-Supporting Information-

EPR study of substrate binding to Mn(II) in hydroxynitrile lyase from *Granulicella tundricola*

Femke Vertregt,† Guzman Torrelo,† Sarah Trunk,§ Helmar Wiltsche,‡ Wilfred R. Hagen,† Ulf Hanefeld† and Kerstin Steiner§**

†Biokatalyse, Afdeling Biotechnologie, Technische Universiteit Delft, van der Maasweg 9, 2629HZ Delft, The Netherlands.

‡Institute of Analytical Chemistry and Food Chemistry, Stremayrgasse 9/III, TU Graz, 8010 Graz, Austria.

§Austrian Centre of Industrial Biotechnology GmbH, Petersgasse 14/4, 8010 Graz, Austria.

Corresponding authors: E-mail: u.hanefeld@tudelft.nl; E-mail: kerstin.steiner@acib.at.

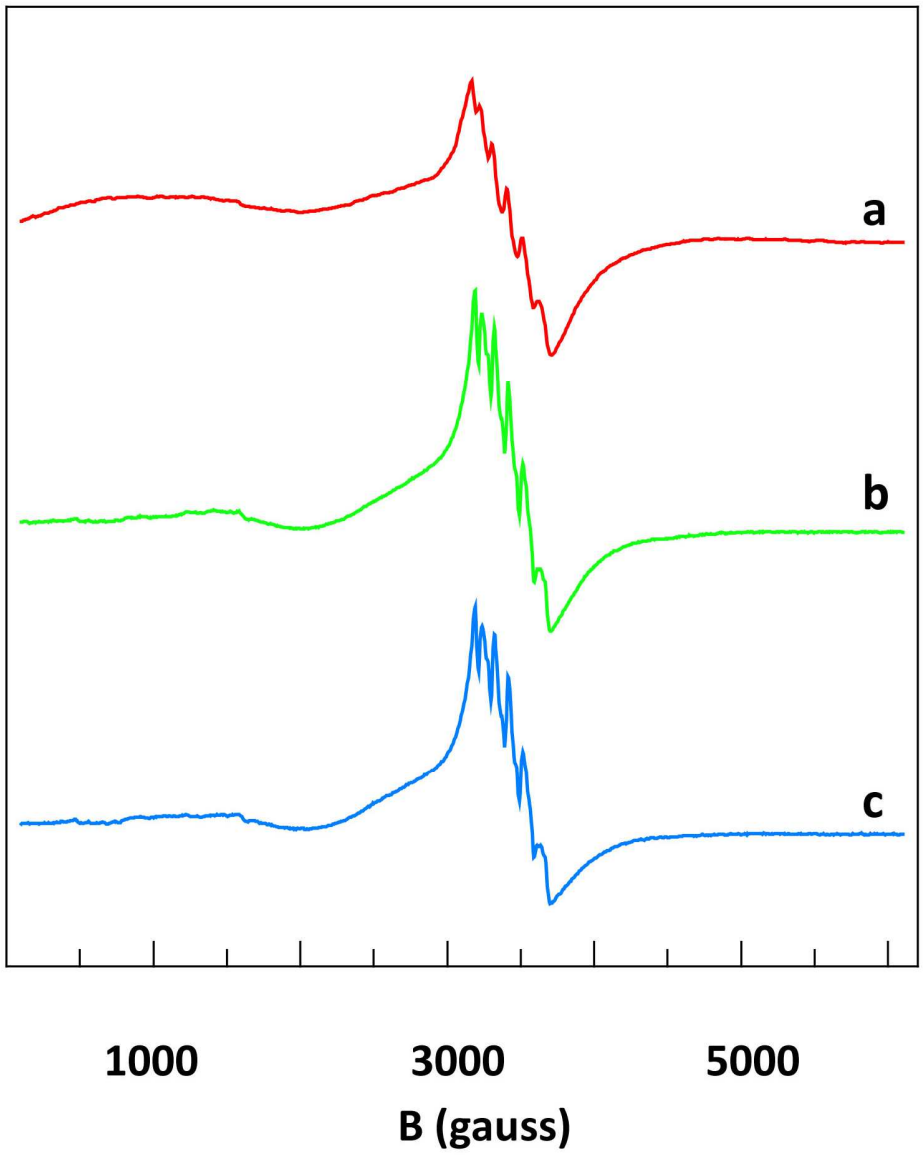

Figure S1. EPR spectra of single variant *Gt*HNL-H96A. Trace a is 0.1 mM $MnCl_2$ in 80 mM sodium oxalate/MES buffer, pH 5.5; trace b is circa 22 mg/mL, i.e. 1.5 mM monomer, variant enzyme in 20 mM Tris-HCl buffer, pH 7.5, and 200 mM NaCl; trace c the enzyme of trace b with 1 volume % benzaldehyde, 78 mM final concentration. EPR conditions were as in Fig. 2.

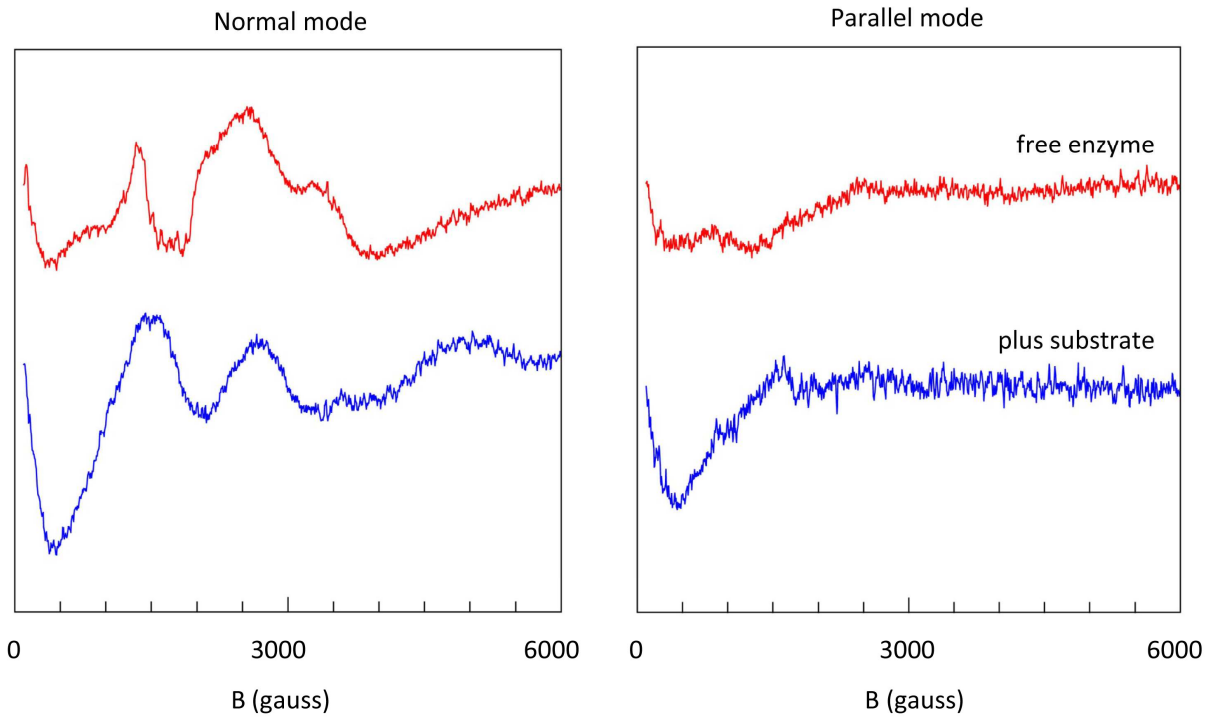

Figure S2. Dual-mode EPR spectra of wild-type *Gt*HNL in the absence and presence of benzaldehyde. The enzyme was circa 5 mg/ml, i.e. 0.33 mM monomer, in 20 mM Tris-HCl buffer, pH 7.5 and 200 mM NaCl. The lower traces are in the presence of 1 volume % benzaldehyde, 78 mM final concentration. In the left panel the microwave is perpendicular to the magnetic field; in the right panel the microwave is parallel to the magnetic field. EPR conditions: microwave frequency, 9.61 (normal) and 9.25 (parallel) GHz; microwave power, 80 mW; modulation frequency, 100 kHz; modulation amplitude, 8 gauss; temperature, 14 K.

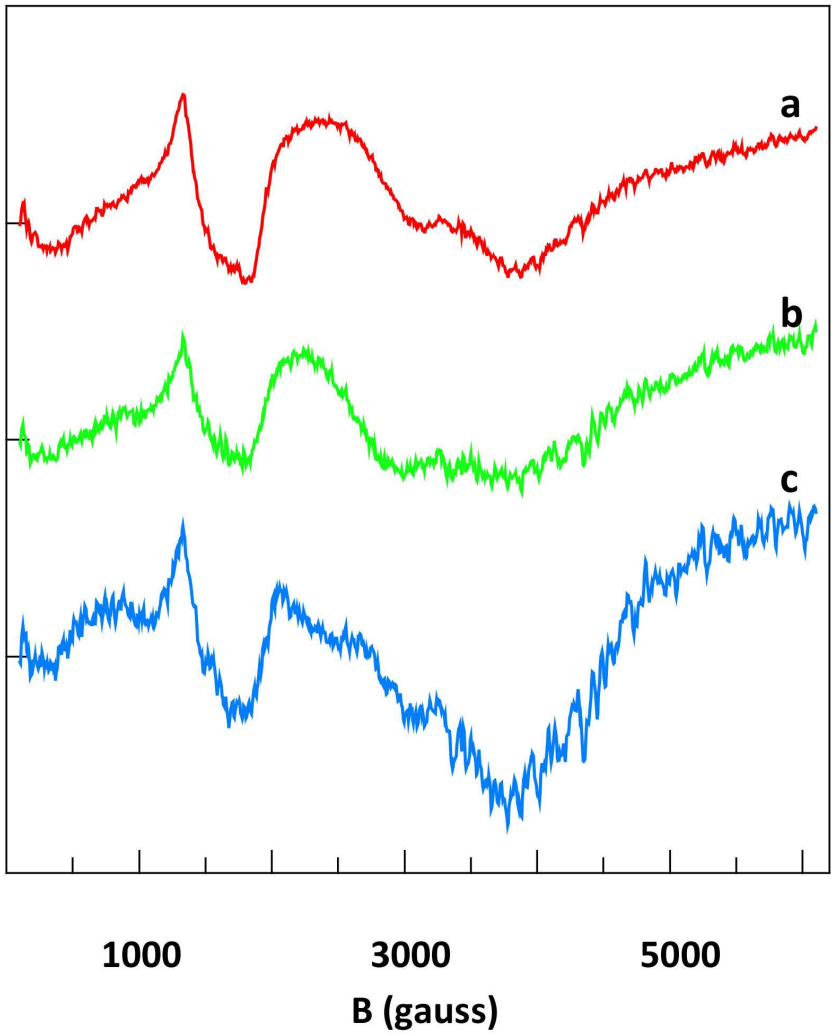

Figure S3. EPR spectra of triple variant *Gt*HNL-A40H/V42T/Q110H. Trace a is circa 5 mg/ml, i.e. 0.33 mM monomer, variant enzyme in 20 mM Tris-HCl, pH 7.5, and 200 mM NaCl; trace b is with 1 volume % benzaldehyde, 78 mM final concentration; trace c is after 1:1 dilution and 5 minutes incubation with 36 mM mandelonitrile in 160 mM Na-oxalate/MES buffer, pH 5.5. EPR conditions were as in Fig 2.

1. Production of *Gt*HNL-WT and variants thereof

*Gt*HNL-WT and the variants *Gt*HNL-H96A and *Gt*HNL-A40H/V42T/Q110H were expressed and purified, and their HNL activity was measured in a plate reader assay as described before.[1] The standard protocol for metal removal and exchange was established by Hajnal et al.[2] and metal analysis using ICP-OES was performed as described in the same paper. Metal analysis using a chemiluminescent and colorimetric assay applying luminol and PAR (4-(2-pyridylazo)resorcinol) in a plate reader was performed according to Höblom.[3]

2. Optimization of apo protein production

In the standard protocol for metal removal of *Gt*HNL-WT purified protein was first dialyzed against 2 L of 10 mM 2,6-pyridinedicarboxylic acid monohydrate (PDCA) in 100 mM sodium acetate and 150 mM NaCl, pH 5.5, for 26 h with one buffer change and subsequently dialyzed against 4 L of 20 mM Tris/HCl, pH 7.5, for 20 h with one buffer change.[2]

For the removal of metal ions from *Gt*HNL-A40H/V42T/Q110H, the protocol had to be adapted due to incomplete metal removal with the standard protocol. PDCA concentration was increased to 20 mM and the time of dialysis was extended to 50 h with one buffer change. Results are given in Figure 4.

3. Enzymes and chemicals used for EPR sample preparation

Four enzyme solutions prepared as described above: apo-*Gt*HNL, i.e. purified apo-*Gt*HNL i.e. without Mn^{2+} (~5 mg/mL in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl); purified *Gt*HNL with Mn^{2+} (~4.9 mg/mL with 60 % Mn^{2+} loading in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl); purified *Gt*HNL-H96A with Mn^{2+}(~ 22 mg/mL with 46 % Mn^{2+} loading in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl) and purified *Gt*HNL-A40H/V42T/Q110H with Mn^{2+} (~5.05 mg/mL with 57 % Mn^{2+} loading in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl).

A 1.5-2 M solution of HCN in MTBE was prepared as described earlier.[4] Benzaldehyde was freshly distilled and stored under nitrogen.

Rac-Mandelonitrile was freshly synthesized via spontaneous chemical reaction of HCN (1.7 M in MTBE, 2 mL) and benzaldehyde (distilled, 150 µL) in potassium phosphate buffer (5 mM, pH 6.5, 1.5 mL) by stirring overnight at room temperature. Extraction with MTBE, drying over $MgSO_4$ and evaporation of the solvent yielded pure *rac*-mandelonitrile that can be used up to 3 days after synthesis, when stored at -20 ºC. Alternatively commercial mandelonitrile (Acros or Aldrich) was purified by column chromatography.[4] MilliQ water was used for preparation of solutions or dilutions. Buffers were prepared as described earlier.[1,2,4]

Reconstitution and desalting of apo-*Gt*HNL: 7 µL of a 100 mM $MnCl_2$ solution (7.10^{-4} mmol or five times excess) was added to 500 µL of apo-*Gt*HNL (~5 mg/mL, $1.4.10^{-4}$ mmol) and shaken at room temperature (300 rpm, 4h). Absorbance at 280 nm (Shimadzu UV-Vis spectrometer, 50 µL quartz cuvette, path length 1 cm) was used to determine protein concentration before and after desalting. A PD minitrap G-25 column (GE Healthcare) was used for desalting against Tris-HCl buffer 20 mM pH 7.5 with 200 mM NaCl according to the

manufacturer's gravity protocol. From eluate fractions of 100 µL, the five with the highest enzyme concentrations were combined (~ 500 µL; final concentration 4.8 mg/mL) and used for EPR measurements.

Sodium dithionite treatment was performed with a 20 mM stock solution in 100mM Tris/HCl buffer pH 8. This was added to 0.33 mM *Gt*HNL (monomer) solution (20 mM Tris/HCl pH 7.5, with 200 mM NaCl) under strictly anaerobic conditions with Ar5.0 to a final concentration of 0.8 mM, i.e. 1.6 mM concentration reducing equivalents. This was incubated for > 5 min.

4. EPR spectroscopy

EPR spectra at 9.6 GHz were recorded on a Bruker ECS106 EPR spectrometer and recorded from 100 to 6100 à 6400 Gauss in 336 s with the time constant set to 328 ms. Further EPR conditions: microwave power 80 mW, modulation frequency 100 kHz, modulation amplitude 8 gauss. Sample composition see table S1. Samples were in principle carefully frozen in liquid nitrogen directly after preparation, unless stated otherwise. During analysis, the samples were cooled within the spectrometer to 13.5 - 14.5 K with liquid Helium.

Table S1. EPR sample composition

Spectrum	Composition
Fig. 2a	apo-*Gt*HNL + Mn^{2+}, desalted, ~4.8 mg/mL, i.e. 0.32 mM monomer, enzyme in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl; sample volume 0.2 mL
Fig. 2b	*Gt*HNL ~4.9 mg/mL with 60 % Mn^{2+} loading in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl; sample volume 0.25 mL
Fig. 2c	As Fig. 2b but with 1 µl 1.7 M HCN in methyl *tert*-butyl ether to a final concentration of 8.5 mM; sample volume 0.20 mL
Fig. 2d	As Fig. 2b but with 2 µl benzaldehyde to a final concentration of 78 mM; sample volume 0.25 mL

Fig. 2e	As Fig. 2b but with 0.5 µl mandelonitrile to a final concentration of 20 mM; sample volume 0.20 mL
Fig. S1a	0.10 mM $MnCl_2$ in Na-oxalate/MES buffer 80 mM pH 5.5; sample volume 200 µL
Fig. S1b	*Gt*HNL-H96A ~ 22 mg/mL, i.e. 1.5 mM monomer, with 46 % Mn^{2+} loading in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl; sample volume 0.2 mL
Fig. S1c	As Fig. S1b but with 2 µL benzaldehyde to a final concentration of 78 mM; sample volume 0.2 mL
Fig. S2 upper traces	As Fig. 2b
Fig. S2 lower traces	As Fig. 2d
Fig. S3a	*Gt*HNL-A40H/V42T/Q110H with Mn^{2+} ~5.05 mg/mL with 57 % Mn^{2+} loading in 20 mM Tris/HCl buffer pH 7.5 with 200 mM NaCl; sample volume 0.25 mL
Fig. S3b	As Fig. S3a but with 2 µl benzaldehyde; sample volume 0.25 mL
Fig. S3c	As Fig. S3a but diluted 1:1 with mandelonitrile solution of 36 mM in sodium oxalate/MES buffer 160 mM pH 5.5, incubate 5 min; sample volume 0.2 mL

References

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2) Hajnal, I.; Łyskowski, A.; Hanefeld, U.; Gruber, K.; Schwab H.; Steiner, K.; *FEBS J*., **2013**, *280*, 5815–5828.

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4) Torrelo, G.; van Midden, N.; Stloukal R.; Hanefeld, U.; *ChemCatChem*, **2014**, *6*, 1096-1102.