H_2O (40 mL) and then with 50 mM NaCl (40 mL). Elution was followed through the absorbance at 280 nm. The fractions containing the inactive cofactor from five separate Resource Q columns were combined (3.4 μmol based on the absorbance at 300 nm, 79 % yield) and divided into four fractions, each containing 12.5 ml, and again passed through a 0.22-μm filter. The inactive cofactor was then applied to a Synergi 4μ Polar RP 80A column (250 mm × 4.6 mm, Phenomenex, Aschaffenburg) equilibrated with H_2O (pH 4, HCl). The inactive cofactor eluted with 10 % methanol, and the combined fractions yielded 3 μmol of product (87 % yield). The purity was checked by HPLC using the same column and a linear H_2O/CH_3OH gradient. The combined, purified inactive cofactor was lyophilized and dissolved in H_2O (0.55 ml), almost all of which was required for analysis by NMR spectroscopy.

Spectra were recorded with the following parameters: ^{31}P,^{1}H HMBC: number of scans (NS) = 16, defocusing delay: 50 ms, 8192 × 1024 points in t_2,t_1;. ^{15}N,^{1}H HMQC: NS = 16, 4096 × 600 points in t_2,t_1; ^{15}N,^{1}H HMBC: NS = 32, defocusing delay: 30 ms, 4000 × 600 points in t_2,t_1; HNN experiment: NS = 48, defocusing and refocusing delay for the N,N coupling: 40 ms and 120 ms, 4096 × 600 points in t_2,t_1; ^{13}C,^{1}H HMBC: NS = 64, defocusing delay: 60 ms, 8192 × 1024 points in t_2,t_1; ^{13}C,^{1}H HSQC: NS = 32, 4096 × 512 points in t_2,t_1. All spectra were recorded at 600 MHz except for the spectra involving ^{31}P that were recorded on a 700 MHz spectrometer at 283.42 MHz. The ^{13}C spectra was recorded on a 500 MHz spectrometer equipped with a ^{13}C-detecting cryoprobe at 125.721 MHz. δ values for ^{13}C ($^{2}H_2O$), ^{1}H ($^{2}H_2O$, DMSO), ^{15}N (DMSO), and ^{31}P ($^{2}H_2O$) in ppm: N(1): 148, H(1): –, 10.73, C(2): 156.3, N(2): 73.6, H(2): –, 6.68, N(3): 173.2, C(4): 154.5, C(5): 118.1, C(6): 161.3, N(7): 249.2, C(8): 140.7, H(8): 7.91, 7.98, N(9): 169.8. C(1′): 90.2, H(1′): 5.77, 5.70, C(2′): 75.3, H(2′): 4.86, 4.55, OH(2′) and OH(3′) (not assigned) [shifts in DMSO: 5.47, 5.56], C(3′): 73.1, H(3′): 4.35, 4.16, C(4′): 86.4, H(4′): 4.21, 3.97, C(5′): 68.3, H(5′/5″): 4.14/4.12, 4.03/3.89, P: –6.18, N(a): 166, H(a): –, 12.60, C(b): 167.3, C(c): 119.1, C(d): 163.1, C(e): 116.2, C(f): 140.2, C(g): 40.2, H(g): 3.44, 3.08, C(h): 177.6, H(COOH) not seen, C(i): 12.4, H(i): 1.78, 1.93; C(j): 13.2, H(j): 1.82, 1.93. Additionally, resonances from contaminants, 2-thioethanol and methanol, were observed.

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