

RESEARCH ARTICLE

# The YHS-Domain of an Adenylyl Cyclase from *Mycobacterium phlei* Is a Probable Copper-Sensor Module

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## Abstract

YHS-domains are small protein modules which have been proposed to bind transition-metal ions like the related TRASH-domains. They are found in a variety of enzymes including copper-transporting ATPases and adenylyl cyclases. Here we investigate a class IIIc adenylyl cyclase from *Mycobacterium phlei* which contains a C-terminal YHS-domain linked to the catalytic domain by a peptide of 8 amino acids. We expressed the isolated catalytic domain and the full-length enzyme in *E. coli*. The catalytic domain requires millimolar  $Mn^{2+}$  as a cofactor for efficient production of cAMP, is unaffected by low micromolar concentrations of  $Cu^{2+}$  and inhibited by concentrations higher than 10  $\mu M$ . The full-length enzyme also requires  $Mn^{2+}$  in the absence of an activator. However, 1–10  $\mu M$   $Cu^{2+}$  stimulate the *M. phlei* adenylyl cyclase sixfold when assayed with  $Mn^{2+}$ . With  $Mg^{2+}$  as the probable physiological cofactor of the adenylyl cyclase  $Cu^{2+}$  specifically switches the enzyme from an inactive to an active state. Other transition-metal ions do not elicit activity with  $Mg^{2+}$ . We favor the view that the YHS-domain of *M. phlei* adenylyl cyclase acts as a sensor for copper ions and signals elevated levels of the transition-metal via cAMP. By analogy to TRASH-domains binding of  $Cu^{2+}$  probably occurs via one conserved aspartate and three conserved cysteine-residues in the YHS-domain.



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## Introduction

Cellular signal transduction is achieved by a complex molecular network to enable the cell to adapt to and react to changes in its environment. Many signal transduction processes involve second messengers. Upon activation of a single second messenger generating protein many of these signaling molecules are produced thereby amplifying the original input signal.

A central second messenger in eukaryotic as well as prokaryotic signal transduction is 3',5'-cyclic adenosine monophosphate (cAMP). cAMP is generated from ATP by adenylyl cyclases. To date six classes of adenylyl cyclases (ACs) have been described which do not share any sequence similarity and are thought to be the product of convergent evolution [1–4]. By far the

largest number of ACs belongs to class III. Class III ACs are found in metazoans including mammals, in protozoans and in eubacteria [1].

The catalytic domain of class III ACs is often termed CHD (cyclase homology domain). It forms head-to-tail dimers with the catalytic centers located at the interface of the dimer, thus making dimerization a prerequisite for catalytic activity [5,6]. Based on analysis of amino acid sequences and structures of their catalytic domains the class III ACs have been classified in four categories, class IIIa through class III d [7]. According to the InterPro protein domain organisation database almost all class III ACs are multi-domain proteins [8]. The general role of many additional domains is that of signal receivers. A stimulus acting on those domains is transmitted to the catalytic domain, usually leading to activation of the CHD [9–12]. Because often regulatory domains associated with ACs are also occurring in other families of signal transduction proteins, the investigation of such ACs can greatly further our understanding of these domains in a more general context [9,12,13].

In the present study we explored the function of the YHS-domain. The YHS-domain is a small cytosolic protein domain of ca. 50 amino acids named after three conserved amino acid residues, *i.e.* tyrosine, histidine, serine. It has first been published as part of the InterPro database (InterPro IPR007029). The YHS-domain shares extensive similarity to the TRASH domain, a protein domain binding transition-metal ions via conserved cysteine residues [14]. Due to the similarity of YHS to TRASH many domains in InterPro are annotated as both, YHS and TRASH (Fig 1B). Thus, the YHS-domain may be regarded as an expansion of the TRASH domain.

Because of its occurrence in a number of bacterial Cu-ATPases it has been proposed that the YHS-domain binds copper ions. However, binding of copper ions to YHS/TRASH has not been experimentally demonstrated in these transporters. A YHS-domain is also found in toluene-4-hydroxylase. The crystal structure of this enzyme has been solved including the YHS-domain [15]. However, neither a metal ion nor any other ligand is bound to YHS in the crystal. Furthermore copper ions have not been implicated in the catalytic mechanism or regulation of the hydroxylase and the function of the YHS-domain is unknown. The prokaryotic ribosomal protein L24e consists of a single TRASH domain. In the crystal structure of the 50S ribosome from *H. marismortui* [16], a  $\text{Cd}^{2+}$ -ion is bound to L24e via the conserved cysteine residues giving experimental evidence of a transition-metal ion binding TRASH-domain and thus corroborating the proposed function of YHS-domains.

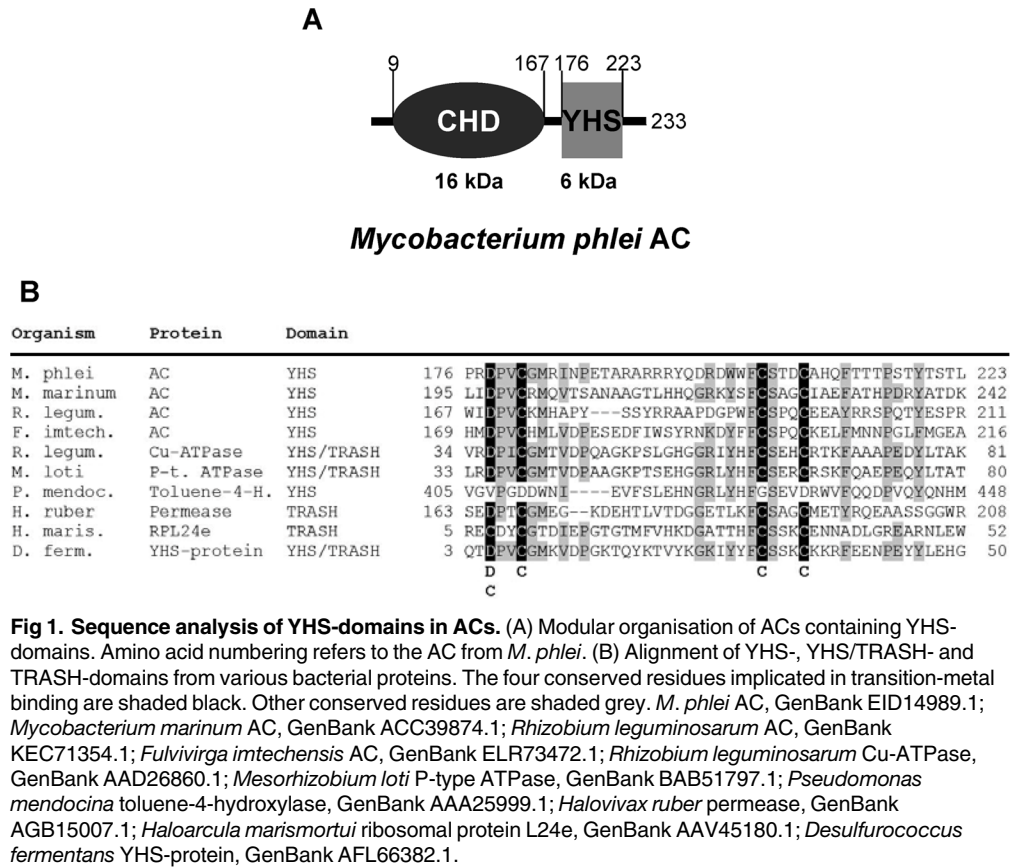
YHS-domains are also present in about 20 putative class III adenylyl cyclases, predicted from whole genome sequencing data. They occur mainly in mycobacterial species, but also in diverse eubacteria like *Gordonia bronchialis* and several *Rhizobium* species. In these ACs the CHD is fused to the YHS-domain located at the C-terminal end.

Here we expressed, purified and characterized the YHS-domain containing AC from *Mycobacterium phlei*. We show that the enzyme is an active AC and is activated by copper ions. Activation relies on the presence of the YHS-domain. Thus the AC-associated YHS-domain appears to act as a sensory module for copper ions.

## Materials and Methods

### Cloning of *Mycobacterium phlei* AC

The gene for *M. phlei* AC (MpAC, GenBank accession EID14989.1) was codon-optimized for expression in *E. coli* and fitted with an N-terminal *Bam*HI and a C-terminal *Hind*III site (S1 Fig). The DNA was synthesized commercially (GeneArt/life technologies). For expression of MpAC<sub>1-182</sub> the synthetic gene was transferred via the respective *Bam*HI and *Hind*III sites into the vector pQE30 (Qiagen). The YHS-domain was then removed by cutting with *Sph*I and



**Fig 1. Sequence analysis of YHS-domains in ACs.** (A) Modular organisation of ACs containing YHS-domains. Amino acid numbering refers to the AC from *M. phlei*. (B) Alignment of YHS-, YHS/TRASH- and TRASH-domains from various bacterial proteins. The four conserved residues implicated in transition-metal binding are shaded black. Other conserved residues are shaded grey. *M. phlei* AC, GenBank EID14989.1; *Mycobacterium marinum* AC, GenBank ACC39874.1; *Rhizobium leguminosarum* AC, GenBank KEK71354.1; *Fulvivia imtechensis* AC, GenBank ELR73472.1; *Rhizobium leguminosarum* Cu-ATPase, GenBank AAD26860.1; *Mesorhizobium loti* P-type ATPase, GenBank BAB51797.1; *Pseudomonas mendocina* toluene-4-hydroxylase, GenBank AAA25999.1; *Halovivax ruber* permease, GenBank AGB15007.1; *Haloarcula marismortui* ribosomal protein L24e, GenBank AAV45180.1; *Desulfurococcus fermentans* YHS-protein, GenBank AFL66382.1.

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*Hind*III, blunting the ends by treatment with *Klenow*-enzyme and recircularization of the product. The resulting open reading frame codes for MRGSH<sub>6</sub>GS-MpAC<sub>1-182</sub>-A. For expression of the full-length enzyme the gene was cloned into the *Bam*HI and *Hind*III sites of a modified pQE30-vector coding for a TEV-protease recognition sequence downstream of the hexahistidine motif. The open reading frame codes for MRGSH<sub>6</sub>GSENYLFQGS-MpAC<sub>1-233</sub> (His-TEV-MpAC<sub>1-233</sub>). Expression plasmids were sequenced for checking the intactness of the expression cassettes.

### Expression and purification of *M. phlei* adenylyl cyclase

MpAC was produced in *E. coli* BL21(DE3)[pRep4]. Batch size was 1 liter of LB-medium supplied with 100 mg/l ampicillin and 25 mg/l kanamycin. Cells were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 5–6 hrs at 20°C. Bacteria were washed with buffer (50 mM Tris/HCl, 1 mM EDTA, pH 8), frozen in liquid nitrogen and stored at -80°C. Purification was started by suspending the cells in 20 ml lysis buffer (50 mM Tris/HCl pH 8), sonicating for 40 s and treating with 0.2 mg/ml lysozyme for 30 min on ice. After addition of 5 mM MgCl<sub>2</sub> and 20 μg/ml of DNaseI the incubation was continued for another 30 min. After centrifugation (31000 x g, 30 min) the solution was supplemented with 250 mM NaCl and 15 mM imidazole (final concentrations). 600 μl Ni-NTA-agarose were added and the mixture was gently shaken for 3 hours on ice. The resin was transferred into a column, washed with 10 ml buffer A (lysis buffer containing 250 mM NaCl, 15 mM imidazole, 5 mM MgCl<sub>2</sub>) and subsequently with 5 ml of buffer B (lysis buffer containing 15 mM imidazole, 5 mM MgCl<sub>2</sub>). Protein

was eluted with 0.6 ml of buffer C (37.5 mM Tris/HCl, pH 8, 250 mM imidazole, 2 mM MgCl<sub>2</sub>). Purified MpAC was stored at -20°C after addition of 40% glycerol. The purity of the recombinant proteins was assessed by 14% SDS-PAGE and staining with Coomassie Blue G250.

### Removal of the hexahistidine-tag of full-length MpAC

Purified His-TEV-MpAC<sub>1-233</sub> was diluted with an equal volume of 20 mM Tris/HCl, pH 8 and 0.12 µg His-tagged TEV-protease per µg AC was added. The mixture was incubated for 12 hours at 4°C. Buffer was rapidly changed by consecutive dilution and ultrafiltration to 20 mM Tris/HCl, pH8; 7 mM Imidazole; 50 mM NaCl; 2 mM MgCl<sub>2</sub>. 250 µl Ni-NTA-Agarose per 100 µg of protein were added and incubation was performed for 3 hours on ice. The resin was removed by filtration. The filtrate was mixed with 25% glycerol and stored at -20°C. The final product carries an N-terminal dipeptide compared to native MpAC. The sequence is GS-MpAC<sub>1-233</sub>.

### AC assay

AC activity was measured for 10 min at 30°C in a volume of 30 µl. Standard reactions contained 2.5 mM Tris/HCl, pH 8.0, 5 mM MnCl<sub>2</sub> or MgCl<sub>2</sub>, 1 mM ATP. cAMP was determined by HPLC as described previously [17]. All data are means of 2 to 6 points and are denoted with their standard deviations.

### Tryptophane-fluorescence spectroscopy

Samples were irradiated in a 1 ml fluorescence cuvette at 295 nm (gap width 5 nm) at 20°C. Fluorescence intensities at 345 nm were recorded with a gap width of 5 nm. Fluorescence data were corrected for the inner-filter effect of protein and reagents and for the dilution factor upon addition of reagents.

## Results and Discussion

### Primary structure of ACs containing a YHS domain

A search of the InterPro protein domain architecture database [8] for protein sequences containing class III adenylyl cyclase catalytic domains (CHDs, InterPro IPR001054) yielded a list of 1369 different architectures present in a total of 19961 protein sequences. Among these, 13 proteins were found which contained a YHS domain. All share the same architecture of a single CHD linked to a single C-terminal YHS domain (Fig 1A). The linker region between the two domains consists of 8–11 residues. Using the AC-YHS protein from *Mycobacterium phlei* (GenBank accession EID14989.1) as a query, a BLAST search of the non-redundant protein data base [18] yielded a set of 24 sequences, partially overlapping the results from the InterPro search. A total number of 26 putative AC-YHS enzymes were detected by the combination of both searches. A representative sequence alignment is given in the supplementary material (S2 Fig).

We have focused our work on the AC form *M. phlei* because of its compact structure with 233 residues compared to a range of 216–473 amino acids (aa) among all AC-YHS sequences and because *M. phlei* is a well characterized species with a history as a mycobacterial model organism [19]. Inspection of the CHD of the *M. phlei* AC revealed that all six canonical catalytic residues [5,7] are present (highlighted in S2 Fig). Furthermore the dimerization arm of the *M. phlei* AC is short with 7 residues, which classifies it as a class IIIc CHD [7]. Analysis of the complete set of AC-YHS proteins shows that all of them belong to that subclass.

An alignment of the YHS domains of ACs with the YHS/TRASH domains of Cu-ATPases and other YHS and TRASH domains is shown in Fig 1B. The mode of metal-ion coordination has previously been uncovered by the crystal structure of the ribosomal TRASH-domain subunit L24e [16]. A  $\text{Cd}^{2+}$  ion is bound to four cysteine residues which are highlighted in Fig 1B. In the YHS domain of ACs the N-terminal cysteine is replaced by aspartate (D178 in *M. phlei* AC), while the other three cysteine residues are conserved (*M. phlei* AC: C181, C204, C208). An aspartate in the position of the first metal-ion coordinating residue appears to be common among YHS and TRASH-domains (Fig 1B). In general the sequence profile of the YHS-domains of bacterial ACs fits that of bacterial Cu-ATPases and other metal-ion binding YHS- and TRASH-domains, which led us to the hypothesis that the AC of *M. phlei* may bind transition-metal ions, probably copper, via its YHS-domain and that it may be regulated by such ions.

### Expression and characterization of the catalytic domain of *M. phlei* adenylyl cyclase

In a first step towards investigation of the YHS-domain the isolated catalytic domain of *M. phlei* AC (MpAC) was characterized to provide a base for the separation of direct effects of metal-ions on the CHD from effects mediated by the YHS-domain. The CHD (aa 1–182) of MpAC was N-terminally tagged with a hexahistidine motif and expressed in *E. coli*. Upon purification of MpAC<sub>1-182</sub> by affinity chromatography the protein migrated at 22 kDa on SDS-PAGE (calculated 20.1 kDa, Fig 2A). The identity of the protein was confirmed by mass spectrometry of a tryptic digest (data not shown). MpAC<sub>1-182</sub> showed robust AC activity of  $196 \pm 12$  nmol cAMP  $\text{mg}^{-1} \cdot \text{min}^{-1}$  with  $\text{Mn}^{2+}$  as a cofactor whereas activity with  $\text{Mg}^{2+}$  was minimal with  $0.37 \pm 0.02$  nmol cAMP  $\text{mg}^{-1} \cdot \text{min}^{-1}$ . With  $\text{Mn}^{2+}$  as a cofactor MpAC<sub>1-182</sub> displayed maximal activity in the range of pH 8.0–8.5. The temperature optimum was at 30°C (data not shown). Subsequently all assays were performed at 30°C and pH 8.0.

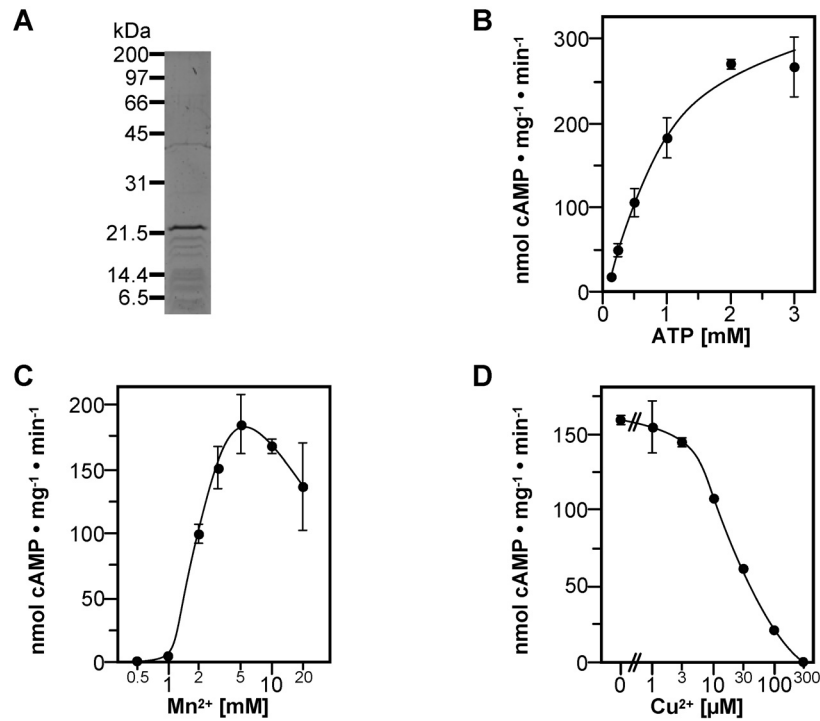
Kinetic analysis showed a  $v_{\max}$  of  $335 \pm 70$  nmol cAMP  $\text{mg}^{-1} \cdot \text{min}^{-1}$  with half-maximal activity ( $\text{SC}_{50}$ ) at  $0.90 \pm 0.22$  mM ATP and a pronounced positive cooperativity for ATP as seen by a Hill coefficient of  $1.5 \pm 0.2$  (Fig 2B, Table 1). The  $\text{Mn}^{2+}$ -dependence of MpAC<sub>1-182</sub> at 1 mM ATP is shown in Fig 2C. Activity is low up to 1 mM  $\text{Mn}^{2+}$  and surging when the concentration of  $\text{Mn}^{2+}$  exceeds that of ATP. This behavior is typical for the two-metal ion mechanism of ACs and demonstrates the requirement of both, MnATP and free  $\text{Mn}^{2+}$  for catalysis to occur [20]. Based on a reciprocal plot the affinity of MpAC<sub>1-182</sub> for the cofactor was calculated yielding an  $\text{EC}_{50}$  for free  $\text{Mn}^{2+}$  of  $2.0 \pm 0.6$  mM.

Because copper ions are activators of full-length MpAC (see below), the effect of  $\text{Cu}^{2+}$  on MpAC<sub>1-182</sub> was tested (Fig 2D).  $\text{Cu}^{2+}$  acted as an inhibitor of the CHD with an  $\text{IC}_{50}$  of 20  $\mu\text{M}$ .

### Expression and characterization of full-length *M. phlei* adenylyl cyclase

The full-length MpAC was fitted with an N-terminal hexahistidine-tag carrying a TEV-protease recognition sequence and expressed in *E. coli*. Purified His-TEV-MpAC<sub>1-233</sub> appeared at 31 kDa on SDS-PAGE, slightly higher than expected (calculated 27.0 kDa, Fig 3A). Subsequently the hexahistidine-tag was removed with TEV protease and the untagged full-length *M. phlei* AC was purified by reverse  $\text{Ni}^{2+}$ -affinity chromatography. Untagged MpAC<sub>1-233</sub> showed a slightly higher mobility on SDS-PAGE compared to His-TEV-MpAC<sub>1-233</sub> demonstrating the success of the tag-removal procedure (Fig 3A).

The specific activity of MpAC<sub>1-233</sub> with  $\text{Mn}^{2+}$  as a cofactor was  $541 \pm 56$  nmol cAMP  $\text{mg}^{-1} \cdot \text{min}^{-1}$ , while no activity was detectable with  $\text{Mg}^{2+}$ . The  $v_{\max}$  of  $1660 \pm 90$  nmol cAMP  $\text{mg}^{-1} \cdot \text{min}^{-1}$  and  $\text{SC}_{50}$  of  $2.11 \pm 0.54$  mM ATP were higher than the corresponding values of the



**Fig 2. Biochemical analysis of the catalytic domain MpAC<sub>1-182</sub>.** (A) SDS-PAGE of MpAC<sub>1-182</sub>. (B) Substrate kinetics measured with 5 mM Mn<sup>2+</sup> as a cofactor at 30°C and pH 8.0. Vertical bars indicate the standard deviation if larger than symbol size. (C) Mn<sup>2+</sup>-dependance assayed at 1 mM ATP. (D) Inhibition by Cu<sup>2+</sup>. Reactions contained 1.6 μM MpAC<sub>1-182</sub>, 5 mM Mn<sup>2+</sup> and 1 mM ATP.

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isolated CHD (Fig 3B, Table 1). In addition, MpAC<sub>1-233</sub> did not show cooperativity with a Hill coefficient of 0.9 ± 0.1. The Mn<sup>2+</sup>-dependance of MpAC<sub>1-233</sub> showed a similar requirement for Mn<sup>2+</sup> in excess of ATP like that of MpAC<sub>1-182</sub>. Yet, the affinity for Mn<sup>2+</sup> was lower for MpAC<sub>1-233</sub> with an EC<sub>50</sub> of 8.3 ± 0.6 mM free Mn<sup>2+</sup> (Fig 3C). The change in the kinetic parameters and the Mn<sup>2+</sup>-affinity by the presence of the YHS-domain indicates that the YHS-domain has an impact on the conformation of the CHD even in the absence of transition-metal ions.

### Stimulation of full-length *M. phlei* adenylyl cyclase by copper ions with Mn<sup>2+</sup> as a cofactor

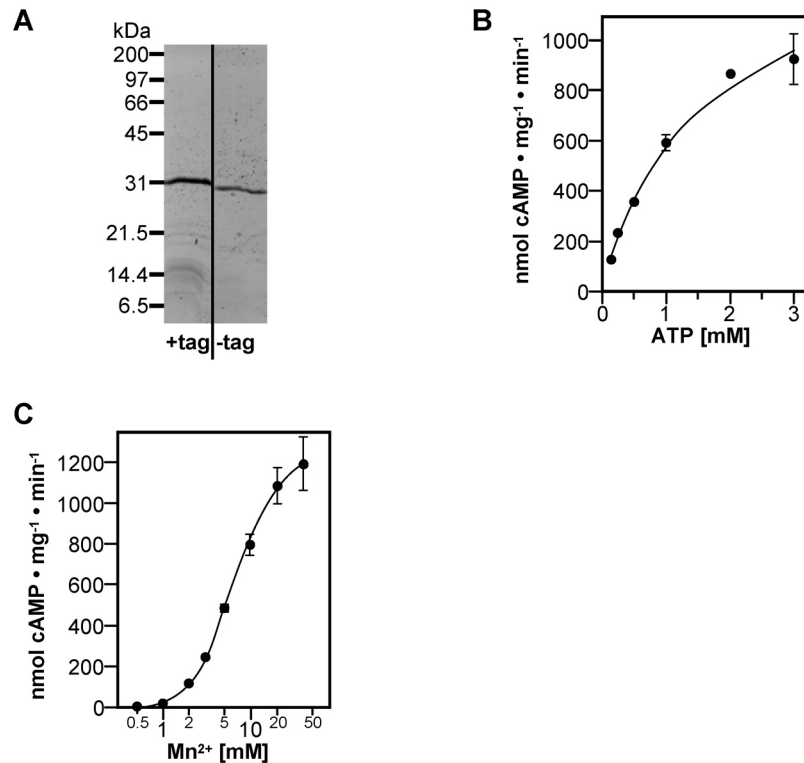
The hypothesis of the YHS-domain of *M. phlei* AC being a transition-metal ion binding domain with similarity to the YHS-domains of CuATPases was tested in assays of the untagged

**Table 1. Kinetic parameters of MpAC<sub>1-182</sub> and MpAC<sub>1-233</sub>.**

Enzyme	V <sub>max</sub> [nmol cAMP · mg <sup>-1</sup> · min <sup>-1</sup> ]	SC <sub>50</sub> [mM ATP]	Hill-coefficient	EC <sub>50</sub> [mM Me <sup>2+</sup> ]
MpAC <sub>1-182</sub> / Mn <sup>2+</sup>	335 ± 70	0.90 ± 0.22	1.5 ± 0.2	1.95 ± 0.58
MpAC <sub>1-233</sub> / Mn <sup>2+</sup>	1660 ± 90	2.11 ± 0.54	0.9 ± 0.1	8.27 ± 0.57
MpAC <sub>1-233</sub> / Mn <sup>2+</sup> /Cu <sup>2+</sup>	3630 ± 1060	0.37 ± 0.15	1.2 ± 0.3	0.68 ± 0.17
MpAC <sub>1-233</sub> / Mg <sup>2+</sup> /Cu <sup>2+</sup>	485 ± 35	1.63 ± 0.23	1.5 ± 0.1	1.84 ± 0.82

Reactions were performed under standard-conditions (30°C, pH 8.0) with 5 mM cofactor (Mn<sup>2+</sup> or Mg<sup>2+</sup>) and with addition of 10 μM Cu<sup>2+</sup> where indicated. EC<sub>50</sub> values for the cofactor ion were determined at 1 mM ATP. Values are means ± SD, n = 2–4.

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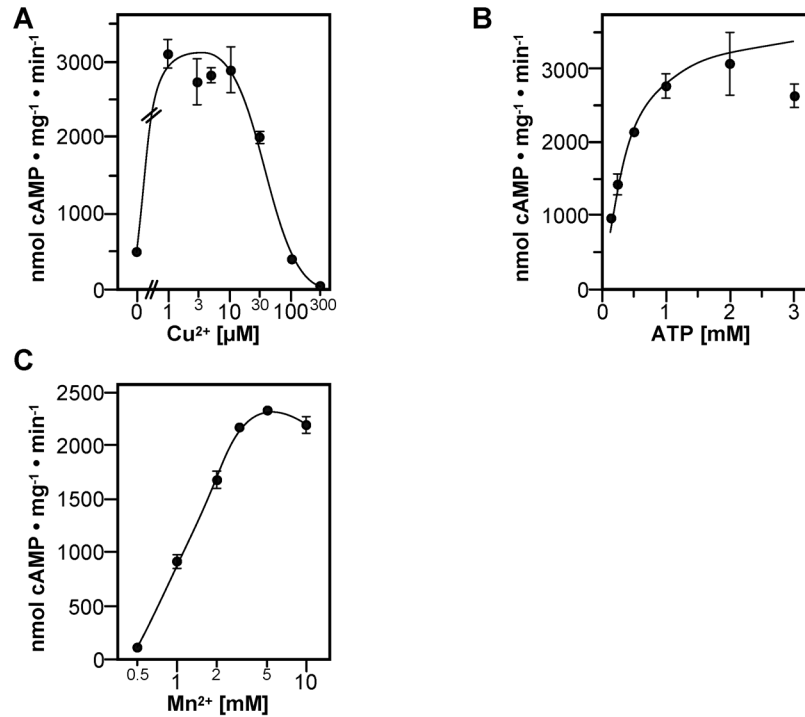
**Fig 3. Biochemical analysis of full-length MpAC<sub>1-233</sub>.** (A) SDS-PAGE of His-TEV-MpAC<sub>1-233</sub> (+tag) and untagged MpAC<sub>1-233</sub> (-tag). Both lanes are from the same gel and are shown in alignment with the molecular weight marker on that gel. (B) Substrate kinetics measured with 5 mM Mn<sup>2+</sup> as a cofactor at 30°C and pH 8.0. (C) Mn<sup>2+</sup>-dependence assayed at 1 mM ATP.

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full-length MpAC<sub>1-233</sub> with micromolar concentrations of Cu<sup>2+</sup> and Mn<sup>2+</sup> as a cofactor (Fig 4A). Cu<sup>2+</sup> maximally stimulated MpAC<sub>1-233</sub> sixfold at concentrations of 1 to 10 μM. Because we used an enzyme concentrations of 0.4 μM to assure efficient dimerization of MpAC<sub>1-233</sub> (see below), assays with Cu<sup>2+</sup> below 1 μM were not conducted. Yet, the data imply that the EC<sub>50</sub> for activation by Cu<sup>2+</sup> is in the nanomolar range. On the other hand, high concentrations of Cu<sup>2+</sup> were inhibitory similar to the results seen with the isolated catalytic domain. Thus, we concluded that the stimulatory effect of low concentrations of Cu<sup>2+</sup> on MpAC<sub>1-233</sub> is mediated by the YHS-domain. The inhibitory effect of higher concentrations of Cu<sup>2+</sup> may be due to general interactions and reactions with protein similar to those exploited in classic protein assays like the Lowry- and the Biuret-method [21]. Kinetic analysis of MpAC<sub>1-233</sub> showed that 10 μM Cu<sup>2+</sup> led to an increase in v<sub>max</sub> and a concomitant decrease of SC<sub>50</sub> for ATP (Fig 4B, Table 1). The Hill coefficient of 1.2 ± 0.3 indicated that Cu<sup>2+</sup> did not induce pronounced cooperativity for ATP. Furthermore, addition of 10 μM Cu<sup>2+</sup> resulted in a 10-fold higher affinity for the cofactor Mn<sup>2+</sup> with an EC<sub>50</sub> of 0.7 ± 0.2 mM for the free ion (Fig 4C). Taken together Cu<sup>2+</sup> appears to increase the catalytic efficiency of the enzyme.

### Activation of full-length *M. phlei* adenylyl cyclase by copper ions with Mg<sup>2+</sup> as a cofactor

Because a requirement of millimolar concentrations of Mn<sup>2+</sup> for AC activity may not reflect the physiological environment of the enzyme in *Mycobacterium phlei* we explored the effect of Cu<sup>2+</sup> on MpAC<sub>1-233</sub> with Mg<sup>2+</sup> as a cofactor. MpAC<sub>1-233</sub> produced 122 ± 20 nmol cAMP mg<sup>-1</sup>



**Fig 4. Stimulation of full-length MpAC<sub>1-233</sub> by Cu<sup>2+</sup> assayed with Mn<sup>2+</sup> as a cofactor.** (A) Effect of Cu<sup>2+</sup>. Reactions contained 0.4  $\mu\text{M}$  MpAC<sub>1-233</sub>, 5 mM Mn<sup>2+</sup> and 1 mM ATP. (B) Substrate kinetics measured with 5 mM Mn<sup>2+</sup> as a cofactor and 10  $\mu\text{M}$  Cu<sup>2+</sup>. (C) Mn<sup>2+</sup>-dependence assayed at 1 mM ATP and 10  $\mu\text{M}$  Cu<sup>2+</sup>.

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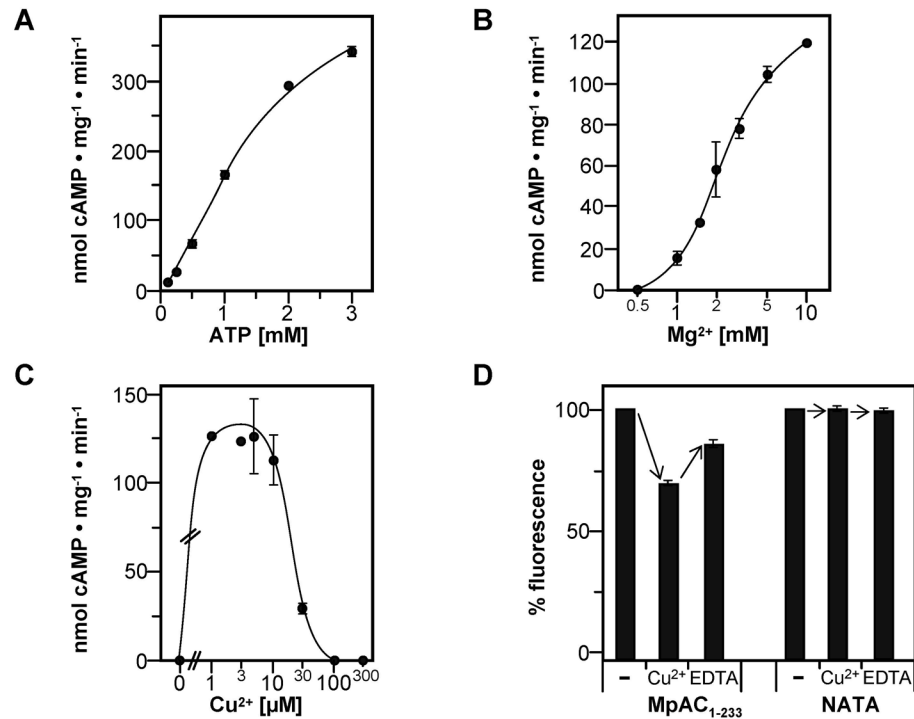
$\cdot \text{min}^{-1}$  upon addition of 10  $\mu\text{M}$  Cu<sup>2+</sup>. Thus Cu<sup>2+</sup> served as an on-switch of MpAC that relies on the YHS-domain of the enzyme.

Kinetic analysis (Fig 5A) yielded a  $v_{\text{max}}$  of  $485 \pm 35$  nmol cAMP  $\text{mg}^{-1} \cdot \text{min}^{-1}$  with an  $\text{SC}_{50}$  of  $1.63 \pm 0.23$  mM ATP. The Hill coefficient of  $1.5 \pm 0.1$  demonstrated a positive cooperativity for ATP with Mg<sup>2+</sup> in contrast to assays with Mn<sup>2+</sup> (Table 1). Mg<sup>2+</sup>-dependence showed the typical characteristics of a two-metal ion mechanism as seen with Mn<sup>2+</sup>;  $\text{EC}_{50}$  for free Mg<sup>2+</sup> was  $1.8 \pm 0.8$  mM (Fig 5B). Activation by Cu<sup>2+</sup> was maximal in the range of 1 to 10  $\mu\text{M}$  with strong inhibition at higher concentrations (Fig 5C).

If the YHS-domain served as a Cu<sup>2+</sup>-sensor the activation of MpAC<sub>1-233</sub> should be specific to copper ions. MpAC<sub>1-233</sub> was assayed with Mg<sup>2+</sup> as a cofactor and addition of Zn<sup>2+</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, Cr<sup>3+</sup>, Co<sup>2+</sup> and Ni<sup>2+</sup>. There was no detectable AC activity with any of these transition-metal ions. Next we checked whether activation of MpAC<sub>1-233</sub> by Cu<sup>2+</sup> occurs by binding of the metal ion to the enzyme or whether it is a consequence of the oxidative properties of Cu<sup>2+</sup>, e.g. by oxidizing the conserved cysteine residues [22] of the YHS-domain. MpAC<sub>1-233</sub> was incubated with 10  $\mu\text{M}$  Cu<sup>2+</sup> for 15 min in the presence of Mg<sup>2+</sup> as a cofactor, then Cu<sup>2+</sup> was selectively quenched by 375  $\mu\text{M}$  EDTA before the reaction was started by addition of ATP. No AC activity was detected, while in a parallel experiment under same conditions, but without quenching by EDTA, AC activity was preserved (data not shown).

Finally, we investigated the effect of Cu<sup>2+</sup> on the YHS-domain by tryptophane-fluorescence spectroscopy, which was facilitated by the only two tryptophane residues of MpAC<sub>1-233</sub> being located there (W201 and W202). The fluorescence emission spectrum showed a maximum at 345 nm, which was unchanged by the addition of Cu<sup>2+</sup> or EDTA (data not shown). However, the fluorescence intensity at 345 nm was significantly reduced by Cu<sup>2+</sup> and addition of EDTA





**Fig 5. Activation of full-length MpAC<sub>1-233</sub> by Cu<sup>2+</sup> assayed with Mg<sup>2+</sup> as a cofactor.** (A) Substrate kinetics measured with 5 mM Mg<sup>2+</sup> as a cofactor and 10 μM Cu<sup>2+</sup>. (B) Mg<sup>2+</sup>-dependence assayed at 1 mM ATP and 10 μM Cu<sup>2+</sup>. (C) Effect of Cu<sup>2+</sup>. Reactions contained 0.4 μM MpAC<sub>1-233</sub>, 5 mM Mg<sup>2+</sup> and 1 mM ATP. (D) Tryptophane-fluorescence spectroscopy. The fluorescence emission signal at 345 nm of 1.15–2.55 μM MpAC<sub>1-233</sub> (n = 3) or 5 μM N-acetyl-tryptophanamide (NATA, n = 2) was taken as 100% (-). Subsequently 25 μM Cu<sup>2+</sup> were added (Cu<sup>2+</sup>) and finally the copper-ions were complexed with 375 μM EDTA (EDTA).

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in essence reverted the effect (Fig 5D). As a control, the fluorescence of N-acetyl-tryptophanamide was unaffected by Cu<sup>2+</sup> and EDTA showing that neither reagent acted as a general quencher of fluorescence (Fig 5D). The data are consistent with a conformational change of the YHS-domain triggered by the binding of Cu<sup>2+</sup>. Thus we favor the view that the *M. phlei* AC acts as a Cu<sup>2+</sup>-sensor enzyme, where binding of Cu<sup>2+</sup> to the YHS-domain in turn activates the CHD.

Although we do not know the mechanism of activation by Cu<sup>2+</sup> at this time, we can exclude an enhanced dimerization as an underlying principle. The dissociation constant of the enzyme is 0.16 μM as calculated from the protein dependence of the AC activity (data not shown). We used at least 0.4 μM MpAC<sub>1-233</sub> in all assays to ensure that the enzyme largely remains dimerized. Crystal structures of class III ACs have shown that upon activation only minor changes in secondary structure occur, whereas often pronounced shifts in the orientation of the CHD monomers to each other are visible [5,10,17]. Similar mechanisms might trigger the activation of the *M. phlei* AC by Cu<sup>2+</sup>.

The physiological role of the Cu<sup>2+</sup>-sensor AC in the bacterium remains open to speculation. It has been known since the 1950's that *M. phlei* is exquisitely sensitive to Cu<sup>2+</sup> with strong growth inhibition occurring with as little as 1 μM Cu<sup>2+</sup> in the medium [23]. For comparison 100 μM Cu<sup>2+</sup> has no effect on the growth rate of *E. coli* [23]. A link between Cu<sup>2+</sup>-sensitivity and Cu<sup>2+</sup>-sensor AC in *M. phlei* therefore appears as a plausible hypothesis.

By structural analogy to the related TRASH-domain the conserved aspartate and cysteine residues marked in Fig 1B are likely to coordinate Cu<sup>2+</sup> in the YHS-domain of *M. phlei* AC

[14]. Our study provides biochemical evidence for a function of the YHS-domain in transition-metal ion binding and enzyme regulation which also impacts on our understanding of the TRASH-domain. Furthermore our data underline the high versatility of class III AC catalytic domains in their interaction with diverse regulatory domains as seen in past studies.

## Supporting Information

**S1 Fig. Sequence of the synthetic gene for *Mycobacterium phlei* AC.** Flanking *Bam*HI and *Hind*III are shown in italics. Differences to the natural gene (GenBank EID14989.1) are underlined.

(TIF)

**S2 Fig. Alignment of ACs carrying a C-terminal YHS-domain.** In the CHD, critical functional residues for metal-cofactor binding (M), adenine-moiety binding (A) and for catalysis (C) are shaded black, as well as the four conserved putative transition-metal ion binding residues of the YHS-domain. Sequences are from *Mycobacterium phlei* (GenBank EID14989.1), *Mycobacterium marinum* (GenBank ACC39874.1), *Williamsia* sp.ARP1 (NCBI reference WP\_045825068.1); Candidatus *Microthrix parvicella* RN1; (GenBank CCM65060.1); *Rhizobium leguminosarum* (GenBank KEC71354.1); *Rhizobium etli* (NCBI reference WP\_040141435.1); *Fulvivirga imtechensis* (GenBank ELR73472.1).

(TIF)

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## Author Contributions

Conceived and designed the experiments: JUL. Performed the experiments: JUL. Analyzed the data: JUL. Contributed reagents/materials/analysis tools: JUL. Wrote the paper: JUL.

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