δ-Amino group hydroxylation of L-ornithine during coelichelin biosynthesis

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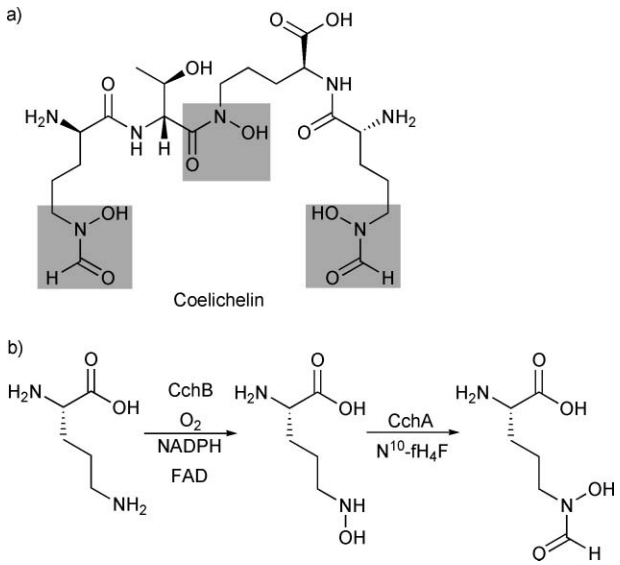
The nonribosomally produced hydroxamate siderophore coelichelin from *Streptomyces coelicolor* contains the nonproteinogenic amino acids N^5-hydroxyornithine and N^5-hydroxyformylornithine that are important for iron assembly. The hydroxylation of the δ-amino group of L-ornithine is catalyzed by the flavin-dependent monooxygenase CchB. During the redox reaction nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen are consumed and flavin adenine dinucleotide (FAD) is needed as a cofactor. During this work the monooxygenase was biochemically characterized and it could be shown that the hydroxylation of L-ornithine is most likely the first step in the biosynthesis of the siderophore coelichelin.

Introduction

For most organisms iron is an essential nutrient, which is mostly available only as ferric iron Fe(III). This forms ferric oxide hydrate complexes in the presence of water and oxygen leading to an extremely low concentration of free iron in the environment. Microorganisms overcome this restriction by producing small-molecule compounds with high Fe(III) affinity termed siderophores responsible for the iron acquisition. Siderophores are synthesized intracellularly, then secreted to chelate Fe(III) with catecholate, carboxylate, or hydroxamate groups and this iron is delivered into the cell *via* membrane transporters.[1]

The tris-hydroxamate siderophore coelichelin from *Streptomyces coelicolor* is a tetrapeptide, which is thought to be assembled by a trimodular nonribosomal peptide synthetase (NRPS) by an iterative mechanism. By sequence comparison the specificity of the three adenylation domains within the NRPS was predicted. The most likely activated amino acids are: N^5-hydroxyformylornithine, L-threonine, and N^5-hydroxyornithine.[2] Since the hydroxylated and formylated form of ornithine is present twice in the structure of coelichelin (Scheme 1), it is proposed that the A-domain of module 1 works iteratively and activates the amino acid twice during the assembly of the tetrapeptide. It is thought that only one C-domain, either of module 2 or module 3, catalyzes the last condensation reaction during the assembly and the other domains are skipped during this iteration.[3] Such a module-skipping process during nonribosomal peptide synthesis has also been proposed for the assembly of myxochromide S from *Stigmatella aurantiaca.*[4] To be able to investigate this uncommon mechanism of nonribosomal peptide assembly it is necessary to examine the biosynthesis of the precursor molecules N^5-hydroxyformylornithine and N^5-hydroxyornithine. Besides this, the hydroxamate groups at the ornithine side chains represent the iron binding groups within this class of siderophores and therefore it is also important to clarify the assembly of these building blocks.

Within the biosynthetic gene cluster of coelichelin two tailoring enzymes were identified, the putative L-ornithine N^5-hydroxylase (CchB) and the L-N^5-hydroxyornithine-formyltransferase (CchA), which are proposed to be responsible for the modification reactions at the ornithine side chains (Scheme 1).[2,3] The first enzymatic step in the generation of N^5-hydroxyornithine and N^5-hydroxyformylornithine, the chemically demanding hydroxylation of the δ-amino group of L-ornithine, is thought to be catalyzed by the ornithine N^5-hydroxylase CchB. Sequence alignments reveal that this monooxygenase is likely to require the cosubstrate nicotinamide adenine dinucleotide phosphate (NADPH), the cofactor flavin adenine dinucleotide (FAD), and molecular oxygen for its catalytic activity.[5] During the catalytic cycle of flavin-dependent monooxygenases, NADPH reduces the flavin, and the reduced flavin forms a putative flavin-peroxo species with molecular oxygen that oxidizes the substrate.[6] These monooxygenases are called external flavin-dependent monooxygenases because they use reduced coenzymes to provide the FAD with two electrons.[7]



Scheme 1 Structure of coelichelin and synthesis of precursor molecules. (a) The branched tetrapeptide of coelichelin with the hydroxamate groups involved in iron binding highlighted in grey. (b) Synthesis of N^5-hydroxyornithine by CchB and the following formylation reaction catalyzed by CchA in the presence of the cosubstrate N^{10}-formyltetrahydrofolate.

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The ornithine N^5-hydroxylase PvdA involved in the biosynthesis of the siderophore pyoverdine,[8] the ornithine N^5-hydroxylase PsbA used in pseudobactin biosynthesis,[9] and the lysine N^6-hydroxylase IucD required for production of the aerobactin siderophore[10] show sequence identities of 39%, 38%, and 34% to CchB from *Streptomyces coelicolor*. It has been proposed that the hydroxylation reaction is the first step in the biosynthesis of the corresponding siderophore. In contrast to this, it has been shown that the lysine residues of the nonribosomally produced siderophore mycobactin are hydroxylated at the N^6-position during the last step of the synthesis.[1] Besides the flavin-dependent monooxygenases involved in the siderophore biosynthesis, the most thoroughly studied hydroxylase of this class of enzymes is the *p*-hydroxybenzoate hydroxylase (PHBH) involved for example in the biodegradation of lignin from wood.[11] Other flavoprotein monooxygenases are involved in cholesterol biosynthesis (human squalene monooxygenase), or in ubiquinone biosynthesis (ubiB).[11] The investigation of flavin-dependent monooxygenases is very revealing because of the versatility and the broad range of possible applications of this group of enzymes.

Results

Production and purification of CchB

The enzyme was overproduced as an N-terminal hexahistidine tag fusion protein in *E. coli* BL21(DE3) cells and purified by Ni-NTA affinity chromatography as soluble protein with >95% purity as displayed by SDS-PAGE analysis (Fig. 1(a)) and with yields of 7–8 mg per litre of bacterial culture.

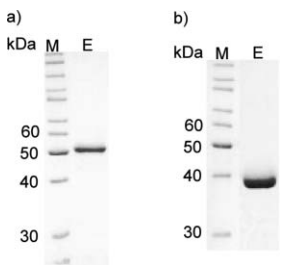

Fig. 1 SDS-PAGE analysis of Ni-NTA affinity chromatography purifications. Abbreviations: M, protein marker (Fermentas, *PageRuler*); E, elution fraction. (a) Monooxygenase CchB; M.W. = 51.3 kDa. (b) Formyltransferase CchA; M.W. = 37.2 kDa.

Activity and pH dependence of CchB

To examine the recombinant protein, the activity of the hydroxylase was determined in a typical NADPH oxidation assay.[12] The enzyme was incubated with NADPH, FAD, and L-ornithine and the reaction was monitored by measuring the decrease in NADPH absorption at 340 nm. Additionally, the reaction mixture was analyzed by HPLC-MS by scanning for the masses of L-ornithine (m/z 133.09 $[M + H]^+$) and N^5-hydroxyornithine (m/z 149.08 $[M + H]^+$). The HPLC-MS chromatogram (Fig. 2) shows quantitative conversion of L-ornithine to N^5-hydroxyornithine by CchB in 12 h at 25 °C. In a control reaction without enzyme, only the substrate could be observed. Chemically synthesized N^5-hydroxyornithine[13] was also analyzed by HPLC-MS as a standard and this compound

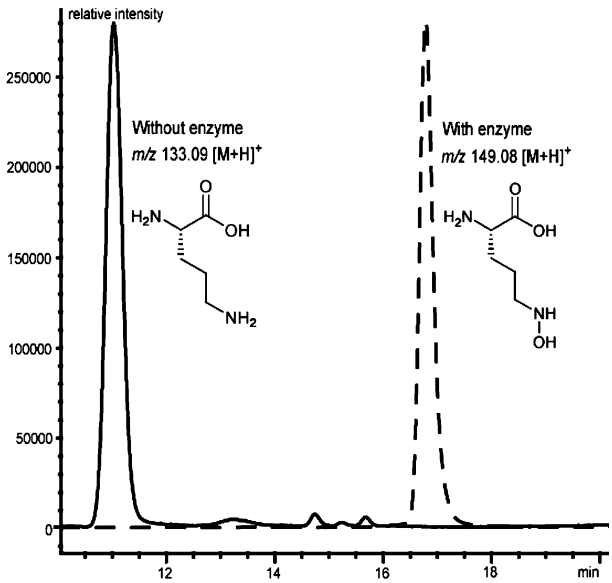

Fig. 2 HPLC-MS analysis of the activity assay. The chromatogram shows the assay without enzyme as a negative control (continuous trace) and the assay with CchB (dashed trace). The analysis reveals that the enzyme quantitatively converts L-ornithine to N^5-hydroxyornithine in 12 h at 25 °C.

coeluted with the product formed during the enzymatic reaction at a retention time of $t_R = 16.7$ min (data not shown).

Concerning the pH optimum for the catalytic activity of CchB, the initial velocities for NADPH oxidation by the hydroxylase were measured at 340 nm in a pH range of 6.0–9.5 in 100 mM TRIS buffer (Fig. 3). The pH range for maximal turnover was found to be 8.0–9.0. Other buffer systems such as 50 mM HEPES buffer or 100 mM phosphate buffer showed lower turnover rates (data not shown) and therefore all further assays were carried out in the 100 mM TRIS buffer pH 8.0.

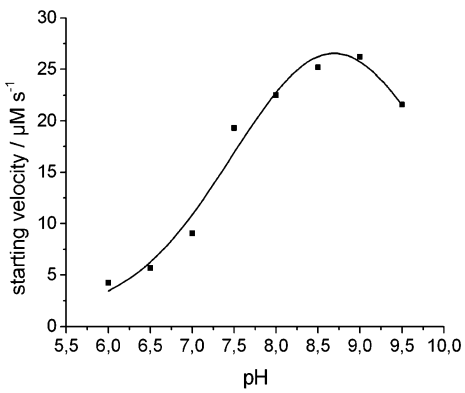

Fig. 3 pH dependence of the activity of CchB. The starting velocity, which was determined spectrophotometrically at 340 nm, is plotted against the pH values of the 100 mM TRIS buffer.

Evaluation of the substrate specificity

Additionally, the substrate specificity of CchB was evaluated by incubating it for 5 h at 25 °C with different amino acids and the unmodified coelichelin tetrapeptide (D-Orn-D-Thr-L-Orn-D-Orn) prepared by Fmoc-based solid phase peptide synthesis. D-Thr has been incorporated into the peptide backbone instead of

Table 1 Determination of the substrate specificity of CchB

Substrate	m/z [M + H]$^+$ substrate	m/z [M + H]$^+$ hydroxylated product	m/z [M + H]$^+$ observed[a]	hydroxylation
L-Ornithine	133.1	149.1	149.1	Yes
N^5-Formylornithine	161.1	177.1	161.1	No
D-Ornithine	133.1	149.1	133.0	No
L-Lysine	147.1	163.1	147.2	No
L-Glutamate	148.0	164.0	148.1	No
L-Glutamine	147.1	163.1	147.1	No
L-Valine	118.1	134.1	118.1	No
SNAC-activated tetrapeptide (D-Orn-D-Thr-L-Orn-D-Orn)	563.3	611.3	563.3	No

[a] Masses observed by HPLC-MS after 5 h incubation of CchB with NADPH, FAD and the corresponding substrate.

D-*allo*-Thr and the molecule has been activated at the C-terminus as a thioester with the leaving group *N*-acetylcysteamine to mimic the ppan-cofactor of a PCP-domain (Table 1). All assays were analyzed by HPLC-MS and hydroxylation only occurs in the presence of L-Orn as substrate. Neither the chemically synthesized N^5-formylornithine[14] nor the tetrapeptide (D-Orn-D-Thr-L-Orn-D-Orn) or other amino acid classes were accepted as substrates for hydroxylation (Table 1). Furthermore, the cosubstrate specificity of the hydroxylase was determined by incubation of CchB with NADH instead of NADPH. No hydroxylation could be detected and therefore it can be concluded that the enzyme is specific for NADPH and L-Orn.

Determination of kinetic parameters

To determine the kinetic parameters of CchB, the NADPH oxidation assay was used and the L-ornithine concentration was varied between 1 mM and 18 mM. Initial velocities were measured as a function of the L-ornithine concentration and the resulting curve was fit to the Michaelis–Menten equation (Fig. 4) to obtain an apparent K_M of 3.6 ± 0.58 mM and a k_{cat} of 17.4 ± 0.87 min^{-1}. The catalytic efficiency of this enzyme could be determined as $k_{cat}/K_M = 4.83 \pm 0.64$ min^{-1} mM^{-1}.

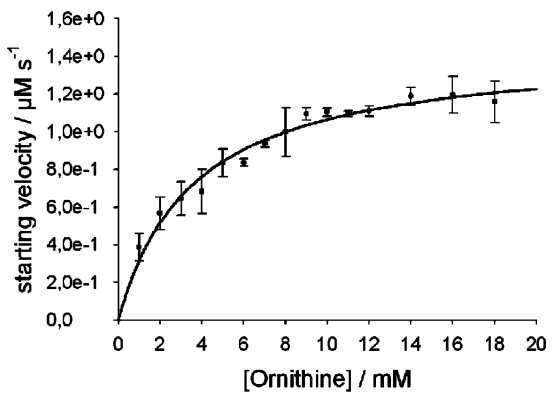

Fig. 4 Michaelis–Menten plot of CchB. The substrate concentration was varied between 1 mM and 18 mM, the enzyme concentration was 5 µM, and the curve represents the best fit to the Michaelis–Menten equation.

Determination of H_2O_2

As described previously for IucD, a slow NADPH oxidation takes place even in the absence of substrate.[15] Due to reduction of FAD to $FADH_2$ and the following oxidation, a flavin 4a-hydroperoxide species is formed which decays to yield FAD and hydrogen peroxide.[6] At different L-ornithine concentrations (0 mM–3 mM) the amount of produced hydrogen peroxide could be measured by a coupled assay with horseradish peroxidase and the dye 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 725 nm (Fig. 5).[16] At very low L-ornithine concentration most of the oxidized NADPH gives rise to the formation of hydrogen peroxide, but with increasing amounts of L-ornithine the concentration of hydrogen peroxide decreases and finally remains steady at the low level of about 6.5 µM. This confirms the prediction that the hydroxylation of the δ-amino group of ornithine takes place through the formation of a flavin 4a-hydroperoxide species which sets hydrogen peroxide free in the absence of an acceptor group. The incubation of CchB with L-lysine also reveals a slow NADPH oxidation activity however no hydroxylated lysine could be detected by HPLC-MS analysis.

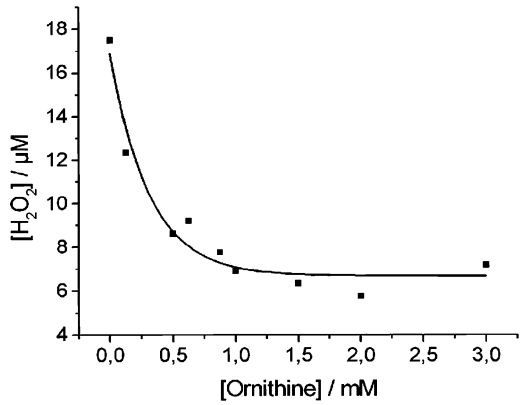

Fig. 5 Generation of hydrogen peroxide. The hydrogen peroxide produced at the given L-ornithine concentrations was determined spectrophotometrically at 725 nm in a coupled assay.

Production and activity assay of CchA

The enzyme was overproduced as an N-terminal hexahistidine tag fusion protein or an N-terminal thioredoxine fusion protein in *E. coli* BL21(DE3) cells and purified by Ni-NTA chromatography as soluble protein with yields of 1.5 mg per litre of bacterial culture (Fig. 1(b)).

To examine the catalytic activity of the recombinant protein, it was incubated with the cosubstrate N^{10}-formyltetrahydrofolate

(N^{10}-fH_4F) and the putative substrate N^5-hydroxyornithine and the reaction mixture was analyzed by HPLC-MS by scanning for the masses of N^5-hydroxyornithine and N^5-hydroxyformylornithine. Unfortunately, no enzyme activity could be detected (see also the discussion section).

Discussion

The flavin-dependent monooxygenase CchB is the third purified enzyme involved in the biosynthesis of hydroxamate groups of siderophores so far. In contrast to the previously described results it could be shown in this work for the first time *in vitro* that the hydroxylation reaction is the first step in the biosynthesis of hydroxamate siderophores.

CchB shows high sequence similarities to the previously purified L-ornithine N^5-hydroxylase PvdA and L-lysine N^6-hydroxylase IucD, but some differences occur, especially in comparison with the L-lysine hydroxylating enzyme IucD. Concerning the substrate specificity of these enzymes, the NADPH oxidation activity of CchB is considerably enhanced in the presence of L-ornithine and one can observe the hydroxylation of the substrate. In contrast to this observation, the one carbon atom longer amino acid L-lysine also enhances the NADPH oxidation activity of the monooxygenase giving rise to hydrogen peroxide formation instead of substrate hydroxylation. Probably, the ε-amino group of L-lysine is not located at an optimal position in the active site to be hydroxylated because of the additional carbon atom of the side chain.[8] This situation is analogous to the substrate specificity of IucD. This enzyme hydroxylates L-lysine and the NADPH oxidation activity is also enhanced by incubating IucD with homolysine, which possesses one additional carbon atom, however in the presence of this compound no hydroxylamine is produced.[6] Contrary to the dependence of the NADPH oxidation rate of CchB on the L-ornithine concentration that follows the Michaelis–Menten kinetics, the enzyme IucD is inhibited due to excess binding of the substrate.[6] Nevertheless, the kinetic parameters of CchB, PvdA and IucD can be compared. The apparent K_M value of CchB for L-ornithine (3.6 mM) is one order of magnitude higher than the reported K_M value of PvdA for L-ornithine (0.58 mM)[8] and the K_M value of IucD with L-lysine as substrate (0.11 mM).[6] Regarding the catalytic efficiencies k_{cat}/K_M of both L-ornithine hydroxylating monooxygenases, PvdA shows a tenfold higher efficiency (44.52 min^{-1} mM^{-1})[17] than CchB (4.83 min^{-1} mM^{-1}).

Concerning the cosubstrate specificity of these flavin-dependent monooxygenases involved in siderophore biosynthesis some differences occur, too. Although the activity of IucD is twofold lower than in the presence of NADPH, hydroxylation by incubating the enzyme with NADH was observed.[18] In contrast to IucD, CchB was not able to utilize NADH instead of NADPH as the reducing cofactor to form the flavin 4a-hydroperoxide species during the catalytic cycle.

Concerning the proximate δ-amino group formylation reaction during the biosynthesis of N^5-hydroxyformylornithine, the most likely involved enzyme L-N^5-hydroxyornithine-formyltransferase (CchA) could be expressed in a soluble, but unfortunately inactive form, although different expression vector systems (pET28a(+) derivative (Novagen), pQ-Tev (Qiagen), pBAD202/D-TOPO (Invitrogen)) were investigated. The sequence comparisons reveal the presence of an N^{10}-formyltetrahydrofolate (N^{10}-fH_4F) binding motif in CchA suggesting this compound as being the cosubstrate for the formylation reaction.[19] Since N^{10}-fH_4F is not commercially available due to instability, it has to be generated *in situ* for each formylation reaction. There exist two different reaction pathways to obtain N^{10}-fH_4F. In dependence of NAD^+ and formaldehyde, the enzyme FolD from the organism *Methanosarcina barkeri* converts tetrahydrofolate to N^{10}-fH_4F.[20] In a second possible reaction pathway the formyltetrahydrofolate synthetase (FTHFS) from *Clostridium thermoaceticum* converts tetrahydrofolate to N^{10}-fH_4F in the presence of sodium formate, magnesium chloride and ATP.[21] Both possibilities have been investigated during this study but we could not detect a turnover of N^5-hydroxyornithine to N^5-hydroxyformylornithine in the presence of N^{10}-fH_4F and CchA. In addition to this approach, the more stable and commercially available N^5-formyltetrahydrofolate (N^5-fH_4F) was also tested as a cosubstrate without success. It has been shown previously that the formylation domain involved in the nonribosomal peptide synthesis of linear gramicidin accepts both cosubstrates (N^{10}-fH_4F and N^5-fH_4F) for the formylation of PCP-bound L-valine.[20] In another experiment, the hydroxylation and the formylation reactions were coupled by firstly incubating L-ornithine with CchB, NADPH and FAD and in the following reaction N^{10}-fH_4F or N^5-fH_4F and CchA were added to the enzymatically generated N^5-hydroxyornithine. Unfortunately, this procedure did not result in the production of N^5-hydroxyformylornithine.

This study reveals that the hydroxylation reaction takes place exclusively at the L-ornithine side chain and that this reaction occurs before the formylation reaction during the biosynthesis of the hydroxamate siderophore coelichelin. The flavin-dependent monooxygenase accepts the unmodified L-ornithine as a substrate, but the enzyme is unable to convert N^5-formylornithine or the assembled tetrapeptide (D-Orn-D-Thr-L-Orn-D-Orn) to the hydroxylated form.

Experimental

Isolation of genomic DNA

Streptomyces coelicolor A3(2), obtained from DSMZ (strain 40783), was inoculated in Media 65 (DSMZ) and grown at 28 °C for 4 days. Genomic DNA was isolated using Qiagen Genomic-tips.

Cloning of CchB

The *cchB* gene was amplified from genomic DNA using Phusion DNA polymerase (Finnzymes) with the synthetic oligonucleotide primers 5′-AAAAAA*GGATCC*TCACAGGTTCTTCCTGCTGACTCAAC-3′ and 5′-AAAAA*AAGCTT*TTAACGCGCGCCGGTGCCGGT-3′ (*BamH*I and *Hind*III restriction sites are italicised). The resulting 1377 basepair PCR fragment was purified, digested with *BamH*I and *Hind*III, and ligated into a pET28a(+) derivative (Novagen), digested with the same enzymes. The identity of the plasmid pCB28a(+)-*cchB* with an N-terminal hexahistidine tag was confirmed by DNA sequencing.

Purification of CchB

The plasmid was used to transform *E. coli* strain BL21(DE3) (Novagen) and the enzyme was overproduced in LB medium

supplemented with kanamycin (50 μg mL^{-1}) (30 °C → 25 °C; induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside; 4 h). After harvesting by centrifugation (7000 rpm, 15 min, 4 °C) and resuspension in 50 mM Hepes pH 8.0 and 300 mM NaCl, the cells were lysed by two passages through an EmulsiFlex-C5 (Avestin) at 10 000 psi, and the recombinant protein was purified by Ni-NTA affinity chromatography using an ÄKTA purifier system (Amersham Pharmacia Biotech) with a linear gradient of 3–250 mM imidazole. Fractions containing the CchB (51.3 kDa) were identified by 10% SDS-PAGE analysis, pooled, and dialyzed against 100 mM TRIS buffer pH 8.0 using HiTrap desalting columns (Amersham Pharmacia Biotech). Protein concentration was determined spectrophotometrically with the help of the calculated extinction coefficient at 280 nm.

Synthesis of N^5-formylornithine

N_α-Fmoc-protected ornithine (391 mg, 1.0 mmol) was dissolved by heating in 98% formic acid (1.0 mL). A hot solution of sodium formate (75 mg, 1.1 mmol) in 98% formic acid (1.0 mL) was added and after cooling down, acetic anhydride (225 μL) was added slowly. The reaction mixture was heated at 70 °C for 30 min and then evaporated under vacuum to remove the formic and acetic acids. The residue was dissolved in acetone (5.0 mL) and again evaporated. For the deprotection of the formylated amino acid (1.0 mmol) it was dissolved in DMF (10.0 mL) and piperidine (2.5 mL, 25 mmol) was added dropwise at 0 °C. After stirring for 1 h at room temperature the solution was evaporated and the residue was dissolved three times in toluene and again evaporated. The product was purified by flash column chromatography on silica gel (MeOH : H_2O 8 : 2) yielding 130 mg (81%) N^5-formylornithine as a light yellow powder. HPLC-MS analysis (Hewlett Packard 1100 series): m/z 161.0848 $[M + H]^+$ calculated, m/z 161.0825 $[M + H]^+$ observed.

Synthesis of SNAC-activated unmodified tetrapeptide (D-Orn-D-Thr-L-Orn-D-Orn)

Fmoc-Orn(Alloc)-OH (438.5 mg, 1 mmol) was coupled to 2-chlorotritylchloride resin (380 mg) by the addition of *N*,*N*-diisopropylethylamine (695 μL, 4 mmol) in 10 mL dichloromethane. After stirring for 2 h at room temperature the resin was washed five times with dimethylformamide and stored at −20 °C for further usage. The cleavage of the Alloc group was performed by addition of phenylsilane (1.3 g, 12 mmol) and the catalyst tetrakis(triphenylphosphane)palladium (57.8 mg, 0.05 mmol) in 5 mL dichloromethane. The mixture was stirred at room temperature for 10 min and then the resin was washed three times with dichloromethane. This reaction was performed twice to optimize the yield. Fmoc-D-Thr(^{t}Bu)-OH was then coupled to the free amino group of the ornithine side chain by solid phase peptide synthesis in an automated peptide synthesizer (Advanced ChemTech APEX396). The synthesis of the tetrapeptide was completed by a double coupling step with Boc-D-Orn(Boc)-OH after removal of the Fmoc groups from the α-amino groups of Orn and Thr. After releasing the peptide from the resin, the tetrapeptide was activated at the C-terminus with *N*-acetylcysteamine (1 mL, 10 mmol) in the presence of dicyclohexylcarbodiimide (413 mg, 20 mmol) and 1-hydroxybenzotriazole (306 mg, 20 mmol) in dichloromethane followed by deprotection of the amino acid residues. After purification with the help of a preparative HPLC (Hewlett Packard 1100 series) a white solid (200 mg) with a yield of 36% was obtained. HPLC-MS analysis: m/z 563.33 $[M + H]^+$ calculated, m/z 563.25 $[M + H]^+$ observed.

Determination of enzyme specificity and kinetic parameters

The recombinant CchB (5 μM) was incubated with L-ornithine (1 mM), the cosubstrate NADPH (2 mM), and the cofactor FAD (20 μM) in 100 mM TRIS buffer (pH 8.0) for 12 h at 25 °C. The reaction was stopped by adding 1.3% (v/v) trifluoroacetic acid. As a control reaction the mixture was incubated without enzyme. With the help of reversed-phase HPLC-MS analysis on a Hypercarb column (Thermo Electron Corporation, pore diameter of 250 Å, particle size of 5 μM, 100% carbon) the assays were analyzed by the use of the following mobile phases: 20 mM aqueous nonafluoropentanoic acid (A), and acetonitrile (B). The applied gradient was: 0–15% B in 15 min and 15–30% B in 10 min with a flow rate of 0.2 mL min^{-1} at 20 °C. Substrate specificity assays were carried out under similar conditions using different amino acids as substrate and an incubation time of 5 h (see Table 1).

Assays to determine the kinetic parameters of CchB towards L-ornithine were carried out as described above, except that the L-ornithine concentration was varied between 1 mM and 18 mM. All kinetic assays were performed with an Ultrospec 3100 pro spectrophotometer (Amersham Biosciences) and the enzyme activity was estimated from the decrease in NADPH absorbance at 340 nm ($\varepsilon = 6300\ M^{-1}\ cm^{-1}$).

The pH dependence of CchB activity was tested spectrophotometrically with the standard assay mixture over a pH range of 6.0 to 9.5 in 0.5 increments.

Stoichiometry of hydrogen peroxide formation

Hydrogen peroxide was determined with an enzyme-coupled assay where the oxidation of ABTS at 725 nm in the presence of horseradish peroxidase was monitored. The assay mixture contained NADPH (300 μM), FAD (10 μM), CchB (5 μM), and L-ornithine (0–3 mM) in a 100 mM TRIS buffer (pH 8.0) in a total volume of 100 μL. After oxidation of NADPH was complete (10 min), 2 N HCl (2 μL) was added to reach a pH of 2.0 and to stop the reaction. Then the solution was diluted with TRIS buffer (725 μL) and 150 μL ABTS (0.2 mg mL^{-1}) were added. The reaction was started by adding 25 μL horseradish peroxidase (1.0 mg mL^{-1}) and the absorbance change of oxidized ABTS was monitored at 725 nm ($\varepsilon = 14\,200\ M^{-1}\ cm^{-1}$). To obtain a standard curve, various concentrations of H_2O_2 from 0 μM to 75 μM were assayed with the described method and values of the absorbance at 725 nm were plotted against the known H_2O_2 concentrations.

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