The oligomeric assembly of the novel haem degrading protein HbpS is essential for interaction with its cognate two-component sensor kinase.

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Abbreviations used:

PAGE: poly-acrylamide gel electrophoresis; **PDB:** protein databank; **rmsd:** root mean square deviation; **SDS:** sodium dodecyl sulphate; **SLS:** static light scattering; **TAT:** twin arginine translocation; **TCS:** two component system; **XAFS:** X-ray absorption fine spectroscopy

Summary (249/300 words)

HbpS, a novel protein of previously unknown function from *Streptomyces reticuli*, is up-regulated in response to haemin and peroxide-based oxidative stress, and interacts with the SenS/SenR two-component signal transduction system. In this manuscript we report the high resolution crystal structures (2.2 Å and 1.6 Å) of octomeric HbpS crystallized in the presence and absence of haem and demonstrate that iron binds to surface exposed lysine residues of an octomeric assembly. Based upon an analysis of the crystal structures we propose that the iron atom originates from the haem group and subsequent biochemical experiments are reported which demonstrate that HbpS possesses haem-degrading activity *in vitro*. Further examination of the crystal structures has identified amino acids that are essential for assembly of the octomer. The role of these residues is confirmed by biophysical experiments. Additionally, we show that while the octomeric assembly state of HbpS is not essential for haem degrading activity, the assembly of HbpS is required for its interaction with the cognate sensor kinase, SenS. Homologs of HbpS and SenS/SenR have been identified in a number of medically and ecologically relevant bacterial species (including *Vibrio cholera*, *Klebsiella pneumoniae*, *Corynebacterium diphtheriae*, *Arthrobacter aurescens* and *Pseudomonas putida*), suggesting the existence of a previously undescribed bacterial oxidative stress-response pathway common to Gram-negative and Gram-positive bacteria. Thus, the presented data provides the first insight into the function of a novel protein family and an example of an iron mediated interaction between an accessory protein and its cognate two-component sensor kinase system.

Keywords

Coupled Oxidation; Haem; Oxidative Stress Response; Static Light Scattering; X-ray crystallography

Introduction

Streptomyces are highly differentiated soil bacteria which produce a wide repertoire of medically relevant antibiotics, antifungal and cytostatics compounds, as well as many extracellular hydrolytic enzymes and enzyme inhibitors^{1; 2}. The cellulose degrader *Streptomyces reticuli* (*S. reticuli*) has recently been shown to secrete a novel haem-binding protein, HbpS³ and a comparative sequence analysis⁴ has demonstrated that HbpS shares a high sequence homology with a number of proteins of unknown function from the Gram-positive bacteria *Corynebacterium diphtheriae*⁵, *Streptomyces coelicolor* A3(2)⁶, *Rhodococcus* sp. RHA1⁷, *Arthrobacter aureescens* TC1⁸ as well as proteins from the pathogenic Gram-negative bacteria *Vibrio cholerae*⁹, *Pseudomonas putida*¹⁰ and *Klebsiella pneumoniae*¹¹.

The production of HbpS is highly enhanced *in vivo* during the cultivation of *S. reticuli* and *S. lividans* transformants in the presence of high amounts of haemin (the Fe³⁺ oxidized form of haem), and HbpS has been shown to directly bind haemin³. Haemin, a natural porphyrin, is known to possess significant antibacterial activity when augmented by physiological concentrations of hydrogen peroxide or reducing agents^{12; 13} and it has been suggested that HbpS-like proteins may play an important role in bacterial defense against haemin-based toxicity³. Studies have also demonstrated that secretions of HbpS is carried out under the control of the twin-arginine translocation (TAT) pathway, through which folded and oligomeric proteins are translocated across the outer cell membrane^{14; 15}. Biochemical analysis using culture filtrates of *S. reticuli* and *S. lividans* transformants revealed the presence of monomeric, dimeric and higher order oligomers of HbpS³.

The disruption of HbpS induces a significant decrease in the production of CpeB, a mycelia-associated haem-containing enzyme, *in vivo*. While the manner of interaction of HbpS and CpeB is currently unknown CpeB has been shown to utilize H₂O₂ to oxidize a number of substrates via an attached haem group (ferricprotoporphyrin) or in a haem-independent reaction, which is coupled to Mn(II)/(III) peroxidation¹⁶. As the haem-dependent catalase activity of CpeB leads to the dissociation of H₂O₂ into H₂O and O₂ it also plays an important part in the detoxification of H₂O₂. CpeB shows a high degree of amino acid identity (62 % identity, 73 % similarity) to the catalase-peroxidase KatG from *Mycobacterium tuberculosis*. The front-line anti-tuberculosis drug isoniazid (isonicotinic acid hydrazide) requires KatG activation before exerting a lethal effect¹⁷ and isoniazid resistant strains of *M. tuberculosis* have been shown to possess no detectable levels of KatG, although they acquire a compensatory mutation resulting in an up

regulation of expression of an alkyl hydroperoxide reductase protein, Ahp¹⁸. Sherman and co-workers suggested that KatG would confer protection against H₂O₂-mediated damage even in the absence of adequate catalase and peroxidase activities, thus promoting survival of the organism in the environment of the phagocyte oxidative burst.

A specific interaction has been identified between HbpS and the sensor kinase SenS from the recently identified two-component sensor system (TCS), SenS/SenR, which negatively regulates the *furS-cpeB* operon and the *hbpS* gene^{19; 20; 21}. Recently published data demonstrates that HbpS regulates the autokinase activity of SenS in a redox-dependent manner and that SenS requires iron to initiate phosphorylation²². The influence of accessory molecules or proteins on the activity of sensor kinases has been shown for other TCS, such as the PhoQ/PhoP system of *Salmonella typhimurium* which is activated by binding of antibacterial peptides to the sensor kinase²³. Disruption of the SenS/SenR TCS also results in an increased sensitivity of *S. reticuli* to haemin and hydrogen peroxide. The closest available annotated homolog of SenS is the sensor kinase ChrS of *Corynebacterium diphtheriae* (34 % identity, 46 % similarity). ChrS has been shown to be involved in the sensing of haem and the transduction of this signal to the response regulator ChrA, which regulates the expression of a haem oxygenase gene²⁴.

This homology in HbpS and HbpS interaction partners across a wide range of bacterial species, including a number of medically and ecologically relevant bacteria, implies a common functional role for the HbpS homologs. Thus, the study of the biochemical function of HbpS could provide an insight on a novel bacterial stress-response pathway that is conserved across a wide range of bacterial species. Based upon the research reviewed above, we have previously suggested that the major role of HbpS-like proteins would appear to regulate the expression of CpeB/KatG-like proteins, via an interaction with the SenS/SenR two-component system, in response to extracellular redox conditions (i.e. the presence of H₂O₂, haemin, etc.) that are detrimental to bacterial survival.

In order to gain deeper insight into the physiological role of HbpS-like proteins we have determined the high resolution crystal structure of HbpS from *S. reticuli*. Analysis of the crystal structures of HbpS crystals grown in the presence and absence of haem led to the proposal that HbpS has a haem degrading activity, a hypothesis that has been confirmed *in vitro*. Disruption of the residues coordinating the liberated iron has implications for the downstream signalling of oxidative stress via the SenS/SenR two-component system. Additionally, we report on *in vitro* biophysical and biochemical experiments that demonstrate that the oligomeric state of HbpS plays an essential

role in the interaction with the sensor kinase SenS.

Results and Discussion

The 2.2 Å native crystal structure of HbpS reveals a novel octomeric architecture

The elucidation of the 2.2 Å crystal structure of HbpS reveals a previously undescribed overall octomeric architecture (Figure 1a-d), as determined by a database search using the DALI server²⁵. A structural homolog of unknown function from *Klebsiella pneumoniae* is available within the protein databank (2a2l) that has an rmsd on backbone carbon alpha positions with HbpS of 1.2 Å²⁶. However, no manuscript or report could be found in the literature that describes this structure or its function.

The core of each protein monomer of 154 amino acids shows some structural homology to the profilin-like protein family (displaying an rmsd of 2.6 Å for the carbon-alphas of 75 aligned residues). However, there are still significant differences between the overall folds of HbpS and profilin I (pdb: 1a0k²⁷; Figure 1a) and no indication could be found in the literature that profilin I may form octomers *in vitro*. The tertiary structure of HbpS consists of a sheet of four anti-parallel beta-strands, which is flanked by two antiparallel helices on the exterior of the octomer and a helix-turn-helix-like motif on the interior of the octomer (Figure 1a & b). The helix-turn-helix motif on the interior of the octomer also shows some similarities to motifs found in the DNA binding domains of transcription factors, although a functional equivalence of this region is highly unlikely, due to the extra-cellular location of HbpS³ and the positioning of this region on the interior and interfaces of the octomeric assembly.

The protein is present as an octomer in the asymmetric unit, with extensive buried surfaces between each individual monomer (Figure 1b-d) and experiments to confirm the presence of octomeric HbpS *in vitro* are described below. The total buried surface of protein within the octomer analyzed using the PISA server²⁸ is 23680 Å², which is over 60 % of the total surface area of the assembly (38780 Å²) and suggests a stable oligomeric state.

In order to identify the most likely lower oligomeric states of HbpS detected in culture filtrates of *Streptomyces*³ we initially compared the buried surfaces between contacting monomers of the native octomer using

the web server PISA²⁸. It should be noted that due to the internal symmetry of the octomer only three non-redundant dimer possibilities exist: AB, AC and BC (Figure 1c). The average contact area between monomers A and B of Figure 1c is $530.6 \text{ \AA}^2$, whereas the average contact areas between of monomers A and C or monomers B and C is $709.1 \text{ \AA}^2$ and $1186.9 \text{ \AA}^2$, respectively. A large proportion of the BC dimer interface is provided by the interaction of the extended N-terminal region of each monomer with its immediate neighbour (Figure 1c). This significantly larger buried interface between monomers B and C leads us to propose that this arrangement of monomers represents a model for the dimers of HbpS that have been described *in vivo*³. Further examination of the interface between monomers B and C in the higher resolution structure described below shows that the N-terminus of each monomer makes extensive interactions with the neighbouring monomer and revealed the presence of an unusual intermolecular interaction between Ser26 and His28 (Figure 1c and Figure 2). While this region possess some sequence homology to a $L(X)_3THL(X)_{10}AA$ motif which has been implicated in haem binding³ by homology with cytochrome c oxidase (HbpS: $L(X)_2TH_{28}L(X)_{10}AA$) the two motifs adopt very different conformations. We describe below experiments to confirm both the *in vitro* presence of HbpS octomers and to determine the effect of the mutation of either of these two residues on the ability of HbpS to form octomers.

1.6 Å resolution crystal structure of HbpS:Iron complex

Our recently reported data showed that HbpS is up-regulated in the presence of high amounts of haemin, resulting in the suggestion that HbpS may play a role in the sensing of extracellular oxidative stress conditions and transmitting this signal to the cell via an interaction with the two-component sensor kinase SenS^{3; 22}. In order to more fully examine the biochemical function of HbpS the purified protein was incubated overnight in the presence of haemin (the Fe^{3+} oxidized form of haem) immediately prior to concentration and then crystallized as described previously⁴. Subsequent X-ray diffraction studies demonstrated that the octomeric assemblies of HbpS pack in a different manner. This change in the packing results in a change of the crystal space group from $P2_13$ to $I422$, with a concurrent change in the number of monomers per asymmetric unit from eight (in $P2_13$) to one (in $I422$). However, the overall structures of the octomer are highly similar, with an overall rmsd for all carbon-alpha positions of $0.29 \text{ \AA}$ and only a few residues changing in carbon-alpha position by over $0.5 \text{ \AA}$ (Lys47, Asp48, Gly61, Glu96, Lys125 and Gly126). As the estimated coordinate error of the lower and higher resolution structures are 0.28 and $0.12 \text{ \AA}$, respectively²⁹ no meaningful conclusions can be drawn from the differences of the two structures. While further analysis of the electron density maps revealed no additional signal that could be interpreted as an ordered haem

moiety, a strong peak was observed in the vicinity of Lys108, which lies close to the four-fold crystallographic axis relating the monomers of one single octomeric assembly in the I422 space group. Due to the incubation of HbpS with haemin (Fe^{3+} oxidized form of haem) immediately prior to crystallization this peak was initially modelled as an iron atom (Figure 3). The close interaction between the amide group of Lys108 and the proposed iron atom suggested that the mutation of Lys108 to alanine would disrupt the formation of the HbpS:iron complex and analysis of the native HbpS complex indicated that this mutation would have minimal effect upon oligomerization. As detailed analysis revealed no other significant differences between the HbpS and HbpS:iron structures it is possible that the coordination of iron by HbpS may provide sufficient change in the electrostatic surface to drive the change in crystal form.

In order to provide an initial confirmation of the identity of this moiety an anomalous dataset was collected from these crystals at a wavelength above the iron absorption edge (1.7 Å). A phase set was calculated from the high resolution structure, after the removal of the proposed iron atom and an anomalous difference Fourier calculated based upon these phases³⁰. The resultant anomalous density map shows a clear peak of 5 σ at the proposed iron position on the four-fold axis (Figure 3), supporting the hypothesis that this atom is an iron. However, unequivocal identification of this atom and the precise determination of its oxidation state await analysis by X-ray absorption spectroscopy (XAFS), which is currently underway.

The effect of mutations on the assembly state of HbpS

SDS-PAGE and Western blot analyses have previously shown that HbpS is present in a number of different oligomeric states *in vivo*³. In order to demonstrate that the observed octomer was an assembly state occupied by HbpS *in vitro*, and not unduly influenced by crystallogenesis, samples were analyzed using static light scattering (SLS) after size exclusion chromatography^{31; 32; 33} (Figure 4). The *in vitro* mass of HbpS, as determined by SLS, is 116.7 kDa $\pm$ 2 % with no detectable levels of monomer, dimer, or tetramer present (Figure 4). The predicted molecular mass, based upon the primary sequence of the protein, is 15237.9 Da – with a theoretical mass of 121,903.2 Da for an octomer. Thus, the measured molecular mass in solution is consistent with that of the octomeric assembly determined from X-ray diffraction studies. While the SLS analysis did not show the presence of lower-order HbpS oligomers, it should be noted that these measurements are performed at protein concentrations in excess of those found *in vivo*, and such concentrations are likely to strongly affect the assembly kinetics.

We then analyzed the effect of a mutation designed to disrupt the interaction between the N-terminal regions of the proposed dimer (BC, Figure 1c). Analysis of the SLS data of the His28Ala mutant of HbpS clearly demonstrated that this construct is present in solution predominantly as a monomer, with almost no octomer present (Figure 4). This data provides confirmation for the proposed *in vivo* dimer described above and also demonstrates that the interaction between Ser26 and His28 is critical for the formation of the final octomeric assembly (Figure 2). Due to limitations inherent in the SLS technique (in terms of sample concentration requirement) we decided to confirm the absence of dimer and octomer HbpS species using native PAGE (Figure 5). Under native conditions (without SDS; Figure 5a) wild-type HbpS clearly migrates slower on the gel than the Ser26Ala and His28Ala mutants. The mutation Lys108Ala, which is proposed to disrupt iron binding without affecting the native oligomeric state, runs similarly to the wild-type protein (Figure 5a). Taken together with the SLS data presented above we interpreted the co-migration of the Ser28Ala and His28Ala mutants to indicate that both proteins are present only as monomers in solution, with no indication of dimer formation. While, the determination of the oligomeric state from native PAGE is inaccurate, the co-migration of the two mutants strongly suggests that both are present in the same oligomeric state. The control experiment in the presence of SDS (Figure 5b) demonstrates that protein concentration has no effect upon assembly within this experiment, as the amounts of the various proteins are approximately equivalent. As sample concentrations are lower in this experiment we propose that the residual octomer assembly seen in the SLS experiments described above is a result of elevated sample concentrations.

Disruption in the octomeric assembly HbpS abolishes its interaction with SenS but not haem

Our previous report indicated that HbpS interacts specifically with the sensor kinase SenS from the two-component sensor system, SenS/SenR²⁰. As a result we performed experiments to examine the effect of HbpS assembly upon its interaction with SenS. Briefly, purified His₆-SenS was first incubated with His₆-free HbpS proteins. Ni²⁺-NTA agarose was then added to the reaction mixtures as described under Material and Methods. His₆-SenS and wild-type and Lys108Ala mutant HbpS could be released from the resin with the addition of 250 mM imidazole. In contrast, the Ser26Ala and His28Ala mutant are not co-eluted with His₆-SenS (Figure 6a). Antibody detection demonstrated that only trace amounts of these two mutants co-elute with His₆-SenS (Figure 6b). Taken together, this data strongly suggests that the HbpS assembly state plays an essential role in its interaction with the sensor kinase SenS.

As HbpS has been previously shown to bind haem³ the haem binding activity of each of the isolated proteins

was then estimated spectrophotometrically. The addition of haemin to isolated HbpS wild-type protein produces a characteristic visible absorption spectrum with a peak in the Soret region³⁴, at 411 nm, whereas maximum absorbance of free haemin under the same conditions is 398 nm (Figure 7a). Surprisingly, the visible absorption spectrum of the mutant proteins Ser26Ala, His28Ala and Lys108Ala is identical to those one of the wild-type (Figure 7a). This indicates that while HbpS assembly is required for an interaction with SenS, assembly is not required for the previously described interaction between HbpS and haem.

HbpS degrades haem *in vitro*

Electron density analysis from the I422 crystal form, which is grown from HbpS previously incubated with haem, shows the presence of a bound iron. We hypothesized that the iron has been released from the haem after its degradation by HbpS. The haem degradation activity of HbpS postulated above was examined spectrophotometrically using 10 mM ascorbate as a reductant. Upon incubation of haem with HbpS a time-dependent decrease in the characteristic haem peak at 411 nm can be observed (Figure 7b). No degradation was observed either in the absence of HbpS or in the presence of ascorbate alone (data not shown). The haem degradation activity of the HbpS mutants Ser26Ala, His28Ala and Lys108Ala is almost identical that of the wild-type protein (Figure 7c), indicating that assembly of HbpS is not required for haem degradation activity.

Previous reports showed that haem binding proteins, such as myoglobin, can degrade haem by a nonenzymatic mechanism known as “coupled oxidation”, in which peroxide is generated during the degradation of haem³⁵. In this process ascorbate and the haem iron provide the two electrons required for the reduction of O₂ to H₂O₂, which then participates directly in haem degradation^{35,36; 37}. No residues from the haem binding protein directly attack the haem group. Therefore, coupled oxidation can be prevented by the addition of a catalase to the reaction mixture, prevent any accumulation of H₂O₂. Indeed, when the reactions described above were carried out in the presence of catalase at a catalase:HbpS proteins ratio of 1:10, no decrease of the haem peak was observed (data not shown).

The data presented above clearly demonstrates that HbpS degrades haem nonenzymatically *in vitro* in a coupled oxidation manner and that the assembly of HbpS into dimers or octomers is not required for this activity.

Mutation of Lys108 reduces the HbpS mediated autokinase activity of SenS *in vitro*

The high resolution crystal structure of the HbpS:Iron complex indicated the presence of an iron coordinated by Lys108 from each monomer. As well as the haem degradation activity described above this observation also led us to examine the role that iron binding would play in the assembly of HbpS and its interaction with SenS. As the iron interacts with the terminal amide group of Lys108 we generated a Lys108Ala mutant of HbpS to disrupt the formation of the HbpS:Iron complex. In the same experiments performed to assess the behaviour of the Ser26Ala and His28Ala HbpS mutants we have also demonstrated that the mutation Lys108Ala has no effect on the ability of HbpS to form the final octomeric assembly (Figure 5), to bind to haem (Figure 7a) or to degrade haem (Figure 7c).

However, we performed an experiment to determine the effect of this mutation upon the interaction between HbpS and its physiological interaction partner, the two-component sensor kinase SenS. Recently published data revealed that the autokinase activity of SenS is considerably enhanced in the presence of iron²². Further analyses also show that the presence of HbpS and haemin alone or haemin in combination with H₂O₂ (oxidative stress conditions) significantly enhanced phosphorylation of SenS (Figure 8). The mutation Lys108Ala reduces HbpS-mediated autokinase activity of SenS in response to haemin and *in vitro* oxidative stress conditions by a factor of approximately three or ten-fold, respectively. As Lys108 has been shown above to play no role in assembly, haem binding, interaction with SenS or the haem degradation activity of HbpS we interpret this loss of function to be a result of loss of iron binding on the surface of the HbpS octomer. Further, the autophosphorylation assays presented in Figure 8 also show that the mutations Ser26Ala and His28Ala also result in a reduction (approximately 1.5 or four-fold, respectively) of the SenS autokinase activity under the same conditions. The reduced influence on SenS phosphorylation of these two mutants can be explained as a consequence of their incomplete assembly (Figure 5), resulting in a strong reduction in the interaction with SenS (Figure 6). As both mutants retain haem degrading activity (Figure 7c) and an unmodified Lys108 they could still present a coordinated iron to SenS, influencing SenS phosphorylation. Whereas the mutation of Lys108 to alanine would completely abolish iron coordination, resulting in a loss of activity.

Thus, we propose the presence of a bound iron on the surface of HbpS is an initial stage in the signalling cascade for extracellular oxidative stress detected by the SenS/SenR two-component system.

Summary

The data presented in this manuscript provide a function for the novel HbpS protein from *S. reticuli* and, by homology, for other HbpS-like proteins that are found in both Gram-negative and Gram-positive bacteria. We have solved the three-dimensional structure of HbpS to high resolution (1.6 Å), which provides a novel structural model for a previously uncharacterized family of proteins. While a representative structure of the HbpS-like proteins is already available in the pdb (2a2l) no description of this structure is available in the current literature. Thus, this is the first report describing the three-dimensional structure and function of this novel class of proteins. Analysis of the crystal structures led us to hypothesis that HbpS not only binds haem as reported previously³, but that HbpS also binds an iron atom liberated from the bound haem moiety. The spectroscopic analysis described above demonstrates that the presence of HbpS alone is sufficient to degrade haem *in vitro*. Thus, we could show that the extracellularly located HbpS protein binds and degrades haem leading to a free iron.

A comparison of the HbpS structures with the structure available in the protein database demonstrates that the overall structures are highly similar. However, while Gln95 of 2a2l replaces Lys108 of HbpS the amide groups of both residues are both available to form the proposed interaction with iron reported above. While we cannot exclude a degree of functional divergence within the HbpS-like family, conservative mutations may provide a means for iron coordination by different sequences in the region of Lys108. Additionally, the interaction between His28 and Ser26 is not conserved in 2a2l, suggesting that other members of the HbpS-like family may require a different inter-subunit interaction for the stabilization of the octomeric assembly. However, the overall octomeric architecture is retained.

As previous reports have shown that HbpS is present in a number of oligomeric states *in vivo*³ we examined the crystal structures to identify residues that could be involved in assembly. Mutation of two residues (Ser26 and His28) leads to an HbpS construct that is unable to form dimers or any other higher order oligomers. The location of these two mutations allows us to suggest a model for the *in vivo* dimers previously identified (Figure 1c). However, assembly into dimers or other higher order oligomers is not a prerequisite for this activity, as demonstrated by the activity of the assembly disruption mutants Ser26 and His28. However, coordination of the iron requires both the presence of Lys108 and an HbpS construct that can assemble into higher order oligomers. It is not clear at this stage which of the oligomeric states is sufficient for iron binding, but the X-ray structure implies that the full octomeric assembly would be required. The proposed dimer does not result in an assembly in which the Lys108 residues are in

close proximity, rather lying at opposite poles of the final octomer. We cannot exclude the possibility that a tetramer exists which would provide all four lysine residues for the iron binding site, but as the initial dimer crosses the boundary between two of these potential “pole” containing tetramers in the final octomer we believe it to be unlikely.

While the degradation of haem does not require a complete assembly of HbpS monomers it seems likely that efficient coordination of the released iron would require either an extremely rapid assembly following degradation, or the internalization of haem within the HbpS core prior to degradation. As the Lys108 residues are located on the poles of the assembly (Figures 1a & 3) such “pre-assembly” would position the released iron in the interior with the only channels through the assembly leading directly to the iron binding sites. However, the presence of multiple oligomeric states *in vivo* clearly indicates that the assembly of HbpS is a dynamic process, which may depend upon the presence of other, currently unidentified, factors. Further experiments are underway in order to clarify this issue.

Once the iron is coordinated by correctly positioned Lys108 residues, HbpS then exerts a significant effect upon the autokinase activity of SenS. This effect would allow the extracellular presence of haem and oxidative stress conditions to be communicated across the cell membrane of *S. reticuli* via the SenS/SenR two-component system. Downstream signalling cascades would then up-regulate the appropriate gene expression levels for bacterial survival in an oxidative stress environment. Since HbpS, and its interaction partners display a high degree of homology with proteins of unknown function from other medically and ecologically relevant bacteria, the inhibition of this stress response pathway may present an additional opportunity to understand and impede bacterial survival mechanisms.

Materials and Methods

Crystal structure determination of HbpS and HbpS:Iron complex

The crystallization and phasing of native HbpS has been previously reported elsewhere⁴ and the data collection statistics are reported in Table I, with all data reduction performed using XDS³⁸. Briefly, the crystals obtained were soaked in 1M NaBr for 1 minute before SAD data collection to 3.4 Å was performed above the bromine edge (13.5keV) on beamline X12, EMBL Hamburg (Table I). Ordered bromine ions were identified and phases calculated in space group P213 using the package SOLVE³⁹. This phase set was transferred to a higher resolution native data set (2.2 Å; Table I) and subsequent density modification and automated model building was carried out using the package RESOLVE^{40; 41}. Examination of the partially built model indicated the presence of an octomer in the asymmetric unit, and all further refinement was carried out using 8-fold non-crystallographic symmetry (NCS) restraints in REFMAC5^{42; 43}, with 5% of data omitted from refinement for free-R factor calculations⁴⁴. Due the presence of high NCS free-R reflections in thin resolution shells were chosen to eliminate NCS-based correlation between the free-R and working reflections⁴⁵. The model (comprising residues 16-156) was built using the package COOT⁴⁶ and the final model has an R-factor/Free R-factor of 0.201/0.264 and possesses good stereochemistry, as indicated in Table II.

Surprisingly, crystals of HbpS grown under the same conditions as previously reported⁴, but in the presence of haem, belong to space group I422, with a single HbpS molecule present in the asymmetric unit. Diffraction data were collected on beamline X12, EMBL Hamburg to a resolution of 1.6 Å. Molecular replacement was carried out using the package AMORE⁴⁷ and refinement was again performed using the package REFMAC5⁴², with manual rebuilding performed in COOT⁴⁶. The model (comprising residues 16-156) was refined to a final R-factor/Free R-factor of 0.154/0.189 and possesses good stereochemistry as indicated in Table II.

Finally, in order to demonstrate the presence of a bound iron in the Hbps:Iron complex a dataset was collected to a resolution of 2.36 Å at a wavelength of 1.7 Å (above the iron absorption edge) at beamline ID14-EH4, ESRF Grenoble. The high resolution model was used to directly phase the anomalous data and calculate the anomalous maps shown in the results section above, no additional refinement of the structure was performed.

Site directed mutagenesis and expression of HbpS mutants

Point mutations in the *hbpS* gene on the plasmid pQH2³ were introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The numbering of the amino acids is in agreement with the sequence of the 3D structure of HbpS. Each of the oligonucleotides was designed to replace the corresponding codon (serine, histidine or lysine, respectively) with an alanine codon. After PCR the products were transformed into *E. coli* XL1-Blue. Subsequently, each of the obtained plasmid constructs was analysed with restriction enzymes and by sequencing. The resulting correct plasmids were named pQH2Ser26A, pQH2His28A or pQH2Lys108A, respectively.

The *hbpS*-coding region of the constructs pQH2Ser26A, pQH2His28A and pQH2Lys108A, respectively, were ligated with cut pETM11 vector and subsequently transformed into *E. coli* XL1-blue as previously described⁴. Having sequenced the resulting plasmids pETHbpSSer26A, pETHbpSHis28A and pETHbpSLys108A, respectively, the correctness of the *hbpS* genes and their in frame fusion with the His6-tag and the TEV-protease cleavage site were confirmed. After transformation of the corresponding plasmids in *E. coli* BL21 [DE3] pLysS, the proteins were over-expressed and isolated as described for the wild-type HbpS⁴.

Measurement of the oligomeric state of HbpS mutants

The solution oligomeric state of wild type HbpS and HbpS mutants were measured using a Wyatt Technologies miniDAWN Tristar static light scattering device connected downstream of a Superdex-200 10/30 gel filtration column (GE Healthcare) previously equilibrated in 20 mM Tris pH 8.0, 150 mM NaCl and 5mM β -mercaptoethanol. Reducing agents were used to stabilize the samples used to calibrate the SLS device and should have no effect on the behaviour of the HbpS samples, due to the absence of cysteine residues in the primary sequence. Data analysis was performed following the manufacturers recommended protocol with the package ASTRA (Wyatt Technologies). In parallel, native PAGE experiments were performed to determine the assembly state of HbpS and HbpS mutants using a separating gel pH of 6.8 under standard conditions⁴⁸.

Estimation of protein and haemin concentration

Concentration of purified HbpS solutions was calculated from their absorbance at 280 nm assuming an ϵ_{280} of $8,250 \text{ M}^{-1} \text{ cm}^{-1}$ ($M_w = 15,498 \text{ Da}$). Haemin (bovine hemin from Sigma) was initially dissolved in a minimal volume of 0.2 M KOH and diluted in the appropriate buffer. The pH was adjusted to 7.5 using 50 mM Tris/HCl. Haemin concentration was calculated from the absorbance at 385 nm using the previously published ϵ_{385} of $58,400 \text{ M}^{-1} \text{ cm}^{-1}$. All absorption measurements were done using a Specord 205 UV-Vis spectrophotometer (Analytik Jena, Germany).

Haem binding

HbpS proteins (10 μM) were incubated with an excess of haemin (50 μM) in buffer W (150 mM NaCl, 20 mM Tris-HCl, pH 8) at 30 °C for 4h. Preliminary experiments (not shown) revealed that 4h was sufficient for all HbpS mutants to achieve a similar protein-haem spectrum and an elevated temperature was chosen to mimic the optimal growth temperature of *Streptomyces*⁵⁰. Haem binding was monitored spectrophotometrically by recording scans in the 300 to 500 nm region of the spectrum. Upon incubation of haem with HbpS the characteristic haem peak at 411 nm can be observed³⁴. All absorption measurements were done using a Specord 205 UV-Vis spectrophotometer (Analytik Jena, Germany).

Haem degradation assays

Haem degradation was monitored spectrophotometrically with the decrease in the haem absorbance at 411 nm in the presence of HbpS proteins using ascorbic acid as the electron donor^{51; 52}. HbpS proteins (10 μM) and hemin (50 μM) were previously incubated in buffer W at 30 °C for 4h. This mixture was subsequently treated with ascorbic acid (10 mM) at 30 °C and the spectral changes between 330 and 800 nm were recorded during 2 h at different time intervals. In parallel control reactions containing additional recombinant catalase from bovine liver (Sigma) were performed. All absorption measurements were done using a Specord 205 UV-Vis spectrophotometer (Analytik Jena, Germany).

Detection of HbpS/SenS interaction by Ni²⁺-NTA affinity chromatography

The co-purification studies of SenS and HbpS were performed as previously described with slight variations³¹.
²². Purified His6-SenS protein was incubated with His₆-free HbpS WT or mutants in phosphorylation buffer (25 mM Tris-HCl, pH 7.5, 5% (v/v) glycerol, 10 mM MgCl₂ and 500 mM NaCl) for 30 minutes at 30 °C before addition of Ni²⁺-NTA agarose and imidazole (25 mM). The samples were subsequently loaded onto a column and washed several times with phosphorylation buffer containing 50 mM imidazole. The bound proteins were eluted using 250 mM imidazole according to the instructions of the manufacturer (Qiagen). As a negative control His₆-SenS was omitted in the assays.

Measurement of the autokinase activity of SenS

To perform the *in vitro* phosphorylation assays HbpS and SenS proteins were first isolated as described previously^{4; 22}. To analyze the effect of HbpS proteins on the autokinase activity of SenS, aliquots (5 µM) of HbpS proteins were first incubated with the indicated reagents (H₂O₂ and/or hemin) in 20 mM Tris/HCl pH 7.0 for 10 min. The treated HbpS proteins were then added to SenS (5 µM) in phosphorylation buffer and incubated for 10 min at room temperature. Subsequently 0.05 µCi [³²γ-P] ATP was added to the reaction mixture and further incubated at 30 °C for 10 min. The reaction mixture was then directly dropped on a polyvinylidene fluoride (PVDF) membrane (Immobilon P, Millipore, Germany). After drying, the membrane was exposed to a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA, USA) and quantification was done using ImageQuant 5.2 software. Control samples of SenS were incubated in the presence of 0.05 µCi [³²γ-P] ATP alone in order to establish a baseline activity for SenS autokinase activity. The amounts of the radioactivity are averaged from three to five dot blots per assay, with at least three independent repetitions. The figures presented show the area-integrated radioactivity of the sample divided by the area-integrated intensity of the control sample.

Table I: Data Collection and Processing Statistics of the Reported HbpS Crystal Structures

	Native HbpS		15' soak in 1M Na Br		HbpS:Iron Complex	
Experimental Conditions						
Beamline	BW7A (EMBL-Hamburg)		X12 (EMBL-Hamburg)		X12 (EMBL-Hamburg)	
Wavelength (Å)	0.9778		0.9197		1.0000	
Temperature (K)	100		100		100	
Detector	Mar-CCD 165mm		Mar-CCD 225mm		Mar-CCD 225mm	
Crystal Parameters						
Space Group	P2 ₁ 3		P2 ₁ 3		I422	
Unit Cell Parameters (Å)	a=b=c=152.5; α=β=γ=90°		a=b=c=151.9; α=β=γ=90°		a=b=77.9;c=80.0; α=β=γ=90°	
Resolution Range (Å)	20-2.20	(2.40–2.20)	20-3.4	(3.70-3.40)	20-1.59	(1.70-1.60)
Mosaicity (°)	0.18		0.33		0.22	
Data Processing Parameters						
Number of Measured Reflections	247495	(59161)	70896	(10638)	335456	(55216)
Number of Unique Reflections	37888	(12979)	29023	(5005)	16582	(2712)
Multiplicity	6.53	(4.56)	2.44	(2.12)	20.23	(20.36)
Completeness (%)	98.5	(94.9)	93.2	(76.1)	99.9	(100.0)
I/σ(I)	12.63	(3.56)	9.77	(3.87)	28.73	(4.70)
R _{meas} [*] (%)	8.1	(38.6)	11.4	(33.5)	7.8	(40.1)
Mean Anomalous Difference **	n/a		1.127	(0.854)	n/a	

A table showing the data collection and processing parameters for the native, sodium bromide soaked crystals of HbpS. Numbers in parentheses refer to the high resolution shell of the respective data set. * Redundancy independent R-factor⁵³; **mean anomalous difference in units of its estimated standard deviation ($|F(+)-F(-)|/s$). F(+), F(-) are structure factor estimates obtained from the merged intensity observations in each parity class. The first two columns of this data are published in ⁴.

Table II: Refinement parameters of Native and Iron Complex HbpS

	Native HbpS	HbpS:Iron Complex
Crystal Parameters		
Resolution Limits (Å)	19.86 – 2.20	18.80 – 1.60
(Outer shell)	(2.256 – 2.200)	(1.641 - 1.600)
Space Group	P2 ₁ 3	I422
Cell Edges (Å)	a=b=c=152.5	a=b=77.9, c=80.0
	$\alpha=\beta=\gamma=90^\circ$	$\alpha=\beta=\gamma=90^\circ$
Number of Molecules per asymmetric unit	8	1
Refinement Parameters		
R-factor/Free R-factor*	0.201/0.264	0.154/0.189
(Outer shell)	(0.377/0.454)	(0.191/0.210)
Number of Residues / asymmetric unit	1120 (8 x 140)	140
Number of atoms / asymmetric unit	8497	1177
Number of Water Molecules / asymmetric unit	513	134
% of residues in Core/Allowed/Disallowed regions of the Ramachandran plot	92.4%/7.2%/0.0%	95.7%/4.3%/0.0%
RMS deviations from Ideality⁵⁴		
Bond lengths (Å)	0.002	0.01
Bond Angles (°)	0.358	1.391
Chiral Volumes (Å ³)	0.021	0.089
Planar Groups (Å)	0.001	0.007

Numbers in parentheses refer to the high resolution shell of the respective data set. * $S_{hkl} = |F_o| - |F_c|$ / $S_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factors for reflection index (h,k,l), respectively.

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Figure Legends

Figure 1

The crystal structure of HbpS

a) A stereo representation of the monomeric structure of HbpS as determined by X-ray crystallography. The position of the iron atom is indicated and the nearby side chain of Lys108 is shown in stick representation. While no close structural homologs can be found in the literature, some structural similarities are found between the structure of HbpS (shown in green) and that of profilin (shown in blue; pdb: 1a0k^{27; 55}). **b)** A stereo representation of the octomeric HbpS assembly viewing directly down the four-fold axis (or pole). Alpha-helices are coloured in blue and beta-strands coloured in purple. A single HbpS monomer is highlighted in green. **c)** A representation of the contacts between the three non-redundant monomeric HbpS molecules within the octomeric assembly. Extensive buried surfaces exist between monomers A (green), B (red) and C (orange), with the proposed model for the in vivo dimer being composed of monomers B and C. The amino acid side chains of His28 for monomers B and C are shown in stick representation in the centre of the image. **d)** A cartoon representation of the proposed solution dimers in the final octomeric assembly. The docking of the green and blue dimers results in the alignment of the central beta-sheet of each monomer, producing a single eight-stranded sheet.

These figures were produced using PyMol⁵⁶ and povray (<http://www.povray.org/>).

Figure 2

A stereo image of the intermolecular interaction between Ser26 and His28 of proposed HbpS dimer in the 1.6 Å crystal form. Residues from the monomers B and C (Figure 1c) are shown in stick representation and the 2mFo-DFc electron density (blue) is contoured at 2 σ . The dashed lines indicated interactions that satisfy geometrical constraints on hydrogen bonds, with the indicated distances between the hydrogen bonding atoms. These figures were produced using PyMol⁵⁶ and povray (<http://www.povray.org/>).

Figure 3

A stereo figure showing the electron density from the 1.6 Å I422 crystal form which is grown in the presence of haem and iron. The 2mFo-DFc map (blue) is contoured at 2 σ and the anomalous map (red) is contoured at 5 σ . The distances between the terminal amide groups and the iron are too large (average of 3.8 Å) for them to coordinate directly with the iron.

Figure 4

Static light scattering (SLS) experiments performed on native HbpS (blue) and the His28Ala mutant (pink) of HbpS. The wild type HbpS (black) has a measured molecular mass of $116.7 \pm 2\%$ kDa, whereas the mutation of His28 to alanine (grey) results in the almost complete loss of higher order oligomers. The measured molecular mass of His28Ala HbpS is $17.1 \pm 9\%$ kDa. The predicted molecular mass of monomeric and octomeric HbpS from the primary sequence is 15.4 kDa and 121.9 kDa, respectively.

Figure 5

Native and SDS-PAGE analysis of wild-type and mutant HbpS proteins. Purified HbpS proteins wild-type (lane 1), Ser26Ala (lane 2), His28Ala (lane 3) and Lys108Ala (lane 4) were analyzed by native PAGE (**a**) or SDS-PAGE (**b**) and subsequently stained with Commassie brilliant blue. The molecular weights of the protein standards are indicated in kDa (lane S).

Figure 6

Interaction of HbpS and SenS. Purified His₆-SenS was first incubated with His₆-free HbpS proteins. Ni²⁺-NTA affinity chromatography was subsequently performed as described under Material and Methods. Bound SenS alone (lane 1), SenS+HbpS WT (lane 2), SenS+HbpSSer26Ala (lane 3), SenSHbpSHis28Ala (lane 4) and SenS+HbpSLys108Ala (lane 5) proteins were eluted in the presence of imidazole (250 mM) and fractions were analyzed by SDS-PAGE and subsequently stained with Commassie brilliant blue (**a**) or transferred to PVDF membrane and treated with anti-HbpS antibodies (**b**). The protein bands marked as a, b or c represents SenS whereas d represents HbpS proteins as shown immunologically, using anti-SenS antibodies (data not shown) or anti-HbpS antibodies (right), respectively. The molecular weights of the protein standards are indicated in kDa (lane S).

Figure 7

Binding and degradation of haem by HbpS wild-type and mutant proteins. (**a**) The haem-binding of HbpS proteins wild-type (WT), Ser26Ala (S26A), His28Ala (H28A) and Lys108Ala (K108A) was monitored spectrophotometrically (upper spectra), with the spectrum of free haemin was monitored as a control (shown in each diagram, lower spectrum). (**b**) The degradation of haem by the HbpS wild-type protein using ascorbate as a reductant was followed using the disappearance of the haem peak at 411 nm at different time points over 2 hrs (see Materials

and Methods). The arrow indicates a decrease in absorption with time. (c) Using HbpS proteins wild-type (WT), Ser26Ala (S26A), His28Ala (H28A) and Lys108Ala (K108A) the absorbance at 411 nm was monitored in the presence (bottom line) or absence of a catalase (top line) at the indicated time. The Y-axis in each spectrum shows the measured absorbance. This figure is representative of three independent assays.

Figure 8

Influence of HbpS proteins on the autokinase activity of SenS. HbpS proteins wild-type (WT), Ser26Ala (S26A), His28Ala (H28A) and Lys108Ala (K108A) were incubated with the indicated reagents for ten minutes with haemin (500 μ M) or H₂O₂ (5 mM) as indicated prior to the addition of SenS and [γ -³²P] ATP (see Material and Methods). The diagram shows the measured activity of the treated sample divided by the basal activity of SenS (untreated sample). The amount of radioactivity was averaged over three to five dot blots per assay, with at least three independent repetitions.

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