Supporting Information

Reaction mechanism of the metallohydrolase CpsB from *Streptococcus pneumoniae*, a promising target for novel antimicrobial agents

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Materials and Methods

Escherichia coli LEMO21(DE3) host cells were purchased from BioLabs New England. All chromatographic devices (FPLC system, chromatographic protein standards and suitable resins) were purchased from GE Healthcare, all chemicals, buffers and substrates, unless mentioned otherwise, were purchased from Sigma Chemical Co.

The CpsB-encoding expression system (SP0347; TIGR4), where the CpsB gene was cloned under the control of a pBAD promoter (pWQ553), was transferred by heat-shock into *E. coli* Lemo21(DE3) for expression as previously described.[1] The cells were inoculated in LB medium containing ampicillin (50 μg/mL) and chloramphenicol (30 μg/mL). The transformed cell culture was incubated at 37 °C until an OD of ~0.4 was reached. Subsequently, induction of recombinant protein expression was achieved by adding L-arabinose (0.1 % m/v) and the incubation was continued at 30 °C under constant shaking (200 rpm) for another 24 h. The cells were harvested by centrifugation and lysed using a French press (1000 psi). Since recombinant CpsB contains an N-terminal hexahistidine tag the soluble fraction was applied to a Ni^{2+} affinity column (Ni^{2+}-IMAC resin), equilibrated with 50 mM Tris.HCl buffer, pH 8.5, containing 5 mM of β-mercaptoethanol, 150 mM sodium chloride and 20 mM imidazole. The protein was eluted with an isocratic gradient using 50 mM Tris.HCl buffer, pH 8.5, containing 5 mM mercaptoethanol, 150 mM of sodium chloride, 200 mM of imidazole and 10% glycerol. The CpsB concentration was measured at 280 nm using ε_{280} = 20,400 $M^{-1}.cm^{-1}$ (monomeric unit). Purification yielded approximately 30 mg of protein per litre of medium.

Apoenzyme preparation and enzyme reconstitution. Apoenzyme was obtained by incubating approximately 3 mg of purified CpsB in a 3 mL solution containing 10 mM of

EDTA in 20 mM HEPES buffer, pH 7.0, at 4 °C.[2] After 24 hours the protein sample was separated from the chelating solution using an Econo-Pac 10DG gel filtration column, equilibrated with the desired buffer that was previously treated with Chelex® resin (Bio Rad). Atomic absorption spectroscopy (AAS) confirmed the absence of metal ions in the protein solutions. Subsequently, CpsB was reconstituted by the addition of a ten-fold excess of the desired metal ion (*i.e.* $MnCl_2$ or $CoCl_2$) and incubated for at least 24 hours at 4 °C. Excess metal ions were removed using a Econo-Pac 10DG gel filtration column. The number of bound metal ions per enzyme monomer was determined by AAS and was close to 2 for both Mn^{2+} and Co^{2+} (2.3 And 2.4, respectively).

Enzymatic assays. All kinetic measurements were carried out with the fully reconstituted Mn^{2+} form of CpsB. In a standard assay reactions were monitored for 60 s at 25 °C with a Cary 50 Bio Varian UV-Vis spectrophotometer. Buffers were treated prior to use with Chelex resin. The pH profiles of the catalytic parameters (k_{cat}, k_{cat}/K_m) were determined using pNPP, bpNPP and nitrocefin as the substrates. The pH for the assays ranged from 5.5 to 11 using a 100 mM acetate, 100 mM MES, 100 mM HEPES, 100 mM CHES, 100 mM CAPS multi component buffer system. Initial velocities with pNPP or bpNPP were measured by monitoring the release of p-nitrophenol at pH independent isosbestic point at 347 nm (ε = 5,176 $M^{-1}cm^{-1}$).[2] Nitrocefin data was measured at 490 nm (1,520 $M^{-1}cm^{-1}$).[3-5]

All data were analysed by non-linear regression using GraphPad Prism 6 Software, Inc. Rate *vs* substrate concentration profiles was fitted to the Michaelis-Menten equation (equation 1).[6]

$$v = \frac{V_{max}.S}{K_m + S} \ (equation\ 1)$$

The *pH* dependence of the catalytic parameters was fitted using equation 2, derived for a system with one relevant protonation equilibrium.[6] Here, H is the proton concentration, *K* represents the acid dissociation constant for either the enzyme-substrate complex (ES) or the free enzyme (E), *c* is the *pH* independent values of *y*, and *y* is the kinetic parameter of interest, *i.e.* k_{cat} or k_{cat}/K_m.[6]

$$\log V_{max} = \log\left(\frac{c}{1+\frac{H}{K}}\right) (equation\ 2)$$

Metal ion binding studies using isothermal titration calorimetry

All isothermal titration calorimetry (ITC) data were collected at 25 °C using an ITC_{200} instrument from MicroCal. The unit employed to report the data was the calorie (1 cal = 4.18 J). The concentration of the metal ion solution was verified against standardized EDTA solutions and measured with AAS. At least three sets of data were collected and the average calculated. The ITC data were fitted using MicroCal® Origin 7.0 software. The software uses a nonlinear algorithm with minimization of the χ^2 values fitting the heat flow released by each injection to an equation corresponding to an equilibrium-binding model. The heat of dilution was subtracted from the integrated data, and the data were fitted using two independent binding sites.[7, 8]

Stopped-Flow Measurements. The experiments were performed using an Applied Photophysics SX-18 spectrometer coupled with a photo-diode array spectrophotometer. Data from at least five reproducible experiments were collected, averaged, and corrected for the instrument dead time (1.5 ms). All stopped-flow experiments were carried out under single turnover conditions in 20 mM HEPES at pH 7.0 and 25 °C. In a typical experiment, a solution of CpsB (40 μM) was rapidly mixed with a nitrocefin solution (25 μM). Absorbance

changes were monitored with photodiode array detector (5s scan intervals) over the wavelength range of 310-725 nm or an absorbance photomultiplier at 390, 490, and 665 nm.[3, 4, 8] The catalytic mechanism of CpsB, according to Scheme 1, was simulated using the program KINSIM.[3, 8-10] The data were then fitted using Reactlab software.[9, 10]

Spectroscopic characterization of CpsB. Protein samples were prepared by adding three equivalents of Co^{2+} to the apoenzyme. The sample was stirred overnight at 4 °C. The excess of Co^{2+} was removed using a desalting column, pre-equilibrated in 50 mM Tris.HCl buffer, pH 8.0; the protein sample was then diluted to a 60%/40% (v/v) mixture of glycerol/buffer and transferred to a 0.62 cm path length nickel-plated copper sample cell with quartz windows (final protein concentration: 0.7 mM). The MCD system used has a JASCO J815 spectropolarimeter and an Oxford Instruments SM4000 cryostat/magnet. Spectra were collected at 7.0 T and 1.3 K. VTVH MCD data were collected at increments of 0.5 T from 0 to 7.0 T and at temperatures of 1.4, 3, 6, and 12 K. The spectral data were converted to wavenumbers and fitted to a minimum number of Gaussian peaks to achieve a composite spectrum using the GRAMS AI software package.[11, 12] The fitting of the VTVH MCD data was carried out using the Fortran software VTVH 2.1.1.[12] The fits were testes for robustness once a complete set of parameters had been obtained. To do this, the initial parameters were set to the best fit parameters and then all allowed to float. Subsequently, one key parameter such as J, D, M_{xy}, M_{xz}, or M_{yz} was chosen and its initial value set differently and fit process repeated. The rhombic zero-field splitting parameter E was shown not to have an effect on the VTVH MCD fits so E/D was set to zero in all fits (general convention $0 \leq E/D \leq 1/3$).

EPR measurements were carried out with the same sample used for MCD experiments. Low temperature X-Band cwEPR spectra were recorded on a Bruker EMX EPR spectrometer equipped with an Oxford Instruments ESR900 helium flow cryostat.

Spectra were recorded at 9.64 GHz ($B_0 \perp B_1$) and 9.38 GHz ($B_0 \,||\, B_1$) using an ER4116DM dual-mode cavity, with 10 G (1 mT) magnetic field modulation at 100 kHz. Spin Hamiltonian parameters were estimated from computer simulations carried out using XSophe (Bruker Biospin), assuming $H_0 = \beta B_0 g\hat{S}/\hbar + \hat{S}D\hat{S}$, where $S = 3/2$, $|D| >> |\beta gBS/\hbar|$, and where $D > 0$ implies the $M_S = \pm 1/2$ Kramers doublet lies lowest and all observed EPR transitions are from this doublet, and $D < 0$ implies the $M_S = \pm 3/2$ Kramers doublet lie lowest and all observed EPR transitions are from this doublet.

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