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Structural variations between small alarmone hydrolase dimers support different modes of regulation of the stringent response

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The bacterial stringent response involves wide-ranging metabolic reprogramming aimed at increasing long-term survivability during stress conditions. One of the hallmarks of the stringent response is the production of a set of modified nucleotides, known as alarmones, which affect a multitude of cellular pathways in diverse ways. Production and degradation of these molecules depend on the activity of enzymes from the RelA/SpoT homologous family, which come in both bifunctional (containing domains to both synthesize and hydrolyze alarmones) and monofunctional (consisting of only synthetase or hydrolase domain) variants, of which the structure, activity, and regulation of the bifunctional RelA/SpoT homologs have been studied most intensely. Despite playing an important role in guanosine nucleotide homeostasis in particular, mechanisms of regulation of the small alarmone hydrolases (SAHs) are still rather unclear. Here, we present crystal structures of SAH enzymes from Corynebacterium glutamicum (RelH_Cg) and Leptospira levetitii (RelH_LL) and show that while being highly similar, structural differences in substrate access and dimer conformations might be important for regulating their activity. We propose that a varied dimer form is a general property of the SAH family, based on current structural information as well as prediction models for this class of enzymes. Finally, subtle structural variations between monofunctional and bifunctional enzymes point to how these different classes of enzymes are regulated.

The stringent response is a fundamental mechanism for bacterial survival (1) necessary to cope with metabolic and oxidative stress (2–6), to increase survival during infection (7, 8) and for the development of drug tolerance and resistance (9–11). This is achieved through the inhibition of transcription of genes involved in bacterial growth (12) as well as suppression of DNA replication and protein as well as nucleotide synthesis in preparation for long term survival (1, 4, 13, 14). The signal molecules mainly responsible for triggering the stringent response are guanosine 3′-diphosphate 5′-triphosphate (pppGpp) and guanosine 3′,5′-bis(diphosphate) (pppGpp) (15, 16). In addition, guanosine 3′-diphosphate 5′-monophosphate (ppGpp) was recently shown to also contribute to the stringent response in some bacteria, such as Enterococcus faecalis and Bacillus subtilis (17–19). Collectively, these molecules are referred to as alarmones or (pp)pGpp. Alarmones are synthesized by transfer of the β and γ phosphates of ATP to the 3′-hydroxyl group of GTP, GDP, or GMP by the synthetase domain of enzymes belonging to the RelA-SpoT homologous (RSH) superfamily (13, 20), generating pppGpp, ppGpp, or pGpp, respectively (21, 22). Subsequent removal of these signal molecules, which is critical for cellular (pp)pGpp homeostasis, is achieved in a pyrophosphohydrolase reaction in which the δ and ε phosphate groups are released in the form of pyrophosphate and the original nucleotide is regenerated (23–25).

The (pp)pGpp hydrolase activity is maintained by enzymes containing a metal-dependent hydrolase (HD) domain (24), which is well conserved within the RSH family of bi-functional synthetases/hydrolases (20). These are so-called “long” bifunctional enzymes that contain both a synthetase and hydrolase domain in addition to several C-terminal regulatory domains required for controlling the activity of the two catalytic domains (20). In these enzymes, the predominant activity (synthetase/hydrolase) is controlled through a combination of external and internal inputs such as ribosome binding (26–28), the state of the regulatory domains (27, 29, 30), and allosteric interaction between the synthetase and hydrolase site (23, 31) ensuring that either synthesis or hydrolysis predominates at any given time. In addition to the bifunctional RSHs, many bacterial species encode monofunctional variants known as small alarmone hydrolases (SAHs) and small alarmone synthetases (SASs) that lack any regulatory domains (20). While long RSHs have been extensively studied (1, 32–36), much less is known about the regulation of the monofunctional variants although they appear to be regulated through higher-order oligomeric structure. For example, the B. subtilis (pppGpp SAS ReIQ is regulated through alarmone binding at an allosteric site inside a higher-order, tetrameric enzyme (37). Moreover, pervasive searches for homologs of these enzymes have revealed intriguing new
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regulatory principles, such as the ability of toxic SAS and some SAHs to act as toxin-antitoxin systems (38, 39).

SAHs contain a well-conserved HD domain (24) for which information was derived by comparison to the corresponding domain of long RSHs (23, 26–28, 31, 40, 41). The exact catalytic mechanism as well as mode of regulation of the SAH enzymes, however, have not yet been described in detail. Generally, six conserved catalytic residues, or motifs (HD1- HD6), are involved in the Mn2+-dependent reaction (23, 25, 42, 43). HD1 and HD6 comprise an Arg and an Asn residue respectively, coordinating the guanine base, while HD4 contains a Gln and Asp pair which is proposed to facilitate the nucleophilic attack on the 3’-phosphate group of the substrate (23, 43). Finally, HD2 (His), HD3 (His and Asp), and HD5 (Asp and Arg/Lys) are involved in manganese ion coordination (23, 42, 43). Curiously, the first SAH structures determined were of the metazoan ortholog Mesh1 from human and Droso phila melanogaster (Mesh1Hs and Mesh1Dm, respectively), which were shown to possess (p)ppGpp hydrolase activity both in vitro and in vivo (43). The discovery of a metazoan alarmone hydrolase was initially surprising, as no role for alarmones had been identified in higher organisms at the time. Later, Mesh1 was shown to function as a NADPH phosphatase required in development and ferroptosis-mediated cell death caused by metabolic starvation (44). Recently, the first structure of a bacterial SAH from Pseudomonas aeruginosa (PaSAH) was reported (42). PaSAH is also capable of degrading (p)ppApp and required for biofilm formation (45), expanding its function to interbacterial growth competition. Finally, members of the Nudix family, which are known for their substrate ambiguity (46), have also been shown to be involved in alarmone degradation and nucleotide homeostasis both in plants (47) and bacteria (19, 48, 49).

In this article, we present crystal structures of two unique SAH enzymes from Corynebacterium glutamicum (RelH_{Cg}) and Leptospira levettii (RelH_{Ll}) and show that both enzymes are catalytically active as dimers. Using this information and through comparison with the bifunctional RSHs, we reveal conserved structural features unique to the monofunctional SAHs. We observe that dimerization is a general feature of bacterial SAHs but that the exact mode of dimer conformation varies, which might influence regulation or stability, or both, in diverse bacterial species. Furthermore, we show that RelH_{Cg} is subject to product-dependent inhibition by GTP/GDP. RelH_{Ll} is less active than RelH_{Cg} and does not seem significantly affected by its products, potentially instead relying on active site accessibility for regulation. Our results suggest that regulatory mechanisms differ between bacterial species and may be controlled by the type of dimer formed. We therefore propose that SAHs evolved to form homodimers with diverse dimerization interfaces and regulation mechanisms, likely linked to the maintenance of GTP/GDP homeostasis in some bacteria.

Results

The SAH of C. glutamicum forms a dimer

To better understand SAH function and regulation, we decided to determine the crystal structure of the most well-characterized member of the SAH family, C. glutamicum, RelH_{Cg} (eg1485/cgl1313, 188 amino acids, UniProt ID: Q8NQV9) (50, 51). The structure was determined using seleno-methionine (Se-Met)–derivatized protein by the single wavelength anomalous diffraction method at 2.3 Å in the primitive hexagonal space group, P6_3. This model was then transferred to a higher resolution 1.8 Å native dataset that was used to build the final model. The final structure has R_{work} = 17.6% and R_{free} = 20.5% (see Table 1 for data collection and refinement statistics). The asymmetric unit contains two nearly identical molecules of RelH_{Cg} (Fig. 1A) of which one (chain A) was fully traced to the C-terminus (A188), including the linker and the first two residues of the histidine affinity tag to H192. The second molecule (chain B) could be traced to L185, except for a loop region (145–148) connecting helix α8 and α9. The structure reveals a core fold of ten α-helices with a cavity open to solvent where the active site of the HD domain is located (Fig. 1A) (24). All conserved residues typically involved in the hydrolase activity (R24, H34, H58, D59, E62, D63, D122, K123, and N126) are located inside this cavity (Fig. 1, A and B) of which R24 and N126 (motifs HD1 and HD6) are expected to coordinate the guanine base of the substrate alarmone. R24, which is responsible for π-stacking interactions with the base (23, 31, 42), is observed in two alternative conformations in our structure, probably reflecting the lack of substrate in the active site (Fig. 1B). At the center of the active site cavity, H34, H58, D59, and D122 (the HD2, HD3 and HD5 motifs) coordinate a divalent cation necessary for hydrolase activity (Fig. 1, B and C) (24). A strong positive electron density in the anomalous difference map at this site suggests this ion is Mn^{2+} (Fig. 1C), consistent with the presence of this ion in the crystallization buffer. Moreover, two well-ordered waters completing the octahedral coordination shell are clearly visible (Fig. 1C). E62 and D63 (motif HD4) are known to be involved in the nucleophilic attack on the nucleotide 3’-phosphate (23). Closer inspection of the interaction surface between the two molecules in the asymmetric unit reveals a pocket containing a set of hydrophobic residues typical of protein interfaces. Analysis of the protein contacts in the crystal using the PISA (Proteins, Interfaces, Structures, and Assemblies) server (52) revealed an interface with a total buried area of ~1286 Å², consistent with a homodimer of biological relevance. Finally, to obtain a ligand-bound structure of the enzyme, we next crystallized both WT protein as well as several inactive mutants (E62N, D63N, N126D, N126L, and R24Q) of RelH_{Cg} in presence of substrate (pppGpp or ppGpp) or product (GTP, GDP) at molar ratios of 1:3 to 1:5 (protein:ligand). However, these experiments produced poorly diffracting crystals of insufficient quality to allow structure solution.

RelH_{Cg} and RelH_{Ll} form structurally distinct dimers

To understand if dimerization is a unique characteristic of RelH_{Cg} or a more general feature of SAHs, we selected a homolog from the gram-negative bacterial species L. levettii (CH368_08595, 196 amino acids, UniProt ID: AOA2N0AXP5) as our target for further study. We obtained crystals of RelH_{Ll}...
in the primitive tetragonal space group P4ₓ2ᵧ2z that diffracted to 1.2 Å resolution and were able to determine the structure by molecular replacement using the RelHᵥCᵥ monomer as a search model and refine the structure to Rₓwork = 15.6% and Rₓfree = 18.3% (Fig. 1D and Table 1). In this crystal form, there is a single monomer of RelHᵥLᵥ in the asymmetric unit, but analysis by PISA (52) reveals a dimer interface across the crystallographic two-fold axis, larger than the one observed in RelHᵥCᵥ with a total buried surface of ~1761 Å². To experimentally confirm the structural observation that both proteins form dimers, we used size-exclusion chromatography–multi-angle light scattering and obtained solution masses of 43.1 kDa for RelHᵥCᵥ and 44.4 kDa for RelHᵥLᵥ (Fig. 1E). As the proteins have monomeric masses of 22.4 kDa (RelHᵥCᵥ) and 23.3 kDa (RelHᵥLᵥ), these results are consistent with homodimers in both cases. Moreover, slightly different elution volumes for RelHᵥCᵥ (11.0 ml) and RelHᵥLᵥ (10.6 ml) are consistent with the different Stokes radii assumed by the two dimers.

Comparison of the RelHᵥCᵥ and RelHᵥLᵥ monomers shows a high degree of structural similarity, with an identical active site conformation (Figs. S1 and S2A). For RelHᵥLᵥ, Mn²⁺ was not added to the purification or crystallization buffers, nevertheless, we still observe an anomalous density peak compatible with a manganese ion in the active site metal coordination pocket (Fig. S2B), suggesting that this ion was carried over from the expression host. As in the case of RelHᵥCᵥ attempts to crystallize WT or inactive mutants (D71A, N137L, or R32Q) of RelHᵥLᵥ in presence of nucleotides did not yield crystals with sufficient diffraction. Both the RelHᵥCᵥ and RelHᵥLᵥ dimers are stabilized by a strongly hydrophobic interface generated by the N-terminal helices and Table 1). In RelHᵥCᵥ, this core is centered on four interfacing residues (Fig. 2A). In RelHᵥLᵥ, this core is centered on four interfacing residues (Fig. 2B). The two tyrosine residues are not conserved in RelHᵥLᵥ where they are substituted by L44 and S48 (Fig. 2A). In RelHᵥLᵥ, the active site is centered on two tyrosine residues and the substrate cavities are exposed on opposite sides of the dimer (Fig. 1F). As the active site is centered on two tyrosine residues and the substrate cavities are exposed on opposite sides of the dimer, the hydrophobic core of RelHᵥLᵥ involves different amino acids and the dimer has an overall different conformation (Fig. 2A, bottom). Moreover, while the substrate cavities open up on the same side of the dimer surface in RelHᵥCᵥ, the monomers are rotated 180° with the substrate cavities exposed on opposite sides of the dimer in RelHᵥLᵥ (compare Figs. 1A and 1D). In RelHᵥLᵥ, F16, L20, and L24 are located at the center of the dimeric helical arrangement and form a hydrophobic core (Fig. 2A, bottom left). As

Table 1

Crystallographic data collection and refinement statistics

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Values in parentheses represent the highest resolution shell. The data was treated and models refined against anomalous data, indicated values account for merged Friedel pairs.
the replacement of Y36 and Y40 of RelHCg with L44 and S48 in RelHLl represents one of the major differences of their hydrophobic dimerization interfaces, we probed the importance of the tyrosine residues for dimer formation by generating a RelHCg Y36E-Y40E double mutant to destabilize the dimer interface by electrostatic repulsion and drive the equilibrium toward monomers. The resulting protein was not soluble, suggesting that dimerization is likely required for protein stability and thus, natural function of the enzyme.

Outside of its hydrophobic core, RelHCg has only one notable dimer interaction between R9 of helix α1 from one monomer and D132 of α7 from the other (Fig. 2A, top right). These residues are replaced by glycine and serine in RelHLl, respectively, and do not interact (Fig. 2B). Instead, the interaction interface of RelHLl extends between helices α7, α10, and α2. This region contains several polar residues creating a network of hydrogen bonds and salt bridges between the monomers (Fig. 2B). At its center, R139 from each monomer forms a π-stacking interaction with the corresponding residue from the other molecule, which is supported and kept in position by E186 of the same monomer (Fig. 2A, bottom right). Except for E52, which is located on α2, all other residues involved in electrostatic interactions for dimer formation are located at the C-terminus of RelHLl (Fig. 2B). Of the nine residues involved in helices α7 and α10, H136, S140, D144, R182, and E186 of RelHLl are conserved between the two hydrolases (Fig. 2B), but intriguingly they do not form dimer contacts in RelHCg. Furthermore, R182 belongs to helix...
Of RelHLl, while R166 of RelHCg belongs to helix α9. The remaining four of the corresponding residues of RelHCg are substituted with side chains that are either hydrophobic or of opposite charge. Thus, the conformational differences of the interaction interfaces of RelHCg and RelHLl allow for the major deviation between the positions of the respective monomers observed and their substrate cavity in the two enzymes.

To test the recent developments in protein structure prediction against our findings, we generated prediction models of currently deposited SAH structures, RelHCg and RelHLl using the AlphaFold-Multimer system of AlphaFold2 (53) under the assumption that they can dimerize. This list includes *P. aeruginosa* SAH (PaSAH, PDB ID: 6YVC) (42), the metazoan orthologs Mesh1 from human (Mesh1Hs, PDB ID: 3NR1 and 5VXA) and *D. melanogaster* (Mesh1Dm, PDB ID: 3NQW) (43, 44), and the unpublished putative SAH from *Listeria monocytogenes* (RelHLm, PDB ID: 4YF1). Five models were created for each SAH (Fig. S3). We observed that the predicted

**Figure 2. Dimerization interfaces and sequence comparisons of RelHCg and RelHLl.** A, comparison of the RelHCg (teal) and RelHLl (amber) dimers and close-up views of their respective dimerization interfaces. A selected subset of residues involved in the interaction are highlighted as sticks and labeled. At the center, the top monomers are aligned to highlight the differences in dimer formation. **Left,** hydrophobic dimer interfaces in RelHCg (top) and RelHLl (bottom), respectively; **right,** inter-subunit electrostatic interactions found in RelHCg and RelHLl, respectively. **B,** pairwise sequence alignment of RelHCg and RelHLl with indication of secondary structure shown in teal (RelHCg) and amber (RelHLl). Black dots indicate every 10th residue of each sequence. Identical residues are shown in white on a brown background, while residues with similar chemical properties are shown in red. Active site residues are shown in white on a gold background, and black arrows indicate hydrophobic residues involved in interactions at the dimer interfaces. Finally, blue arrows indicate polar residues involved in electrostatic interactions.

α10 of RelHLl, while R166 of RelHCg belongs to helix α9. The remaining four of the corresponding residues of RelHCg are substituted with side chains that are either hydrophobic or of opposite charge. Thus, the conformational differences of the interaction interfaces of RelHCg and RelHLl allow for the major deviation between the positions of the respective monomers observed and their substrate cavity in the two enzymes.

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models of RelHC₈₉, RelH₁₃₁, PaSAH, Mesh₁₄₁₁, and Mesh₁₄₁₃ had very high local and global confidence prediction (Fig. S4, A–C, E and F) and converged to dimers that aligned to PISA-predicted dimers from experimental crystallographic data with low RMSD values for the Ca backbone ranging from ~0.3 to ~0.8 Å (Table S1 and Fig. S3). The predicted RelH₁₃₁₃ models, however, had good local confidence but showed poor predicted alignment error scores instead, indicating that the relative orientation of the domains is unreliable (Fig. S4D). Furthermore, the predicted RelH₁₃₁₃ dimers did not converge toward any singular dimer conformation and all predicted conformations were different from those observed in the crystal structure (Fig. S3 and Table S1). We believe that these results highlight that AI-based protein structure prediction, particularly when involving intermolecular contacts, should go together with experimental verification to avoid pitfalls.

RelHC₈₉ is a highly efficient hydrolase limited by product-dependent inhibition

To understand if the SAH enzymes are active as dimers, we purified the proteins on their dimer forms and carried out in vitro hydrolysis reactions in which we separated the substrate alarmones (p)ppGpp from their GTP/GDP products by ion exchange chromatography (Fig. S5). In time course experiments, the concentrations of each enzyme were adjusted to 0.05 μM for RelHC₈₉ and 2.5 μM for RelH₁₃₁ to achieve measurable hydrolysis curves and better estimate initial rates. For RelHC₈₉, we observed specific activities of 0.59 μmol/min/mg (pppGpp) and 2.23 μmol/min/mg (ppGpp). Notably, only ~50% pppGpp was hydrolyzed after 180 min (Fig. 3A, left). In contrast, RelH₁₃₁ showed similar rates of pppGpp and ppGpp hydrolysis but was overall much less efficient than RelHC₈₉. In this case, we observed specific activities of 0.039 μmol/min/mg (pppGpp) and 0.026 μmol/min/mg (pppGpp). Unlike RelHC₈₉, RelH₁₃₁ was able to hydrolyze up to 90% of total pppGpp, at a slightly higher rate for pppGpp compared to ppGpp (Fig. 3A, right).

It has previously been observed that >500 μM substrate (alarmone) concentrations can inhibit RelHC₈₉ (50), however, those findings do not explain the observations in our experimental setting, where we observe a clear reduction of pppGpp hydrolysis activity over time in RelHC₈₉ starting from an alarmone concentration of 50 μM (Fig. 3A, left). To check for regulation by nucleotides, we next decided to study the effect of the presence of the products GTP and GDP on the hydrolysis reaction. Upon increasing the concentration of GTP, we observed a clear inhibition of alarmone degradation by RelHC₈₉ (Fig. 3B, left). GDP also seems to have an inhibitory effect, although weaker than GTP, most noticeable against ppGpp hydrolysis in presence of 400 μM GDP (Fig. 3C, left). In the case of RelH₁₃₁, only ppGpp hydrolysis appeared to be affected by 400 μM GTP, while addition of GDP had only a minimal effect (Fig. 3C, right).

To determine if product inhibition of RelHC₈₉ and RelH₁₃₁ is competitive or allosteric, we used the differential radial capillary action of ligand assay (DRaCALA), which measures small molecule affinity in the presence of an antagonist (54–56). A one-way ANOVA was conducted to assess if cold antagonists influenced the fraction of binding of radiolabeled nucleotides. A post-hoc Dunnett’s multiple comparison test was then performed where an effect was observed, to determine if there were significant differences between each cold nucleotide and the buffer control. After binding of radiolabeled GTP (α-32P-GTP) to RelHC₈₉, we observed reduced binding fractions of α-32P-GTP upon incubation with cold GTP, GDP, pppGpp, or ppGpp (p < 0.0001) (Fig. 4A, left) suggesting that these compounds can displace bound α-32P-GTP and thus compete directly with GTP for binding. RelH₁₃₁ showed no evidence of binding α-32P-GTP in the first place (Fig. 4A, right), consistent with the lack of product inhibition in Figure 3B, right. Moreover, pppGpp (p = 0.0104) and ppGpp (p = 0.0111), but not GTP or GDP, significantly reduced the binding of radiolabelled pppGpp (α-32P-pppGpp) to RelHC₈₉ (Fig. 4B, left). Similarly, pppGpp (p = 0.0378) and ppGpp (p = 0.0050) significantly reduced binding of α-32P-pppGpp binding to RelH₁₃₁ (Fig. 4B, right). It should be noted, however, that the fraction of α-32P-pppGpp binding might be influenced by the enzymatic activity of RelHC₈₉ and RelH₁₃₁. Nevertheless, these data suggest that the nucleotide products, GTP and GDP, compete for the active site binding with alarmone to RelHC₈₉ while they did not show a significant effect on RelH₁₃₁ in this setup.

Monofunctional SAHs contain structural elements absent in bifunctional RSHs

Despite their differences, dimerization of RelHC₈₉ and RelH₁₃₁ in both cases involves the N-terminal part of the protein. The hydrophobic pocket formed by helices α₁, α₂, and α₃ at the dimerization interface of SAHs is structurally present in bifunctional RSHs but is obscured by an additional N-terminal helix, as observed in example Rel from Streptococcus dysgalactiae subsp. equisimilis (RelSeq, PDB ID: 1VJ7) (23). This N-terminal helix buries the hydrophobic residues that would otherwise be exposed and thus compensates for the lack of a dimeric interface to shield these residues from solvent (Fig. S6).

Similar structural patterns are observed in the structures of Rel₁₇₃ from Thermus thermophilus (PDB ID: 6S2T, 6S2U, 6S2V) (31), BsRel from B. subtilis (PDB ID: 6YXA) (40), and MtRel from Mycobacterium tuberculosis (PDB ID: 5NXN) (41). The positions of helices α5 and α6 (corresponding to α6 and α7 in bifunctional Rεd enzymes) also differ between monofunctional SAHs and bifunctional RSHs. Upon substrate binding in the synthetase domain of the bifunctional Rel₁₇₃, helix α7 undergoes a massive conformational rearrangement, switching the entire enzyme from the hydrolase-ON/synthetase-OFF to the hydrolase-OFF/synthetase-ON state (31). This shift results in movement of a loop (residues 108–121 of Rel₁₇₃) toward the hydrolase active site, placing Y111 at the guanine base position, effectively preventing switching the entire enzyme from the hydrolase-ON/synthetase-OFF to the hydrolase-OFF/synthetase-ON state (31). This shift results in movement of a loop (residues 108–121 of Rel₁₇₃) toward the hydrolase active site, placing Y111 at the guanine base position, effectively preventing switching the entire enzyme from the hydrolase-ON/synthetase-OFF to the hydrolase-OFF/synthetase-ON state (31).
to the downward facing α9 helix via the perpendicular α8 (residues 137–148 in RelHCg), is found in the monofunctional RelHCg, RelHLl, and RelHLm structures. In the bifunctional RelSeq, RelTh, BsRel, and MtRel, on the other hand, the corresponding α7 helix is shorter and is connected to the downward helix by a flexible loop, instead of another helix (Fig. 5B). This is not unique to bifunctional RSHs, though, as the metazoan SAH orthologs Mesh1Ds (PDB: 3NR1, 3NQW), Mesh1Hs (PDB: 5VXA), as well as the bacterial PaSAH (PDB: 6YVC), all are reminiscent of the bifunctional enzymes, with a short helix and a connecting loop.

The active site of SAHs is conserved with bifunctional bacterial Rel enzymes

Sequence-wise, bifunctional RSHs and SAHs show great differences with the exception of the core active site residues, which are highly conserved as would be expected given that they catalyze identical reactions (Fig. 6) (20). Examination of the key differences in residues reported to be involved in substrate coordination and activity (23) reveals that the HD1 motif (R44) of bifunctional RelSeq (23), which is involved in π-stacking with the guanine base of (p)ppGpp, is well conserved in both monofunctional and bifunctional enzymes, with the notable exception of SAH from P. aeruginosa (PaSAH), where it is substituted for leucine (42) (Fig. 6). Interestingly, while mutation of this arginine to glutamate (R44Q) inactivates the hydrolase activity of RelSeq (23), PaSAH remains active with a Leu at this position (42). In the selected set of SAH sequences, the HD1 Arg is often substituted for Asp, which frequently coincides with another substitution in the HD5 domain, where a D:R/K pair is replaced by D:Φ (where Φ is any hydrophobic amino acid) (Fig. S7). It is currently unknown whether these differences influence
activity, regulation, or both. Notably, while containing the leucine mutation at the HD1 position, the HD5 motif of PaSAH is intact (Figs. 6 and S7). In RelSeq, the side chain of R44 (HD1) is held in place by direct H bonds to residues N148 (which forms the HD6 motif of the HD domain) and T151 when coordinating a nucleotide. The HD6 motif is clearly conserved in all examined RSHs, while T151, conserved in the bifunctional RSH we have included, is conservatively substituted for a Ser in RelHCg (S129), RelHLl (S140), and RelHLm (S120), but not in PaSAH or the Mesh1 orthologs (Fig. 6). This interaction is not present in our structures in absence of a nucleotide in the active site. Although a Ser substitution appears widespread, it is not necessarily shared by all SAHs (Fig. S7).

K45 of RelSeq, which forms an H bond with the substrate, appears to be maintained or conservatively substituted with Arg in most hydrolases, as in the case of bifunctional RelTh (31). S46 from RelSeq is described to stabilize R44 via a water-mediated H bond, as well as forming another H bond with the guanine base of (p)ppGpp. While RelSeq S46 seems well conserved in bifunctional hydrolases (23) and is also found in the bifunctional RelTh (31), it seems often substituted by hydrophobic residues or glycines in monofunctional SAHs (G26 of RelHCg and G34 of RelHLl, Figs. 6 and S7). Additionally, the loop of which the residue is a part assumes a different position in monofunctional hydrolases compared to bifunctional RSHs, as the result of a residue insertion where G26 is followed by an additional residue (Gly in RelHCg, Thr in RelHLl, and Ser in PaSAH) (Figs. 5C and 6). Due to this insertion, this loop assumes two slightly different conformations when comparing monofunctional and bifunctional hydrolases (Fig. 5C). Furthermore, metazoan hydrolases Mesh1Hs and

**Figure 4. Nucleotide competition assay.** A, measurement of the binding fraction of radioactive α-³²P-GTP to RelHCg (left) and RelHLl (right) in the presence of buffer or when challenged by 100 μM cold competitor, as indicated. The bars show the average of two independent experiments with SD. Single experiments are shown as black dots. B, as in A but measured for the binding of α-³²P-pppGpp. Statistically significant differences in the fraction of binding between cold competitors and buffer controls are shown as indicated: *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. pppGpp, guanosine 3’diphosphate 5’triphosphate.
Mesh1Dm present a third distinct conformation reflected by an additional insertion compared to the bacterial SAHs (Fig. 5C). Although the loop containing the HD1 motif can adopt at least three distinct geometries, the position of the HD1 motif (R44 in RelSeq) itself does not differ significantly and occupies virtually the same position in all the observed geometries of the loop from the currently known structures.

Discussion

**SAHs have diverse oligomerization interfaces across species**

Our studies clearly show that RelHc8 and RelHlL are active dimers in solution and since disruption of the interface causes the proteins to become unstable, this is most likely also the case inside cells (Figs. 1 and 2). In both cases, the dimerization interface mainly involves a hydrophobic region formed by the first three N-terminal helices (Fig. 2A). A similar hydrophobic region is also present in bifunctional RSHs but is buried by an additional N-terminal helix (Fig. S6), which prevents bifunctional enzymes from making similar interactions through this side of their hydrolase domain. Furthermore, although the hydrophobic core is still central for dimerization in RelHc8 and RelHlL, we observed two distinct dimers (Fig. 2A). Based on this observation, we further extended our analysis to the other SAHs currently deposited in the PDB (PaSAH, Mesh1Dm, and RelHlM). Analysis of these structures via PISA (52) confirmed the presence of strong dimer interfaces ranging from 1200 to 1350 Å², except for RelHlM, which only has a surface of ~800 Å² but also shows other atypical characteristics, as detailed below. All dimers, except RelHlM, contain a hydrophobic region surrounded by three N-terminal α-helices and in some cases electrostatic interactions. Comparison of the dimers shows that they assume five distinct conformations, with the two Mesh1 homologs as the only pair of identical dimers (Fig. S8). In the unique case of RelHlM, we note that the position of the active site residues in the deposited model does not allow for coordination of a metal ion, which is accordingly absent. Furthermore, the first N-terminal α-helix, which is an integral component of the interaction interface in other SAHs, is not present in the deposited RelHlM model, where the first 20 N-terminal amino acids that are likely to form this helix are unmodeled. Therefore, we believe that the dimer observed in the crystal structure of this protein might...
be an artefact, possibly due to degradation (or unfolding) of the N-terminal region.

**In silico structure prediction should be used with caution**

AlphaFold2 models support the experimentally determined dimers in all cases with high confidence, except for RelH1m (Figs. S3 and S4). In the RelH1m AlphaFold2 dimer prediction in particular, the proposed dimer conformations cannot be considered reliable due to poor predicted alignment error scores (Fig. S4D). Overall, these observations suggest that the dimers predicted by PISA in the other deposited SAHs represent the biologically relevant configuration of the proteins. While prediction models should be used with considerable caution, AlphaFold2 has already been shown to be a powerful tool that is able to predict folded protein domains with great accuracy (53, 57–60). Moreover, application to multidomain or oligomeric proteins has shown great promise, although their reliability depends on the target and critical evaluation of their confidence metrics (61, 62).

**The activity of RelHCG is controlled by a substrate-dependent competition mechanism**

We observed that both SAHs were able to effectively hydrolyze ppGpp, although at different rates (Fig. 3A).
Interestingly, RelH_{Cg} was much more efficient than RelH_{Ll} but was unable to fully hydrolyze pppGpp (Fig. 3A). When RelH_{Cg} was first characterized, inhibition of the hydrolysis reaction at alarmone concentrations above 300 to 500 μM was interpreted as substrate-dependent inhibition (50). Although our tests were conducted at only 50 μM alarmone concentration, 6- to 10-fold less, we observe a marked reduction of pppGpp hydrolysis over time (Fig. 3A, left). Based on this, we hypothesize that the products GTP and GDP, rather than pppGpp, might be responsible for the reduction in activity. In support of this, our DRaCALA data suggest that both GTP and GDP have an inhibitory effect on the binding of pppGpp and ppGpp by RelH_{Cg}, but not RelH_{Ll} (Fig. 3, B and C). Furthermore, competition experiments suggest that this inhibition is driven by substrate displacement in the active site (Fig. 4, A and B). It was previously shown that synthesis and hydrolysis of alarmones directly contribute to the homeostasis of GTP levels in *B. subtilis* (3, 65) and that perturbation has a wide range of consequences on bacterial viability (36, 66), including the possibility of causing "death by GTP" due to excessive accumulation of this nucleotide (3). Here, product-dependent inhibition might be an effective mechanism of limiting the complete and sudden degradation of alarmones, allowing to maintain control of GTP/GDP levels and fine tune the wide range of alarmone effectors with which they interact.

### Active site access as a possible controller of RelH_{Ll} hydrolysis

RelH_{Ll} not only did not significantly respond to GTP or GDP concentrations in our experiments (Fig. 3, B and C, right and Fig. S3, A and B, right) but had considerably lower activity than RelH_{Cg} (Fig. 3A), possibly requiring even higher concentrations of GTP/GDP for regulation, or relying on a different level of control altogether. Curiously, the remaining 10% of total pppGpp could not be hydrolyzed (Fig. 1A, right), although RelH_{Ll} did not show clear sensitivity to GTP. Considering that RelH_{Ll} and RelH_{Cg} active sites are virtually identical (Fig. S2A) and folds are highly similar (Fig. S1), we believe that the position of helix α8 of RelH_{Ll} might influence substrate binding (Figs. 5B and S9). Comparison of the position of ppGpp as seen in the active site of Rel_{Th} (PDB ID: 6S2T) highlights how helix α8 of RelH_{Ll} partially covers the active site, bringing S157 within ~4 Å of the β-phosphate at the 5′ position of the substrate (Fig. S9A). This potentially creates both steric hindrance and electrostatic repulsion at the active site access point, which would lower the affinity of (p) ppGpp. The position of helix α8 in RelH_{Cg} even accounting for differences between the two monomers in our structure, allows for greater accessibility without occluding entry for the substrate (Fig. S9B).

### Monofunctional SAHs show structural similarity to the inactive hydrolase state of bifunctional Rel enzymes

An intriguing observation is that the position of helices α5 and α6 in the SAH monomers closely resembles the described hydrolase-OFF state of bifunctional Rel_{Th} (Fig. 5A), despite the SAHs being active as dimers *in vitro*. In this conformation, the hydrolase active site of Rel_{Th} is occluded by a long loop and inaccessible, while the synthetase domain is active (31). However, the corresponding loop at this position in SAHs is generally shorter and, although rather flexible, it points away from the active site. Since monofunctional enzymes do not require the type of allosteric control necessary to regulate the activity of bifunctional RSHs (23, 40), a more compact and stable form might have been favored over time. Regulation of the SAHs could instead come from the α8 helix we observe in RelH_{Cg} and RelH_{Ll}, which is not present in bifunctional Rel_{Th}, Rel_{seq} (BsRel, and MtRel) that have a simple loop (Fig. 5B). This, however, does not appear to be a universal feature of SAHs. In the currently known structures, three SAHs contain the α8 helix at the top of the active site (RelH_{Cg}, RelH_{Ll}, and RelH_{Lm}), while another three have a loop like the RSHs instead (OtaSAH, Mesh1_{Eco} and Mesh1_{Dm}) (Fig. 5B). However, we do not yet have sufficient information to determine whether this region significantly impacts the activity of these enzymes. Finally, a potentially important detail is found at the loop where the HD1 motif is located (R44 of Rel_{seq}). We note that while the HD1 motif position does not change, bacterial SAHs have an insertion right after, while the metazoan SAHs contain two insertions making the loop progressively longer (Fig. 5C). It would be highly relevant to study whether these features could affect function or regulation. Considering the importance of nucleotide homeostasis and the observation that SAHs appear constitutionally active *in vitro*, multiple levels of control might need to be investigated depending on the bacterial species involved. As for many enzymes of this type, transcriptional regulation might be the first level. Substrate accessibility, affinity, and feedback mechanisms, such as the product-dependent inhibition of RelH_{Cg}, are equally likely to be involved and should be studied in further detail.

### Experimental procedures

#### Strains and plasmids

The coding sequences of WT *C. glutamicum* ATCC 13032 RelH (Q8NQV9) and *L. levettii* SAH (WP_100728644.1) were codon optimized for *Escherichia coli* expression, synthesized, and subcloned into pET22b expression vectors tagged with a C-terminal non-cleavable 6x polyhistidine tag (GenScript Biotech). Point mutants of RelH_{Cg} (E62N, D63N, N126D, N126L, R24Q, Y36E-Y40E) and of RelH_{Ll} (R32Q, D71A, N137L) were created by site-directed mutagenesis using Phusion high fidelity polymerase (New England Biolabs) with the primers listed in Table S2, using RelH_{Cg} and RelH_{Ll} WT clones as templates.

#### Protein expression and purification

All constructs were transformed into chemically competent *E. coli* Lemo21(DE3) cells and grown on LB agar plates in the presence of 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The RelH_{Cg} construct was also transformed into the methionine auxotroph *E. coli* B834(DE3) strain for production of the Se-Met derivative protein. A 20 ml overnight culture of
Small alarcone hydrolase dimers

each strain was diluted in 1 l of LB medium with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and grown at 37 °C until OD600 reached ~0.6. With the exception of the Se-Met derivative protein, expression was induced by addition of 1 mM IPTG and incubation continued at 37 °C for 3 h. Cultures were harvested by centrifugation at 6000 g for 15 min and the pellets collected and resuspended in a lysis buffer composed of 50 mM Hepes, pH 8, 500 mM KCl, 20 mM Imidazole, 5 mM β-mercaptoethanol.

To prepare the Se-Met derivative of RelHCg bacteria were harvested by centrifugation at 3500g after reaching OD600 ~0.6 and resuspended in minimal Selenomethionine Medium Base (Molecular Dimensions) supplemented with methionine-less Selenomethionine Medium nutrient mix (Molecular Dimensions), 100 μg/ml ampicillin, and 34 μg/ml chloramphenicol. The cultures were incubated at 37 °C for 3 h to deplete leftover methionine before adding Se-Met at a final concentration of 0.04 mg/ml and induction of expression with 1 mM IPTG. After 24 h, cultures were harvested and treated as previously described.

For all constructs, lysis was performed by sonication and the total lysate centrifuged at 20,000g for 45 min to remove cell debris. The supernatant was applied to a 5 ml HisTrap FF column (Cytiva) and washed with a buffer composed of 50 mM Hepes, pH 8, 500 mM KCl, 50 mM Imidazole, 5 mM β-mercaptoethanol. Elution was performed by increasing the imidazole concentration to 500 mM. Protein fractions were concentrated to the desired concentration for storage or need additional cryoprotection and were frozen directly.

Data collection and structure solution

All data was collected from frozen crystals at 100 K at the P13 (67) or P14 beamlines of Petra III (68) using a PILATUS 6M or EIGER 16M detector, respectively at the Se x-ray peak absorption wavelength of 0.97624 Å. Data were processed in XDS (69) with XDSSGUI. The first structure of RelHCg at 2.3 Å resolution was determined from a Se-Met derivative crystal using the single wavelength anomalous diffraction phasing method with the CRANK2 pipeline (70) of the CCP4 suite (71, 72). High-resolution native structures of RelHCg and RelHLl (1.8 and 1.2 Å respectively) were then subsequently determined by molecular replacement via PHASER (73) of the CCP4 suite using the Se-Met structure as a search model. To build the final structures, successive iterations of refinement with phenix.refine (74) using the Phenix graphical interface (75) or Buster (76, 77) and manual building with Coot (78) were performed. At each iteration, geometry quality was validated with MolProbity (79, 80). To improve model quality of the native and Se-Met RelHCg structures, translation-libration-screw rotation (TLS) domain decomposition was used to account for vibrational motion of discreet regions (81). Boundaries of each TLS group per model were determined by analysis through the TLS motion determination webserver (82). Ordered water molecules were added to all models. A density we interpret as a PEG ligand fragment was found in the map of native RelHCg. The ligand structure was retrieved from the PDB (PDB ID: PG4) and used in refinement along with restraints generated using Grade from the Grade Web Server (http://www.globalphasing.com) (83). In the case of the RelHLl model, atomic displacement parameters for the whole model including waters were anisotropically refined. In the final rounds of refinement, hydrogen atoms in riding positions were added to this model using Reduce (84) as implemented in Phenix to improve the geometry. Finally, ordered solvent occupancies were refined. Figures of protein structures were

Native RelHCg crystals were cryoprotected by dipping in a drop composed of mother liquor supplemented with 25% (v/v) PEG 400 and 2 mM MnCl2. Crystals of RelHLl were soaked in drops of a fresh buffer of the same composition as the mother liquor with the addition of 20% (v/v) glycerol. All crystals were vitrified in liquid N2 for storage and transport before data collection.

WT and inactive mutants of RelHCg and RelHLl were co-cryocrystallized or soaked in presence of GTP, GDP, ppGpp, or ppGpp. A molar ratio of 1:3 or 1:5 protein to nucleotide was used in both type of experiments. In cocryocrystallization experiments, protein and nucleotides were mixed and incubated 5 to 30 min before setting up crystallization drops. Upon successful crystallization, crystals were cryoprotected as previously described or in a suitable buffer with 0.5 mM of the respective nucleotide added. In soaking experiments, nucleotides were directly added to the crystallization drop or to the mother liquor, which was then applied to the crystallization drop. Finally, 0.5 mM of the respective nucleotide was added to the respective cryoprotection buffer before harvesting.

Data collection and structure solution

All data was collected from frozen crystals at 100 K at the P13 (67) or P14 beamlines of Petra III (68) using a PILATUS 6M or EIGER 16M detector, respectively at the Se x-ray peak absorption wavelength of 0.97624 Å. Data were processed in XDS (69) with XDSSGUI. The first structure of RelHCg at 2.3 Å resolution was determined from a Se-Met derivative crystal using the single wavelength anomalous diffraction phasing method with the CRANK2 pipeline (70) of the CCP4 suite (71, 72). High-resolution native structures of RelHCg and RelHLl (1.8 and 1.2 Å respectively) were then subsequently determined by molecular replacement via PHASER (73) of the CCP4 suite using the Se-Met structure as a search model. To build the final structures, successive iterations of refinement with phenix.refine (74) using the Phenix graphical interface (75) or Buster (76, 77) and manual building with Coot (78) were performed. At each iteration, geometry quality was validated with MolProbity (79, 80). To improve model quality of the native and Se-Met RelHCg structures, translation-libration-screw rotation (TLS) domain decomposition was used to account for vibrational motion of discreet regions (81). Boundaries of each TLS group per model were determined by analysis through the TLS motion determination webserver (82). Ordered water molecules were added to all models. A density we interpret as a PEG ligand fragment was found in the map of native RelHCg. The ligand structure was retrieved from the PDB (PDB ID: PG4) and used in refinement along with restraints generated using Grade from the Grade Web Server (http://www.globalphasing.com) (83). In the case of the RelHLl model, atomic displacement parameters for the whole model including waters were anisotropically refined. In the final rounds of refinement, hydrogen atoms in riding positions were added to this model using Reduce (84) as implemented in Phenix to improve the geometry. Finally, ordered solvent occupancies were refined. Figures of protein structures were
created using Pymol (Schrödinger LLC) (85). The RelH$_{L1}$ dimer was reconstructed through the crystallographic symmetry operation across a two-fold crystallographic symmetry axis (Fig. S10).

**SEC-MALS analysis**

For each sample, 20 μl of purified protein at 5 mg/ml was injected on a Superdex 75 Increase 10/300 GL column (GE Healthcare) and MALS spectra recorded using inline DAWN-HELEOS light scattering and Optlab T-Rex refractive index detectors. Prior to the runs, a bovine serum albumin control was used to calibrate the instrument. The mass was determined by analyzing the differential refractive index using the Debye model for proteins (86) integrated in the ASTRA VI software.

**Time course and inhibition hydrolysis experiments**

Hydrolysis reactions were prepared in 50 mM Hepes, pH 8, 200 mM KCl, 1 mM MnCl$_2$, with pppGpp or ppGpp (Jena Bioscience) added to each test tube to a final concentration of 50 μM. Due to the large differences in hydrolysis rates, RelH$_{Cs}$ was added to a final concentration of 0.05 μM, while RelH$_{L1}$ was at a concentration of 2.5 μM. The reactions were conducted at 30 °C in triplicate at 0, 2, 5, 15, 30, 60, 120, and, additionally for pppGpp, 180 min. At each time point, 100 μl of the reaction was removed and 5 mM EDTA added to quench the reaction. This sample was then injected into a 1 ml MonoQ S/50 GL column (GE Healthcare) equilibrated in 50 mM Hepes, pH 8, 10 mM KCl. Separation of the substrates pppGpp and ppGpp from their respective products GTP and GDP was performed in a 50 mM Hepes, pH 8 buffer with a salt gradient ranging from 10 to 380 mM KCl. Product inhibition experiments were performed as above but in duplicates. Samples were incubated for 60 min with the addition of GTP or GDP to the reaction mix at concentrations of 0, 50, 100, 200, and 400 μM, before adding active enzyme. The concentration of remaining alarmone was determined based on the normalized (p)ppGpp peak area compared to time 0 controls, as calculated by the UNICORN evaluation software. Graphs were plotted using GraphPad Prism. Specific activity was calculated based on the initial rate of hydrolysis from time course measurements up to the 15-min mark, which produce a straight line.

**DraCALA binding and competition assays**

DraCALA binding and competition assays were performed essentially as previously described (49, 56). Briefly, purified RelH$_{Cs}$ or RelH$_{L1}$ (at final concentrations of 100 μM) were incubated with 2 nM α-$^{32}$P-pppGpp or α-$^{32}$P-GTP in the presence of 100 μM cold competitor nucleotide, that is, GTP, GDP, ppGpp, or ppGpp. The binding solutions were incubated for 5 min at room temperature before 2 μl were spotted onto a nitrocellulose membrane. The fraction bound was quantified as described (56). Two biological replicates were performed, and the average and SD of the binding fractions were plotted via GraphPad Prism. One-way ANOVA and Dunnett’s multiple comparison tests were performed to assess the presence of statistically significant differences.

**AlphaFold2 prediction models**

Full-length protein sequences of RelH$_{Cs}$, RelH$_{L1}$, PaSAH, RelH$_{Dm}$, Mesh1$_{Hs}$, and Mesh1$_{Dm}$ were used to create prediction models of homodimers using the AlphaFold2 pipeline (53) with the mutliner procedure (62) via Colabfold (87). Multiple sequence alignment search was performed using the MMseq2 API (88).

**Sequence alignments**

Pairwise protein sequence alignment and multiple protein sequence alignment were performed using EMBOSS Needle and Clustal Omega, respectively (89). The structure-based sequence alignment was performed using PROMALS3D (90). All alignments were rendered with the aid of the ESPript 3.0 server (91).

**Data availability**

Atomic coordinates and structure factors of RelH$_{Cs}$ and RelH$_{L1}$ structures presented in this article have been deposited at the RCSB Protein Data Bank (PDB) under the following identifiers: 7QOC (RelH$_{Cg}$, Se-Met form), 7QOD (RelH$_{Cg}$, native), and 7QOE (RelH$_{L1}$, native).

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**Supporting information**—This article contains supporting information (23, 41–43).

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of the article.

**Abbreviations**—The abbreviations used are: DraCALA, differential radial capillary action of ligand assay; HD, hydrolase domain; PDB, protein data bank; pGpp, guanosine 3′-diphosphate 5′-monophosphate; ppGpp, guanosine 3′,5′-bis(diphosphate); pppGpp, guanosine 3′-diphosphate 5′-triphosphate; RSH, RelA-SpoT homolog; SAH, small alarmone hydrolase; SAS, small alarmone
References


Small alarmone hydrolase dimers


Small alarmone hydrolase dimers
