

Structural basis for the regulatory interaction of the methylglyoxal synthase MgsA with the carbon flux regulator Crh in *Bacillus subtilis*

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ABSTRACT

Utilization of energy-rich carbon sources such as glucose is fundamental to the evolutionary success of bacteria. Glucose can be catabolized via glycolysis for feeding the intermediary metabolism. The methylglyoxal synthase MgsA produces methylglyoxal from the glycolytic intermediate dihydroxyacetone phosphate. Methylglyoxal is toxic, requiring stringent regulation of MgsA activity. In the Gram-positive bacterium *Bacillus subtilis*, an interaction with the phosphoprotein Crh controls MgsA activity. In the absence of preferred carbon sources, Crh is present in the nonphosphorylated state and binds to and thereby inhibits MgsA. To better understand the mechanism

of regulation of MgsA, here we performed biochemical and structural analyses of *B. subtilis* MgsA and of its interaction with Crh. Our results indicated that MgsA forms a hexamer (*i.e.* a trimer of dimers) in the crystal structure, whereas it seems to exist in an equilibrium between a dimer and hexamer in solution. In the hexamer, two alternative dimers could be distinguished, but only one appeared to prevail in solution. Further analysis strongly suggested that the hexamer is the biologically active form. In vitro cross-linking studies revealed that Crh interacts with the N-terminal helices of MgsA and that the Crh–MgsA binding inactivates MgsA by distorting and thereby blocking its active site. In summary, our results

indicate that dimeric and hexameric MgsA species exist in an equilibrium in solution, that the hexameric species is the active form, and that binding to Crh deforms and blocks the active site in MgsA.

INTRODUCTION

The rapid and selective utilization of energy-rich carbon sources is essential for the evolutionary success of bacteria. For many bacteria, including the Gram-positive model organism *Bacillus subtilis*, glucose is the preferred source of carbon and energy (1). Glucose can be catabolized via glycolysis and thus feed into the intermediary metabolism. The availability of glucose results in a variety of regulatory events in order to transport and metabolize this sugar efficiently as well as to prevent the simultaneous utilization of secondary carbon sources. In *B. subtilis*, several regulators are involved in these processes. The RNA-binding transcription antiterminator GlcT is required for the expression of the glucose-specific transporter of the phosphotransferase system (PTS)¹ (2). Two regulators, CggR and CcpN, control the expression of enzymes involved in the lower part of glycolysis and gluconeogenesis, respectively (3,4). Finally, the global transcription factor CcpA represses the expression of genes involved in the utilization of less preferred carbon sources and activates genes for overflow metabolism and amino acid biosynthesis (5-8). The DNA-binding activity of CcpA is controlled by an interaction with a regulatory protein that acts as co-factor. This protein, the HPr protein of the PTS, can be phosphorylated on a serine residue (Ser-46) in response to the availability of preferred carbon sources by a dedicated kinase, HPrK. In the presence of glucose, the intracellular pools of ATP and fructose-1,6-bisphosphate are high, and these metabolites activate the kinase activity of HPrK (9-12). The resulting HPr-Ser-P acts as the cofactor for CcpA (13,14). The CcpA/HPr complex is central to the global process of carbon catabolite repression in Gram-positive bacteria with a low-GC content (Firmicutes) (1,15-17).

B. subtilis and closely related bacilli contain a paralog of HPr, Crh (18). In contrast to HPr, which has its main function as a phosphotransferase in the PTS, Crh does not participate in PTS-mediated sugar transport (18). The role of this protein has long been enigmatic.

The HPrK also phosphorylates Crh in a carbon-source-dependent manner (18,19), and it had been proposed that it acts as another cofactor for CcpA (20). However, both the weak expression of Crh and its low affinity to CcpA (21,22) suggest that the physiological role of the protein might be different. To gain more insight into the function of this protein, we have searched for Crh interaction partners, and found that it binds the methylglyoxal synthase MgsA (23).

MgsA is a key protein of a bypass of glycolysis, the formation of methylglyoxal from dihydroxyacetone phosphate. Methylglyoxal has been shown to regulate cell elongation (24) and this bypass acts as an essential overflow mechanism if phosphorylated glycolytic intermediates accumulate in the cell. These phosphorylated intermediates are toxic for the cell (25,26); however, methylglyoxal at elevated concentrations is toxic as well (27). Thus, the bacteria have a choice that is comparable to the one between *Scylla* and *Charybdis*. Therefore, the formation of methylglyoxal has to be tightly controlled, and the bacterium needs efficient mechanisms for its disposal. Indeed, three pathways for the degradation of methylglyoxal to lactate or acetol have been discovered in *B. subtilis* (28).

The *mgsA* gene encoding the methylglyoxal synthase, is part of a seven-gene operon in *B. subtilis*. In addition to MgsA, this operon encodes enzymes for bacillithiol production, which are required for the major pathway of methylglyoxal degradation, as well as essential enzymes for cell wall biosynthesis and protein biotinylation. This operon including the *mgsA* gene is constitutively expressed, but expression is increased by thiol depletion (29, 30). Thus, regulation of MgsA enzymatic activity seems to be the major mechanism to control the production of the toxic metabolite methylglyoxal. In both *Escherichia coli* and *B. subtilis*, this enzyme is inhibited by inorganic phosphate, which is generated by the conversion of dihydroxyacetone phosphate to methylglyoxal (23,31,32). In addition, non-phosphorylated Crh inactivates MgsA by direct protein-protein interaction in *B. subtilis* (23). This form of Crh is present if *B. subtilis* grows with poor carbon sources (23). Thus, MgsA activity is inhibited if *B. subtilis* faces nutrient limitation.

It is interesting to note that MgsA interacts specifically with non-phosphorylated Crh, but not with the phosphorylated form of the protein.

Moreover, HPr, which exhibits 45% identity with Crh, is also unable to bind MgsA. In a previous study, we have shown that the N-terminal α -helix of Crh is essential for the interaction of the two proteins. Moreover, replacement of the four differing amino acids in HPr in this helix by those found in Crh allowed efficient binding of HPr to MgsA (23).

In this work, we have studied the molecular details of MgsA multimerization and the interaction between Crh and MgsA. Our findings suggest that MgsA is active only as a hexamer and upon binding of Crh the active site is deformed and thus blocked. Crh binding might result in decreased flexibility of the active center assemblies of MgsA hexamers, and thus, in loss of MgsA activity.

RESULTS

Effect of Crh on the status of MgsA

In order to gain insight into the molecular mechanism by which Crh controls MgsA activity, we performed a SEC-MALS analysis of recombinantly expressed and freshly purified proteins. The results using MgsA identified exclusively dimers when a low concentration of MgsA (13 μ M) was used (see Fig. 1A). This concentration is still about tenfold increased in comparison to the cellular concentration (about 1 μ M as calculated from ref. 33). This is in contrast to the known oligomeric states of MgsA from other organisms showing a multimer exhibiting predominantly a tetrameric state in solution (32,34-36). Moreover, in MgsA from *Thermus sp.* GH5 only a hexameric state has been found in solution and in all the crystal structures available up to date, (see also Fig. S1) (37-39). In order to mimic the conditions in the crystallization experiments, the concentration was increased by a factor of about ten (135 vs 13 μ M), resulting in MgsA eluting as a dimer, and a broader peak width was observed for MgsA when the protein was injected at a higher concentration, indicating a higher number oligomerization state (Fig. 1B). These results, using MgsA alone, strongly suggest an equilibrium of dimers (corresponding to 38 kDa as determined by MALS) and multimers (by calculation using the MALS data a tetramer, which would correspond roughly to the calculated 62 kDa). Interpretation of the MALS data and the shape of the elution profile from the gel filtration experiments argue in favor of a highly dynamic assembly and disassembly of MgsA between a stable dimeric and an instable

hexameric state, resulting in an average multimeric state of about 4. This would explain the observed differences between the in solution and *in crystallo* MgsA oligomerization states best.

Moreover, our experiments with MgsA revealed that the stability of the hexameric complex strongly depends on the surrounding medium, as e.g. phosphate or glycerol strongly increase the percentage of MgsA in a hexameric state (Fig S2; see also discussion).

The purified regulator of MgsA, Crh elutes in two peaks corresponding to the dimer and monomer form in a varying ratio, as has been already observed earlier (40) (Fig. 1, peaks corresponding to a calculated mass of 20 and 10 kDa, respectively).

In the presence of a three-fold excess of Crh over MgsA, the formation of larger complexes was observed for MgsA at higher concentration (Fig. 1B). The elution volume of this corresponds to a mass of 143 kDa, which can be explained best by a hexamer of MgsA (105 kDa) bound to six Crh molecules (9.6 kDa each). This calculation is supported by the SDS-PAGE analysis of the respective fractions, which show the presence of equal amounts of both proteins (see Fig. 1, inset). Interestingly, at low concentration only a slight shift of the first peak was observed in the elution volume (Fig. 1A) which, together with mass determined by MALS, could be interpreted as either a weak interaction between MgsA and Crh or even as a MgsA dimer and as a lack of interaction with Crh. The results from the SDS-PAGE support this conclusion (Fig. 1A inset).

The crystal structure of MgsA

In order to obtain structural information on *B. subtilis* MgsA, purified MgsA was crystallized and X-ray diffraction data were collected. The crystals diffracted to a resolution of 2.34 Å and the phase problem was solved by means of molecular replacement using the methylglyoxal synthase from *Thermus sp.* (PDB 2XW6) as a search model. The crystal structure was refined at 2.34 Å resolution (see Table 1 for data statistics).

The core of the MgsA monomer is composed of the prototypic five β -strand / α -helix repeats resulting in a protein with a centered all parallel β -sheet and the arrangement flanked by three α -helices on one side and two on the other (Fig. 2A). Short helical turns (denoted η 2 and η 1) precede helix α 4 or connect directly to helix α 1,

respectively. In contrast to the canonical motif, helix $\alpha 1$ is followed by a short α helix, numbered as $\alpha 2$ before the next β -strand ($\beta 2$) (Fig. 2A). Surprisingly, C-terminally around 17 residues, which are thought to be involved in interaction to neighboring MgsA molecules and allosteric regulation of the active site (37,39) are missing, most likely they are disordered due to an increased flexibility (see below and also discussion).

The search for homologous proteins performed using the DALI server (41) identified other methylglyoxal synthases deposited in the PDB as having the highest degree of similarity to MgsA (Z-Score of 24-19 and a root mean square deviation (r.m.s.d.) ranging between 0.7 and 1.7 Å over 120 - 150 residues) (Fig. S3). The superposition reveals that the central cores of all enzymes are highly similar, with the structures of the methylglyoxal synthases from *E. coli* and *T. maritima* bearing an additional domain formed by N- and C-terminal regions visible in the crystal structures (Fig. S3). The second-best match are the large subunits of carbamoyl phosphate synthetases (Z-Score of 14 -10) with an r.m.s.d. of 2.3 – 2.9 Å over 108 - 114 residues in the C-terminal region. The most prominent difference to the large subunit of the carbamoyl phosphate synthetase from *E. coli* is a major structural rearrangement in the region of the transition from strand $\beta 3$ to helix $\alpha 3$.

The structure based sequence alignment of MgsA from *B. subtilis* and the other four organisms from which structures have been deposited in the Protein Data Bank helped to identify structurally conserved regions in MgsA (Fig. S4) in all of these structures. In general, they are mostly localized in the region of the C-terminal ends of the β -strands, the adjacent loop regions or the beginning of the following α -helices (Fig. S4).

The superimposition of MgsA and the methylglyoxal synthase from *E. coli* (50% identity and 73% similarity (PDB 1B93) (37) as well as the structures from *Thermus thermophilus* (PDB 1WO8) and *Thermus sp.* (PDB 2XW6) allowed to deduce the localization of the active site residues in MgsA. The other proteins have been crystallized in the presence of phosphate (PDB 2XW6), sulfate (PDB 1WO8), formate ions (PDB 1B93) (37), malonate (PDB 2X8W), phosphoglycolohydroxamic acid (PDB 1IK4) (42), or the competitive inhibitor 2-phosphoglycolate (PDB 1EGH) (43). The active site region of MgsA is formed by the residues located in the loop regions

subsequent to the C-termini of β -strands 1- 4, harboring also most of the highly conserved residues. (Fig. 2B left, see also Fig. S4). The residues involved in the active site formation almost perfectly match in position in the *B. subtilis* and *E. coli* enzymes, in particular residues His-8, Asp-9, Lys-12, Thr-34, -36, -37, Ser-54, Asp-60 and His-87 (according to the numbering of *B. subtilis* MgsA). Their arrangement results in the formation of a well-defined deep cavity that is blocked by residues of the loop connecting strand $\beta 4$ with helix $\alpha 5$ (termed w-loop), which harbors the conserved His-87 at its N-terminal end (Figs. 2, S4). Two histidines, *i. e.* His-8 and His-87 are located at the base of this steep and prolonged wall of the cavity formed by the loop connecting strand $\beta 3$ with helix $\alpha 4$ (termed η -loop, as it harbors the $\eta 2$ -helix). On the opposing side, a shallow saddle is formed by a patch of three threonines (residues 34, 36, 37) and Gly-35. This quasi-contained region harbors the active center of the enzyme formed by residues Asp-9 and Lys-12 on one side as well as Ser-54 and Asp-60 on the other. As the active site has been identified by localization of products and an inhibitor in it, the binding mode of the educt was investigated by *in silico* docking experiments. Using a relaxed MgsA molecule and having the educt 1,3-dihydroxy-acetonephosphate docked into it, the obtained model exhibits an arrangement of the ligand similar to that observed for 2-phosphoglycolate in *E. coli* MgsA (Fig. 2B right). Surprisingly, docking failed completely if the obtained crystal structure was used omitting the relaxation step prior to the docking experiment (data not shown), suggesting that the minute structural changes in the active site arrangement caused by the relaxation have a dramatic effect on binding and perhaps also activity.

As described for the *E. coli* protein, the *B. subtilis* MgsA forms hexamers in the crystal structure (Fig. 3). Overall, the hexamer can be described as a trimer of dimers (chains AB, CD, EF, Fig. 3). The resulting alternating and antiparallel arrangement of the individual subunits in the hexamer results in six active centers, with three on each side (Fig. 3) and a high connectivity between the individual molecules. Each subunit interacts with four of the five other subunits of the hexamer. Subunit A forms a “triangle” with two other subunits (C, E; as defined by orientation of their active centers) with a rotation of 120° resulting in interaction of the w-loop and η -loop with the

identical loops of the other two subunits, covering about 150 \AA^2 surface area each, resulting in 300 \AA^2 surface area per subunit. Moreover, both the w- and the η -loop regions interact with the central scaffold of the subunits from the second triangle (subunits B, D, F), i. e. helices $\alpha 4$ and $\alpha 5$. Interestingly, both loops harbor highly conserved residues that take part in the active site formation and function. As loops are usually quite flexible, their interaction to this rigid core of the neighboring molecule entity might positively or negatively influence active site pocket accessibility and function.

The majority of interactions within the hexamer are formed by two directly neighboring subunits within the hexamer by surface areas of $\sim 850 \text{ \AA}^2$ and $\sim 750 \text{ \AA}^2$ (Fig. 4; see below for details). The smaller of the two surfaces (e.g. molecule A and F, denoted AF) is formed by helices $\alpha 4$ and $\alpha 5$ of both molecules that are oriented in opposing direction with an almost 45° tilt with respect to each other (Fig. 4). The C-terminal ends of these helices are followed by the two loop regions, the η -loop and the w-loop. The larger surface (denoted AB) is formed by helices $\alpha 5$ and $\alpha 6$ as well as $\beta 5$ of both molecules. Strands $\beta 5$ are oriented antiparallel and thus form the continuation of the distorted central β sheet from one subunit to the β sheet of the other. The two strands are in the center of a rhombus with the four helices forming their sides (Fig. 4B). Taken together, in the crystal structure the hexamer of MgsA is stabilized by an intricate interaction pattern involving contacts between almost all subunits present in the molecular assembly.

The functional MgsA dimer

Based on the fact that MgsA at low concentrations forms dimers in solution but hexamers in the crystal structure, we asked which of the two dimer interfaces observed in the crystal structure is physiologically relevant in dimer formation. One possible dimer might be formed by interaction of helices $\alpha 4$ and $\alpha 5$ (dimer AF in Fig. 4). Alternatively, MgsA may be present as an AB type dimer. The latter potential dimer exhibits the larger interface by interaction of helices $\alpha 5$, $\alpha 6$ and strand $\beta 5$. Both potential dimers would lack specific interactions present in the hexamer thought to affect accessibility of the active site and potentially inhibit their function.

To understand which of the two dimer assemblies is more likely to be present in the cell,

we performed an analysis of the properties of the two alternative interfaces. The analysis revealed that the contact surface area AF is around $757.5 \pm 8.4 \text{ \AA}^2$, whereas the other interaction surface (AB) is around $849.1 \pm 6.1 \text{ \AA}^2$. The AB dimer interface is formed by 16 hydrogen bonds (13-17 for the other interfaces in the complexes) and only 4 salt bridges (3-4), whereas the AF dimer interface is formed by 8 hydrogen bonds (8-10 for the other interfaces in the complexes) and 8 salt bridges (8 for all), also indicating a weaker interaction for the latter. Moreover, the calculated solvation energies derived from the respective interaction surfaces (-6.6 ± 0.1 for the smaller AF interface and -10.2 ± 0.4 for the larger AB interface) support the idea that the larger interaction surface is physiologically relevant. Taken together, this analysis suggests that MgsA forms dimers of the AB type.

In order to discriminate experimentally between the two possibilities of MgsA dimer arrangements, various mutant forms of MgsA were generated and tested for their ability to form multimeric assemblies (see Table 2). One of them, MgsA-T107R/T111R, is supposed to interfere with the AB complex formation without having a major impact on the overall folding of MgsA. Interestingly, the corresponding proteins variants formed aggregates, suggestive of either misfolding or a severe defect in dimer formation. A possible explanation would be that the amino acid substitutions cause improper interactions, resulting in large aggregates. Three other mutant proteins, i. e. MgsA-Q62R and the double mutant forms MgsA-Q62R/A65R as well as MgsA-A93W/L97W are predicted to interfere with the formation of the AF dimer. In contrast to the MgsA variants described above, these proteins were soluble. According to the elution volume determined by gel filtration and mass determination by SEC-MALS using concentrations where wild type MgsA still assembles in the multimeric form (see Fig. 5, Table 2), they formed predominantly dimers. The location of these substitutions on the surface of MgsA reveals that the corresponding residues of the two subunits are arranged opposing to each other (Fig. 4, presented as sticks). Thus, these results indicate that the mutations do not interfere with the formation of functional AB dimers. To assess whether this dimer arrangement is enzymatically active, the activity of purified MgsA-Q62R was determined and compared to wild type MgsA. A specific activity of $0.15 \text{ mol min}^{-1} \text{ g}^{-1}$ was

determined for wild type MgsA. In contrast, no activity was observed for MgsA-Q62R (activity below $0.005 \text{ mol min}^{-1} \text{ g}^{-1}$).

Taken together, these results support the idea that the stable dimer formed is of the AB type and confirms that the hexameric state is required for activity.

Identification of the site of interaction between Crh and MgsA

To get a more precise idea of the structural basis for the regulation of MgsA by Crh, we performed an *in vitro* chemical cross-linking analysis. The wild type proteins were purified, and incubated with the cross-linker BS3, either individually or in a 1:1 ratio of MgsA to Crh. The resulting complexes were analysed by SDS-PAGE (Fig. 6A). In the presence of crosslinker, MgsA and Crh homodimers and multimers as well as the putative complex of MgsA and Crh were detected (Fig. 6A, red box). Subsequently, the cross-linked protein complexes were digested with trypsin and cross-linked peptides identified by mass spectrometry. This analysis revealed the presence of intra- and intermolecular cross-links. Intramolecular cross-links derived from SDS-PAGE bands containing exclusively cross-linked MgsA or Crh may result from subunit interactions of oligomeric proteins, but they can also simply reflect the spatial proximity of two amino acids within one protein molecule.

For Crh cross-linked alone (Fig 6A), the lysine residue 5 forms intramolecular cross-links with Lys-11, Lys-37, Lys-40, and Lys-41. Moreover, Lys-11 is arranged close to Lys-37, Lys-40 and Lys-41. Finally, Lys-41 cross-links to Lys-45. These intramolecular cross-links are in good agreement with the observed proximity of these residues in the Crh structure and thus represent intracross-links in monomeric Crh (Fig S5A; PDB: 1K1C, 69). Additional cross-links of Lys-11 to Lys-45 and of Lys-11 to Lys-11 reflect cross-links between two molecules of Crh dimers (Fig S5B; PDB: 2AK7).

In contrast, for MgsA alone (Fig. 6A), we observed only a few intramolecular cross-links within the monomer, predominantly located in the η 1-loop with Lys-10 cross-linked to Lys-12. In MgsA dimers Lys-10 – Lys-10 and Lys-12 – Lys-12 cross-links were detected. These cross-links are in excellent agreement with the structure of MgsA (Fig. S6).

For the MgsA-Crh complex we observed several additional intra- and intermolecular cross-links (Table 3). For MgsA, intramolecular crosslinks between Lys-40 and Lys-10, -11 and -12 were detected that have not been observed in the analysis of the monomeric or dimeric forms of MgsA (Fig. 6). This suggests that the region of helix α 1 and α 3 is arranged quite differently upon interaction of MgsA with Crh. Both helices participate in the formation of the active site cavity, and their altered positioning would also add to the explanation why the dimeric form of MgsA is inactive (see above). Importantly, we observed several intermolecular cross-links (Table 3). The lysine residues 10, 11, and 12 of the η 1-loop in MgsA were found to be cross-linked predominantly to the lysine residues 11, 40 and 41 of Crh (Fig. 6B). In addition to the stretch of these three consecutive lysine residues located in one region of the complex, Lys-40 located in the neighbouring helix α 3 of MgsA was linked to Crh Lys-11. These findings highlight (i) the importance of the N-terminal region of MgsA for the interaction with Crh, and (ii) the relevance of the region around the phosphorylation site Ser-46 in Crh. It should be noted that MgsA contains six lysine residues that are all located in the N-terminal part of the protein. The other two lysines, Lys-2 and Lys-27 are most likely too distant to be accessible for crosslinks after complex formation. This suggests that the stretch of the three consecutive lysine residues and the Lys-40 are all located close to the interaction surface of the two molecules and thus were linked to Crh. Taken together, the biochemical cross-linking experiments indicate that the N-terminal region of MgsA is required for the interaction with Crh (Fig. 6B).

In a complementary approach to understand MgsA regulation, we used computational modeling to predict where Crh might bind onto MgsA (23,44). Blind docking was performed as described in Experimental Procedures. The only constraint used, is based on the observation that interaction of Crh requires a narrow region formed by helices α 1 and α 2 which contains a residue, Ala-20, shown to be important for interaction to MgsA (23). In the solution obtained, Crh is positioned in the vicinity of a hydrophobic patch formed by helices α 1 and α 6 of MgsA with Crh helices α 1 and α 2 oriented in a perpendicular fashion, also generating a conserved hydrophobic patch on Crh (44) (Fig. 7, S7A). The

obtained arrangement positions the region encompassing residue Ala-20 at a prominent position in close proximity to MgsA (Fig. 7A). Any increase in side chain size or charge would strongly interfere with the interaction properties of Crh and thus explains the results obtained (23).

The following criteria were used to confirm, and nicely fit to, the obtained model. First, the cross-linking results, in particular the distances of the lysines cross-linked between MgsA and Crh agree with the docking model within the required limits (Fig. 7A). In agreement with the model derived from the docking, the interaction interface between Crh and MgsA is defined by intramolecular cross-links on two distant regions of the interaction surface (Fig 7A). Second, a region with negative charge should be located on MgsA in the vicinity of Ser-46, to allow for inhibition of interaction if Crh is phosphorylated (23). In the model, Glu-113 is located in perfect spatial distance that a phosphorylation of Ser-46 in Crh would strongly interfere with this interaction (Fig. 7A). Third, a remarkable cluster of highly conserved residues in helices $\alpha 1$ and $\alpha 2$ in Crh is found (23). Similarly, on MgsA, although less conserved, Ala-112 followed by an acidic residue are conserved as well as two small patches of hydrophobic residues located in helices $\alpha 1$ and $\alpha 6$, respectively, are involved in formation of the two opposing hydrophobic surfaces (Figs. 7B, S3).

In sum, the docking model of Crh to MgsA seems quite reasonable as it fulfills the multiple criteria described in this work and in the literature. The possible impact of Crh binding on MgsA activity will be discussed below.

DISCUSSION

In this work, we have unraveled the molecular mechanism that links carbon source availability to the activity of *B. subtilis* methylglyoxal synthase.

Surprisingly, the results of the SEC-MALS analysis and crystal structure determination provided ambiguous results concerning the oligomeric state of MgsA: In agreement with the crystal structures of *E. coli* (37), *Th. thermophilus* and *Thermus spec.* MgsA, only a hexameric state was observed in crystals. In contrast, dimers and multimers (tetramers/hexamers) were found in solution, and at low concentrations, only dimers were detected. This discrepancy is not unprecedented for MgsA: for the *E. coli* protein, elution volumes corresponding to tetramers were

observed rather than hexamers (32,34-36). Given the low concentration of MgsA in *B. subtilis* (about 1,000 molecules per cell, about 1 μ M) (33), the dimeric form of the AB type is also likely to be present in the cell and the conversion into the hexameric form is dependent on the cellular environment. Moreover, our data suggest that the hexamer is the functional form and the formation of hexamers is a prerequisite for MgsA activity. The hexamer stabilization is increased if phosphate ions and presumably also when substrate levels are increased. Mechanistically this could be explained by structural alterations in the C-terminal residues present in MgsA, forming an arm that reaches from one subunit to a neighboring subunit and interacts in the region of and in some cases with the active site (Fig. S3) (37,43). In the latter case an arginine is required for the proposed interaction with a phosphate in the active site, which is also present in the C-terminal region of *B. subtilis* MgsA (Fig. S3). Moreover, the residues that have been shown to be important and highly conserved for another allosteric pathway are conserved in MgsA as well (Fig. S3; 37,45). Interestingly, *Thermus sp.* GH5 MgsA bears a shortened C-terminal extension that lacks this C-terminal Arg-residue but has been shown to still form a stable hexamer in solution independent of an inorganic phosphate (39).

Under conditions of nutrient limitation, MgsA activity is inhibited by a regulatory interaction with the carbon flux regulator, Crh. In contrast, under conditions of good nutrient supply, Crh becomes phosphorylated on Ser-46 by the HPr kinase (19). The phosphorylated form of Crh is unable to bind MgsA (23), and thus the methylglyoxal bypass of glycolysis is operative under these conditions. This is of physiological relevance to prevent the accumulation of toxic phosphorylated glycolytic intermediates.

Protein-protein interactions are a common theme in the regulation of a variety of biological processes such as nutrient transport, gene expression, or metabolism. Usually the interactions affect the activity of the target protein by preventing access of a molecule or by modifying the local stability of important domains. In bacterial signaling, these paradigms have been well established for the control of ammonium uptake by the regulatory PII protein, as well as for the global control of carbon catabolite repression (14,46). In its phosphorylated state Crh has been shown to interact with the global transcription factor CcpA

and here the phosphate moiety on Ser-46 is charge-compensated by two basic residues (Arg-303 and Lys-307) on CcpA (47). Our docking studies suggest that the charge of the hydroxyl-group of Ser-46 forms a strong hydrogen bond with Glu-113 of MgsA.

The *in silico* protein-protein docking studies, supported by the cross-linking data and the positioning of Ser-46 opposite to a negatively charged residue (Glu-113), suggest that Crh binding to MgsA interferes with the structural properties of the active site of MgsA and reduces or shuts down its activity. This may result from allosteric effects upon binding, which influence the active site structure, especially by interaction with helices $\alpha 1$, $\alpha 3$ and $\alpha 6$, and hereby allosterically delocalize the active site loops towards the center of the active site and thus prevent activity (Fig. S7B). Only minute movements seem to be sufficient, as suggested by the ligand docking experiments. As mentioned, docking was successful only in case of a model relaxation procedure prior to docking, which resulted only in slight structural alterations in the active site.

The bacterial phosphotransferase system is a key component in the control of carbon metabolism in bacteria. In *E. coli*, the phosphorylation state of the Crr protein determines the activity of adenylate cyclase and of a variety of sugar transporters and catabolic enzymes. In the presence of preferred carbon sources, non-phosphorylated Crr bind to several sugar permeases as well as to the glycerol kinase to inhibit the activity of these proteins. In contrast, phosphorylated Crr, which indicates the absence of preferred carbon sources, binds the adenylate cyclase and triggers the synthesis of the second messenger cAMP, and thus the expression of catabolic genes and operons. In Gram-positive bacteria, the HPr protein has multiple functions in sugar transport, control of general catabolite repression via interaction with the global transcription factor CcpA, and the regulation of individual pathways through regulatory interactions with operon-specific regulators. All these interactions are controlled by the phosphorylation state of HPr and may involve the phosphorylation of the target proteins as observed for the glycerol kinase and multiple transcriptional regulators. Alternatively, HPr(Ser-P) binds to transcription factors such as CcpA and RbsR to trigger their DNA-binding activity, and HPr(His-P)

binds to the transcription activator RhgR to stimulate its activity (15,16).

With this work, we have added another facet to the control of carbon catabolism by the PTS: The Crh protein, a regulatory paralog of HPr, binds the metabolic enzyme methylglyoxal synthase in the presence of preferred carbon sources to prevent the accumulation of toxic intermediates. Binding of Crh results in inhibition of MgsA via the deformation of the active site which in consequence is no longer accessible for the substrate molecules. Taking into account the cellular concentrations of both interaction partners, the control of MgsA activity is likely to be the major function of Crh.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

E. coli strains XL1-Blue (48) and BL21(DE3) (Stratagene) were used for cloning experiments and expression of recombinant proteins, respectively. *E. coli* was routinely grown in lysogeny broth (LB) at 37°C. Ampicillin (100 μ g/ml) was used for selection of recombinant *E. coli* strains. LB plates were prepared by the addition of 17 g Bacto agar/l (Difco) to LB medium.

DNA manipulation and transformation

Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (49). Restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified by using the QIAquick PCR purification kit (Qiagen, Germany). *Phusion* DNA polymerase was used for the polymerase chain reaction as recommended by the manufacturer. DNA sequences were determined using the dideoxy chain termination method (49). All plasmid inserts derived from PCR products were verified by DNA sequencing.

Protein purification

His-tagged Crh was purified from *E. coli* BL21 (DE3) carrying plasmid pAG1 (18) as described previously (23).

For expression and subsequent purification of Strep-MgsA and its variants, *E. coli* BL21(DE3) carrying plasmid pGP1301 (wild type; 23) and its mutant derivatives were used. Plasmids expressing the mutant *mgsA* alleles were obtained as follows.

The *mgsA* gene was amplified using primers SK59 and SK60 (23) and the appropriate mutagenic primers by PCR (50,51). The PCR fragments were inserted between the SacI and BamHI restriction sites of the expression vector pGP172 (52). Cleared lysates of cells expressing MgsA were prepared as described (23) and subsequently passed over columns containing 1 ml Strep-Tactin matrix (IBA, Germany). The columns were washed with 2.5 ml buffer W (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) and subsequently Strep-MgsA was eluted using 1 ml buffer W containing 2.5 mM desthiobiotin. An S75 Sepharose gel filtration column (GE-Healthcare) equilibrated in 50 mM Tris/Cl pH 7.5, 150 mM sodium-chloride was used for final purification.

If required for particular experiments, HEPES buffer (150 mM NaCl, 50 mM HEPES, pH7.5) was used for the purification procedure. The Bio-Rad dye-binding assay was used to determine protein concentrations. Bovine serum albumin was used as standard.

Methylglyoxal synthase activity assay

The activity of MgsA was determined spectrophotometrically by a direct assay as described previously (32). Briefly, the reaction mixture (total volume of 0.5 ml) contained 40 mM imidazole buffer pH 7.5, 3 mM dihydroxyacetone phosphate, and 1 μ g (1.12 μ M) purified Strep-MgsA. After incubation at 30°C for 10 min, 100 μ l of each sample was removed and added to a solution containing 0.9 ml H₂O and 0.33 ml 2,4-dinitrophenylhydrazine (0.1% 2,4-dinitrophenylhydrazine in 2 M HCl). After incubation at 30°C for 15 min, 1.67 ml of 10% (w/v) NaOH was added and the OD₅₅₅ was determined after an additional incubation at 30°C for 15 min. The readings were converted into μ mol of methylglyoxal using a molar extinction coefficient of 4.48×10^4 .

Protein-protein cross-linking

To determine interacting regions of the proteins, we cross-linked the proteins either alone or as complexes using BS3 (bis-sulfosuccinimidyl suberate), digested the cross-linked complexes (53), and identified the linked peptides by mass spectrometry. The optimal cross-linker to protein ratio was determined by using 2.5 μ g aliquots of the MgsA/Crh complex and a series of cross-linker molar excesses of 5, 10, 25, 50, 100 and 200 as well

as a control without cross-linker (see Fig. S8A). Samples were allowed to react with freshly prepared BS3 (20 mg/ml in dimethyl sulfoxide) for 30 min at room temperature. The cross-linked samples were analyzed by SDS-PAGE on a 4-12% gel (Invitrogen) or on a freshly prepared 15% SDS gel. The gels were analyzed by Coomassie staining. The identity of the proteins in the complexes was verified by Western-Blot analysis using antibodies against Crh and the Strep-Tag (for the detection of Strep-MgsA) (data not shown).

For MS analysis, MgsA and Crh were cross-linked with freshly prepared BS3 in a 50:1 and 100:1 cross-linker to protein ratio, allowed to react and separated as described above. Bands corresponding to the monomeric protein, the homooligomers of various states, and the crosslinked complex were excised from the gel (see Fig. S8B). In-gel digestion and extraction of peptides was achieved as described elsewhere (53). The solution of extracted peptides was concentrated on a vacuum evaporator and redissolved in 20 μ l injection buffer (5 % v/v acetonitrile, 0.1 % v/v trifluoro acetic acid), of which 6 μ l were loaded in technical duplicate onto an in-house packed C18 column (75 μ m x 300 mm; Repronil-Pur 120C18-AQ, 1.9 μ m, Dr. Maisch GmbH). Chromatographic separation was achieved on an UltiMate 3000 RSLC nano system (ThermoFisher Scientific) from 9% to 42% buffer B (80% v/v acetonitrile, 0.08% v/v formic acid), 300 nl/min flow rate, and 38 min duration. Eluting peptides were sprayed into a QExactive HF-X (ThermoFisher Scientific) mass spectrometer. MS1 scans were performed with a scan range from m/z 350 to 1600, a resolution of 120,000, 1e6 AGC target, and 50 ms maximum injection time. Each survey scan was followed by MS2 scans of the 20 most abundant precursors fragmented with a normalized collision energy of 30, a resolution of 30,000, 1e5 AGC target, and 128 ms maximum injection time. Only charge states from 3 to 8 were considered and no dynamic exclusion was set. Raw files were converted to mgf format with Proteome Discoverer 2.1 (ThermoFisher Scientific) and submitted to cross-link database search with plink 1.23 (54). Briefly, spectra were searched against the sequences of MgsA and Crh considering 3 missed cleavages, cysteine carbamidomethylation as fixed, and methionine oxidation as a variable modification, 10 ppm precursor mass deviation, and 1% FDR cut-off. Subsequently, identified cross-link matches were examined manually.

Western blotting

For Western blot analysis, proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by electroblotting. Rabbit anti-Crh (19) or anti-Strep polyclonal antibodies (PromoCell, Heidelberg; 1:10 000) served as primary antibodies. The antibodies were visualized by using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega) and the CDP-Star detection system (Roche Diagnostics), as described previously (55).

Determination of the oligomerization state of MgsA and the MgsA/Crh complex

The oligomerization status of MgsA and the MgsA/Crh complex was determined by size-exclusion chromatography and multi-angle light scattering (SEC-MALS). For this purpose, purified proteins were applied onto the column either alone or as a pre-mixed and pre-incubated sample in gel filtration buffer (50 mM Tris/Cl pH 7.5, 150 mM sodium-chloride, if not indicated otherwise.) The buffer was filtered (0.1 μm filters) and degassed in line (Model 2003, Biotech AB/Sweden) prior to protein separation on a S75/S200 Superdex 10/300GL column on an Äkta Purifier (both GE Healthcare). Subsequently, the eluate was analyzed in line with a miniDawn Treos multi angle light scattering system followed by an Optilab T-rEX RI detector (both from Wyatt Technology, Europe) before fractionation. Data analysis was performed using the ASTRA 6.1 software (Wyatt Technology) and also compared to a gel filtration standard (Bio-Rad).

Crystallization and data collection

Purified MgsA at a concentration of 11.5 mg/ml was crystallized in SwissCI 96-well plates using the sitting drop vapor diffusion method. Initial screening of protein crystallization conditions in a nanoliter-scale droplet array was performed automatically using a pipetting robot (PhoenixRE, Rigaku) for several commercially available crystallization screens. Best diffracting crystals grew in a condition of the Morpheus screen (56) composed of 10% w/v PEG 4000, 0.1 M M-buffer, 20% w/v glycerol and 30 mM M-Divalent Cations buffer as an additive (M-buffer: 0.1 M bicine/Trizma base pH 8.5, M-Divalent Cations

contained 0.3 M magnesium chloride, 0.3 M calcium chloride). Prior to diffraction experiments, crystals were transferred into a cryo-solution composed of reservoir solution supplemented with 10% (v/v) glycerol. Oscillation images were collected at PETRA III (EMBL, Hamburg, Germany) and processed with XDS (57,58). The scaling process revealed an orthorhombic lattice with unit cell parameters of $a=108.93\text{\AA}$, $b=109.71\text{\AA}$, $c=199.95\text{\AA}$, $\alpha=\beta=\gamma=90.00^\circ$. Systematic absences indicated the presence of screw axes along a, b and c resulting in the P2(1)2(1)2(1) space group. The Matthews coefficient (V_m 3.5 $\text{\AA}^3/\text{Da}$) suggested 12 molecules in the asymmetric unit corresponding to a solvent content of 65%. The data collection statistics are summarized below (see Table 1).

Structure determination and refinement

The phase problem was solved by means of molecular replacement (MR) using PHASER and the structure of the methylglyoxal synthase from *Thermus sp.* (PDB 2XW6) as a search model. The search model was identified based on HHPRED (59) search for homologous proteins and trimmed to the last common atom using the sequence alignment and tools from the ROSETTA package (60) prior to the MR search. The initial MR solution was first rebuilt using Rosetta model completion and relaxation (60) followed by alternating cycles of refinement using Phenix (61) and manual rebuilding with Coot (62). Standard parameters including weights optimization, TLS parameterization and building of solvent molecules were used during refinement. A random set of 5% of reflections, selected in thin resolution shells, was excluded from refinement to monitor R_{free} (63). The final model consisting of two MgsA hexamers, 526 water molecules, 9 glycerol molecules and 23 Cl ions has been refined at a resolution of 2.34 \AA to R and R_{free} factors of 17.0% and 20.49%, respectively. Each of the twelve MgsA monomers is made up of 121 residues of which two or three are resulting from the N-terminally placed Strep-tag (sequence Val-Ser-Ser). Seventeen residues at the C-terminus as well as 20 residues of the Strep-tag could not be localized in the electron density map and are most likely disordered. The refinement statistics are summarized in Table 1. The quality of the model was assessed using MOLROBITY as implemented in Phenix (61). Secondary structure predictions were performed using DSSP (64). Coordinates

were superimposed with LSQKAB (65) from the CCP4 program suite (66) or as implemented in Pymol (<http://www.pymol.org>). Exploration of macromolecular interfaces was performed using ‘Protein interfaces, surfaces and assemblies’ (PISA) services at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) using standard settings (67). Structure based sequence alignments were performed using Espresso (68,69) and the respective graphs were prepared using ESPript (70).

Computational modelling

In silico docking experiments of the substrate dihydroxyacetone-phosphate into MgsA was performed using Autodock Vina (71). The MgsA active site was identified based on superposition with the structure of the *E. coli* methylglyoxal synthase complexed with phosphoglycolohydroxamic acid (PDB 1IK4), serving also as the reference for ligand binding mode. The substrate molecule as well as side chains forming the active site were kept flexible during the docking trials: Ile-6, His-8, Asp-9, Lys-12, Thr-34, Thr-36, Thr-37, Ser-54, Asp-60, Phe-77, His-87 and Val-91. Interestingly, successful docking results were obtained only for the receptor model which was relaxed using Rosetta application (72). Relax protocol in Rosetta performs a simple all-atom model refinement in the Rosetta force-field which searches the local conformational space around the starting structure (RMSD calculated for C α atoms forming the MgsA hexamer amounted to 0.06 Å). This indicates, that the receptor molecule was tiny a bit too compressed and very small model-

alterations were necessary to make the narrow active site capable of accommodating the ligand molecule. The final docking model, representing the lowest energy decoy, reveals a very similar position and orientation of the docked dihydroxyacetone-phosphate to the reference phosphoglycolohydroxamic acid (distances between the carbonyl oxygen and the phosphate atoms are 0.5 and 2.0 Å, respectively). Blind protein-protein docking experiments of Crh molecules (PDB 1ZVV, chain W) to a hexameric form of MgsA have been performed using Rosetta (73) in a two-stage protocol. The first stage, where aggressive sampling is done, was performed in the centroid (low resolution) mode with a single site constraint defined based on our previous study (23): Crh residue Ala-20, located in the middle of helix 1, should be in contact with the receptor (MgsA). The 10.000 decoys were sorted by Rosetta score in order to select the 300 best preliminary docking models, which were clustered by pairwise RMSD calculation. The central decoy from the cluster with the lowest score was subjected to all-atom refinement stage (high resolution) that optimizes both rigid-body orientations and side-chain conformations of the docked molecules. The 1,000 docked models were scored based on the Rosetta total energy score as well as on the interface score *I_{sc}* which represents the energy of the interactions across the interface. Best scoring decoys were clustered and the central model from the best cluster was chosen as the final docking model. This model was subsequently used for the further analysis and visual inspection.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

BG, JS, AD, and RF conceived the study. BG, AD, JS, and HU designed the experiments. AD, CPZ, JG, JA, IP, and RH carried out the experiments. PN and AD performed computational structure modeling and protein docking studies. AD, CPZ and JS wrote the manuscript. All authors approved the manuscript.

FOOTNOTES

¹ The abbreviations used are: PTS, phosphotransferase system; SEC, size-exclusion chromatography; MALS, multi-angle light scattering; SDS, sodium dodecylsulphate; PAGE, polyacrylamide gel electrophoresis; PDB, Protein Data Bank; RMSD, root mean square deviation; BS3 bis-sulfosuccinimidyl suberate; cAMP, cyclic adenosine monophosphate; LB, lysogeny broth; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MS analysis, mass spec analysis; RI detector, refractive index detector; FDR, false discovery rate; PVDF, polyvinylidene difluoride; PEG, polyethylene glycol; MR, molecular replacement; PISA, Protein interfaces, surfaces and assemblies; I_sc, interface score; ESRF, European Synchrotron Radiation Facility; Wt, wild type

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Table 1. Data collection and refinement statistics

Data collection	
Wavelength (Å)	0.7732
Resolution range (Å) *	48.99 - 2.34 (2.423 - 2.34)
Space group	P 21 21 21
Unit cell (Å), (°)	108.93 109.71 199.95 90 90 90
Total reflections	478161 (48123)
Unique reflections	101094 (10027)
Multiplicity	4.7 (4.8)
Completeness (%)	99.41 (99.72)
Mean I/sigma(I)	15.94 (2.41)
Wilson B-factor (Å ²)	46.98
R-merge	0.06155 (0.6581)
R-meas	0.06938
CC1/2	0.999 (0.739)
CC*	1 (0.922)
Refinement	
R-work	0.170 (0.252)
R-free	0.205 (0.277)
Number of non-hydrogen atoms	11898
macromolecules	11278
ligands	77
water	526
Protein residues	1464
RMS (bonds) (Å)	0.007
RMS (angles) (°)	1.079
Ramachandran favored (%)	99.2
Ramachandran allowed (%)	0.28
Ramachandran outliers (%)	0
Clashscore	2.00
Average B-factor (Å ²)	54.7
macromolecules	54.6
ligands	71.8
solvent	53.8
PDB	6F2C

*The numbers in brackets refer to the highest resolution shell.

Table 2. Calculated and predicted molecular mass of the various (partially) soluble MgsA forms tested in SEC-MALS.

MgsA	Experimental [kDa]	Predicted* [kDa]
Wt (peak)	62	69.9 (4-mer)
		104.8 (6-mer)
Wt (shoulder)	38	34.9 (2-mer)
T107R/T111R	Aggregate	35.1 (2-mer)
Q62R/A65R	34.1	35.1 (2-mer)
Q62R	41.3	34.9 (2-mer)
A93W/L97W	34.4	35.3 (2-mer)
Crh	Experimental [kDa]	Predicted* [kDa]
Wt	20.5/19.8	21.0 (2-mer)
	10.3/9.6	10.5 (1-mer)

*All weights calculated via ExPASy ProtParam as expressed, including tags.

Table 3. Intermolecular cross-links between MgsA and Crh

MgsA [residue]	Crh [residue]	MgsA peptide	Crh peptide
K10	K40	IALIAHDK*KK	DGK*K
K10	K41	IALIAHDK*K	K*VNAK
K11	K40	IALIAHDKK*K	DGK*K
K12	K41	K*QDMVQFTTAYR	K*VNAK
K10	K11	IALIAHDK*K	LK*TGLQAR
K12	K11	KK*QDMVQFTTAYR	LK*TGLQAR
K40	K11	NHDLYATGTTGLK*IHEATGLQIER	LK*TGLQAR

* cross-linked lysine residues

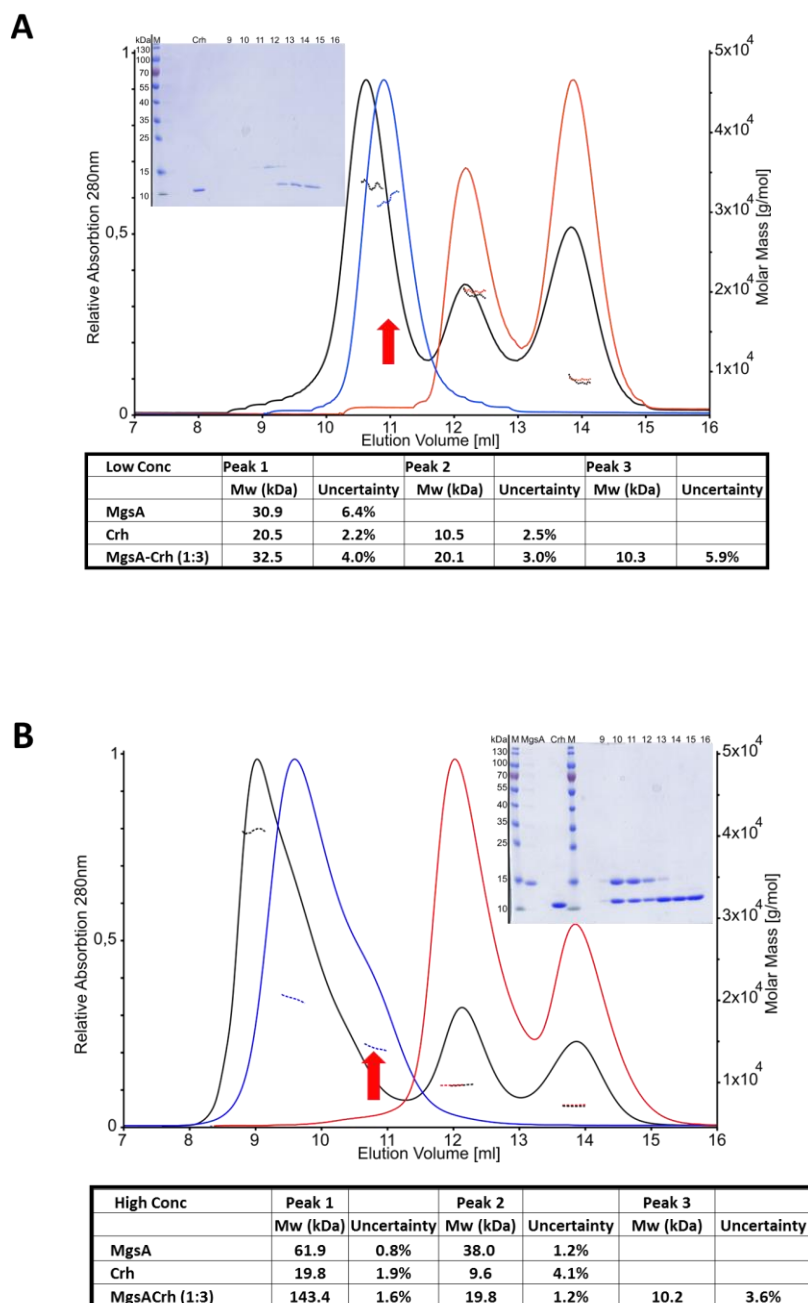


Figure 1. The MgsA binds to Crh only if MgsA is present in hexamers. SEC-MALS experiments suggest that only the MgsA hexamer binds Crh resulting in a 1:1 stoichiometry. **A.** Low MgsA concentration (13 μ M) **B.** High MgsA concentration (135 μ M). In each case Crh was used in a three-fold molar excess. Insets: Coomassie stained gel of the respective fractions (equivalent to the ml of elution volume) obtained in the SEC run of the MgsA-Crh mixture.

The red arrows indicate the elution volume of dimeric MgsA. Below each chromatogram the calculated molar masses determined by MALS are listed. Blue lines, MgsA; red lines, Crh; black lines, mixture of MgsA and Crh (concentrations as indicated). Dotted line, molecular masses as determined by MALS.

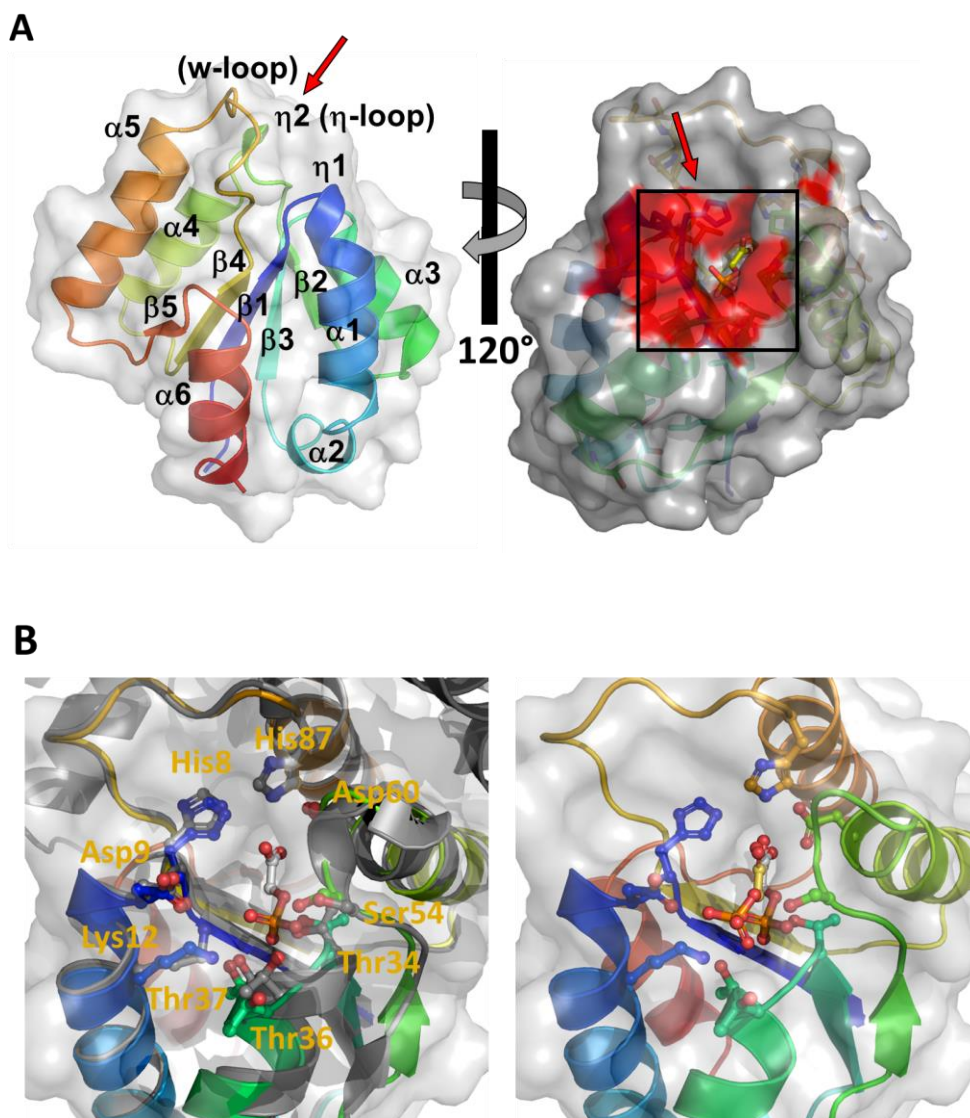


Figure 2. The structure of *B. subtilis* MgsA exhibits an overall globular shape. A. The central five-stranded β -sheet is flanked by six α helices. Coloring in rainbow from N- (blue) to C-terminus (red). The surface structure is depicted in gray; the individual secondary structure motifs are indicated and numbered as α -helices (α) or β -strand (β) from the N to the C-terminus. The panel on the right shows a surface representation of an MgsA monomer in gray with the surface region of important active site residues highlighted in red. **B. Left:** Magnification of the active site as deduced from an overlay with the *E. coli* MgsA (gray) with 2-phosphoglycolic acid (ball and stick mode, PDB 1EGH) bound, serving as reference ligand. Cavity conformation and arrangement of active site residues are highly conserved (See also Fig. S3, S5). Identical residues of *B. subtilis* MgsA within the Mgs-family involved in ligand binding are depicted with their side chains in ball and stick mode with the C-atoms labelled according to Fig. 2A, with oxygen in red and nitrogen in blue. Residue numbering according to *B. subtilis* MgsA. **Right:** Result of a docking experiment with the substrate dihydroxyacetone phosphate (Carbons in yellow, phosphorus in orange) and phosphoglycolohydroxamic acid (PDB 1IK4) as reference.

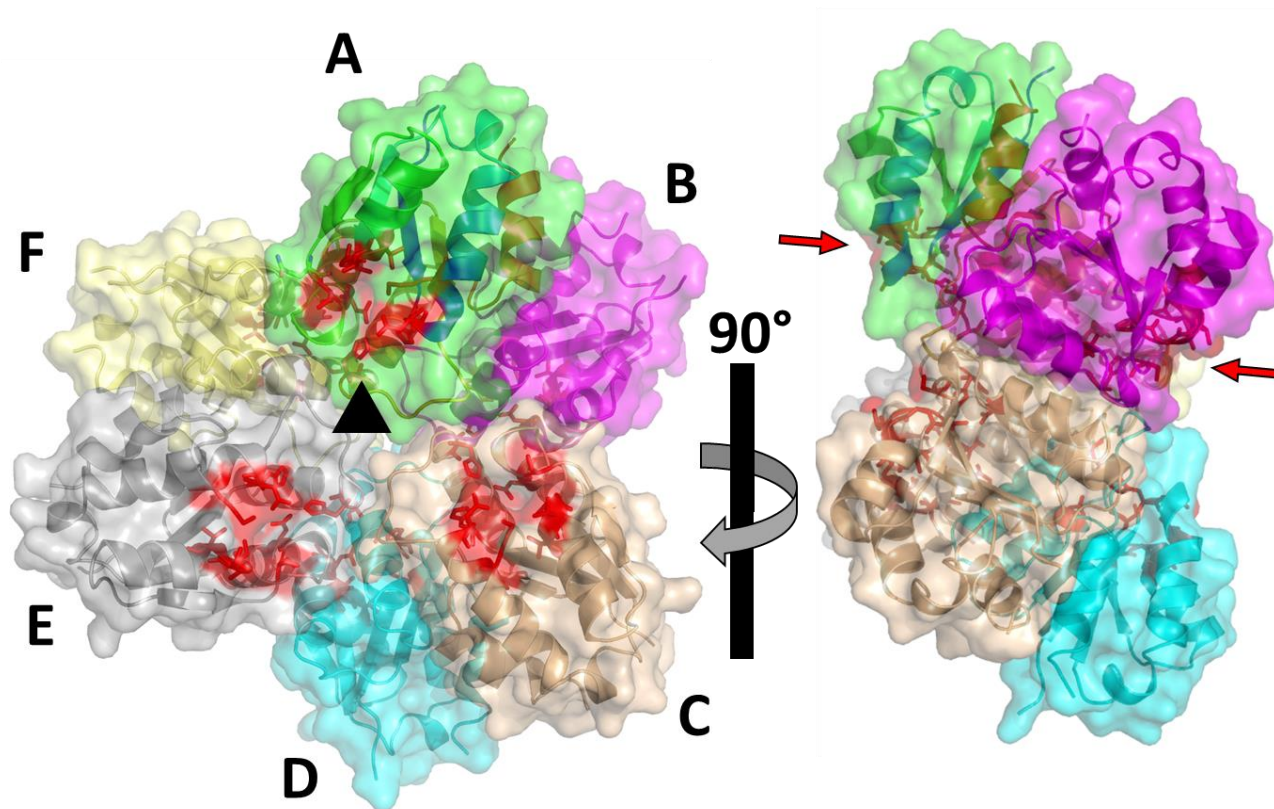


Figure 3. Structure of the *B. subtilis* MgsA hexamer. The hexamer is composed of three dimers of MgsA, which arrange in the hexamer with three active sites (active site residues indicated in red) arranged by 120° rotations (indicated by the black triangle in the center) on either side of the hexamer. The individual molecules are indicated in different colors and labelled A-F (top right panel). Bottom right panel side view of the hexamer. The arrows indicate the active sites of molecules A and B.

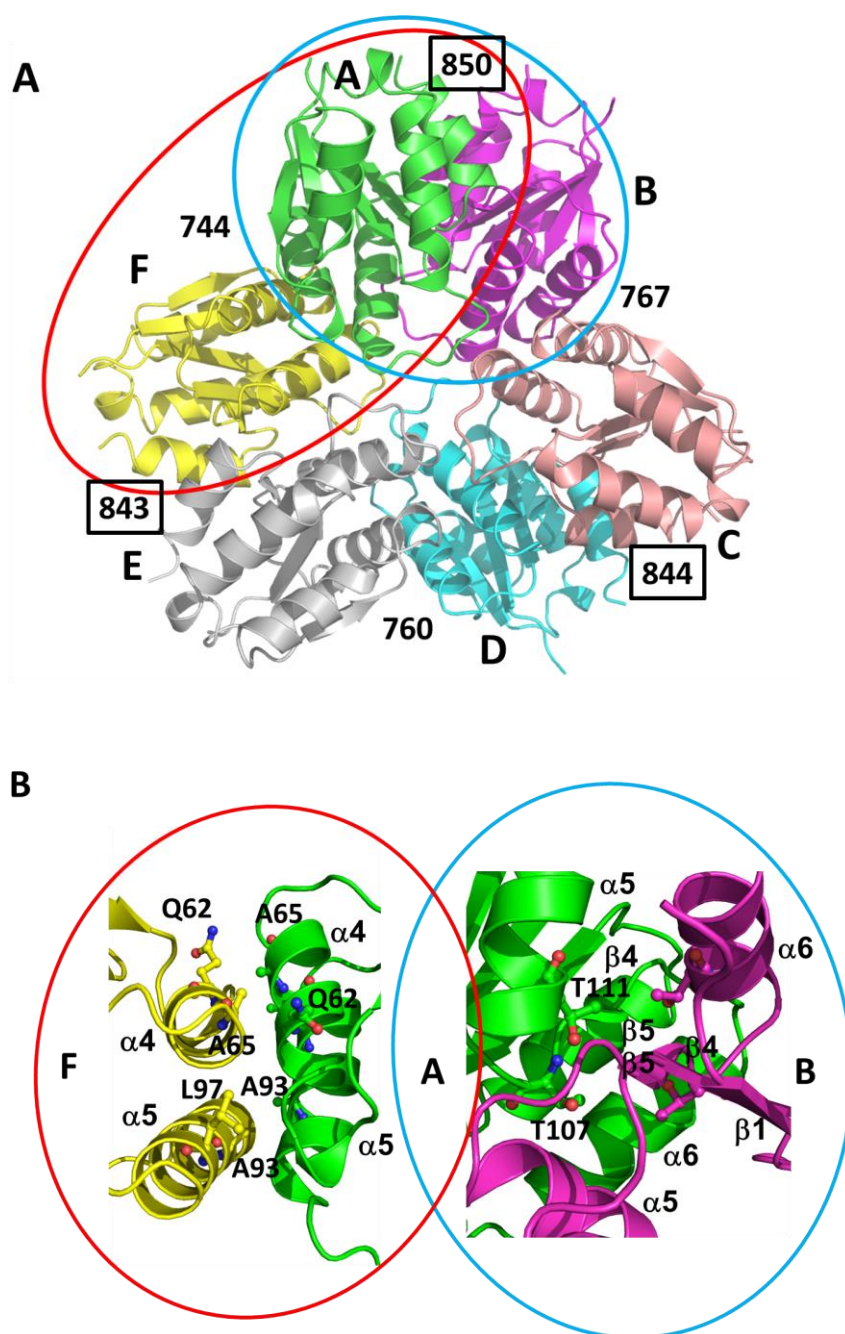


Figure 4. Mutational and structural analysis suggests hexamer disassembly into MgsA dimers. **A.** The disassembly of the hexamer (molecules colored and labeled as in Fig. 3) into dimers could result in two different dimers one leaving the active sites of one subunit in contact with the neighboring molecule (AF, indicated by a red ellipsoid) or disrupt the active site connecting surface (AB, indicated by a cyan ellipsoid). The numbers depicted indicate the average surface area (in \AA^2) of the individual molecules involved in interaction with the neighboring molecule. See text for details. **B.** Magnification of the interfaces AF and AB. The point mutations tested for interference with hexamer-formation of MgsA are indicated and only those located at the interface formed by the MgsA molecules A and F prevent hexamerization. Thus the monomers that interact to form the stable dimer require the other dimer interface (e.g. AB).

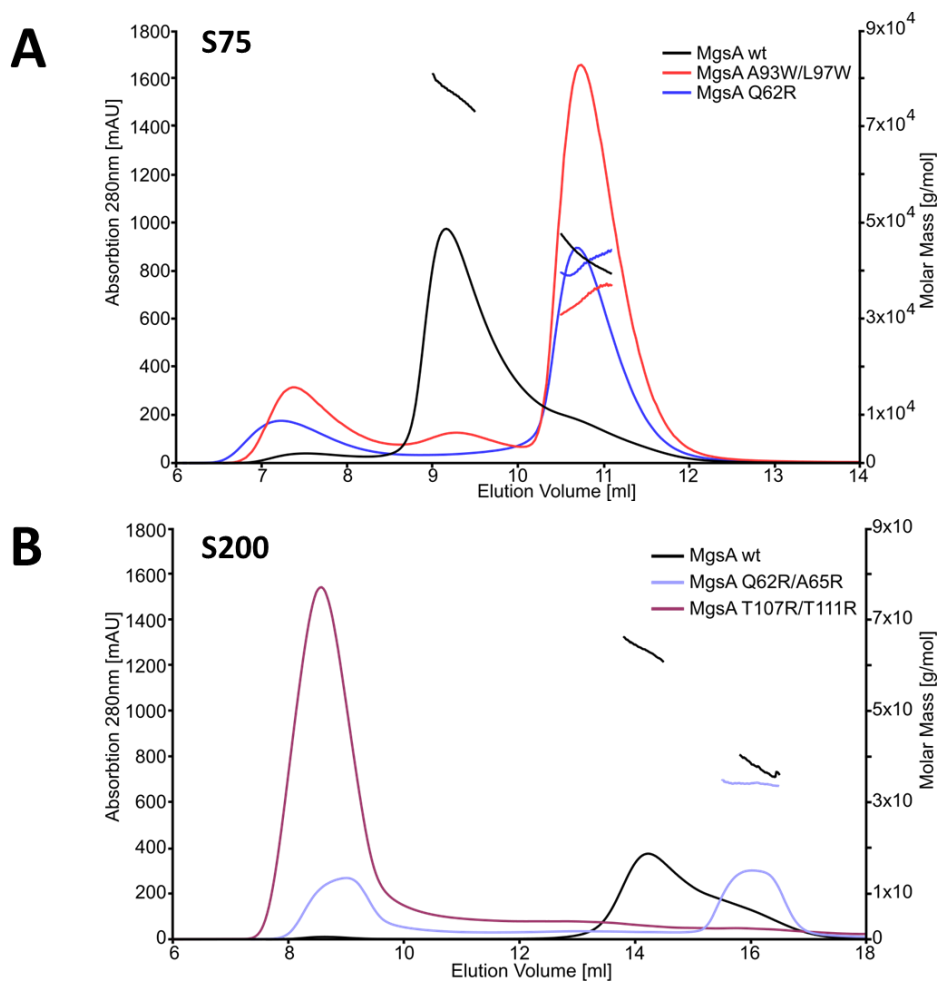


Figure 5. The analysis of the different MgsA variants suggests that the AB-type dimer is the one that exists in solution not the AF-type dimer. SEC-MALS experiments suggest that only the wild-type MgsA is folded correctly and capable of forming hexamers. The solid lines indicate the absorption profile of the SEC run, whereas the stray lines indicate the molecular mass observed in the MALS measurement at the respective elution volume. **A.** In contrast to wild-type MgsA the point mutants A93W/L97W and Q69R form predominantly soluble dimers. **B.** Opposing the wild-type MgsA the point mutant Q62R/A65R forms mostly dimers and T107R/T111R aggregates. See text for details.

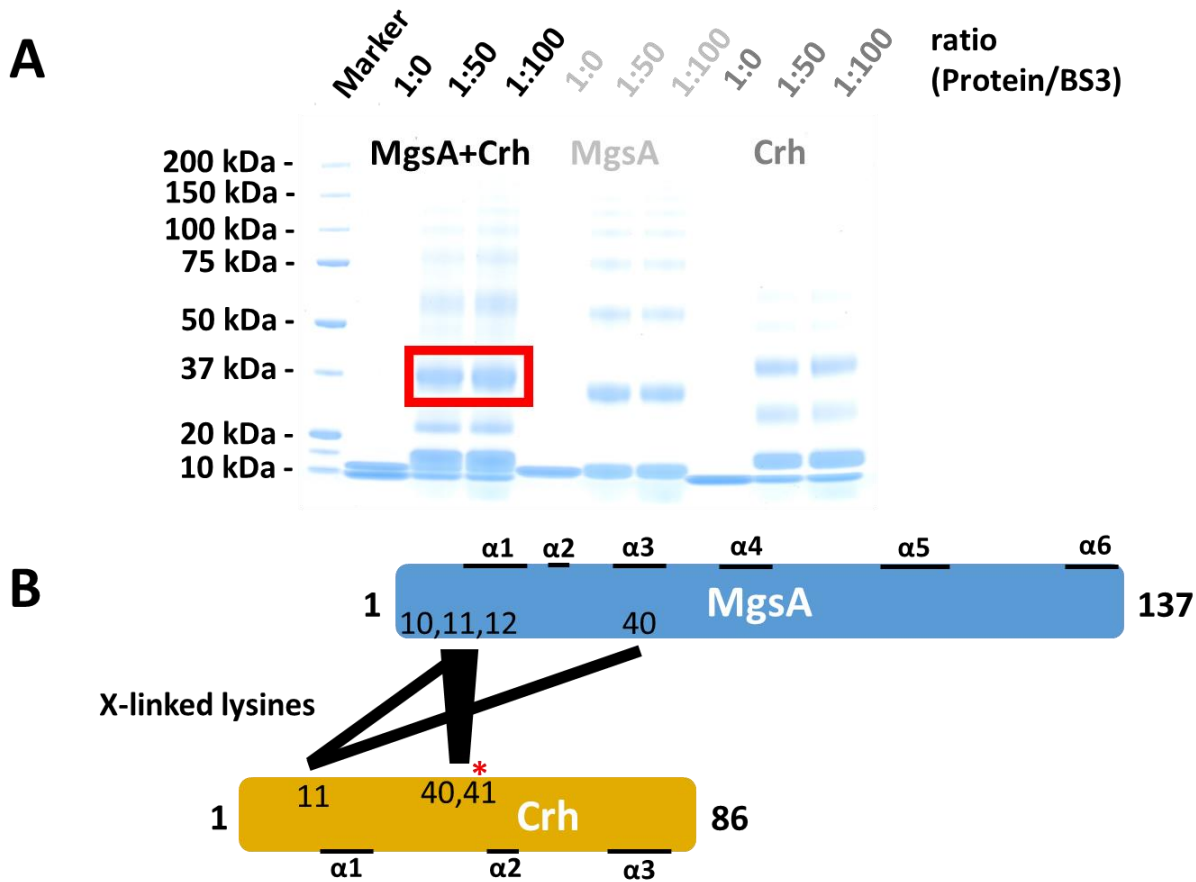


Figure 6. Determination of Crh crosslinks to MgsA. **A** *In vitro* chemical cross-linking of MgsA and Crh either alone or in a complex formed in a 1:1 ratio. The samples were treated with the predetermined (Fig. S8) optimal amounts of BS3 using a 1:50 and 1:100 protein:cross-linker ratio. According to the size, bands that represent the complex of MgsA and/ or MgsA-Crh were analyzed by LC-MS. **B.** Schematic representation of the intermolecular crosslinks determined (See also Table 3 and Figs. S5, S6).

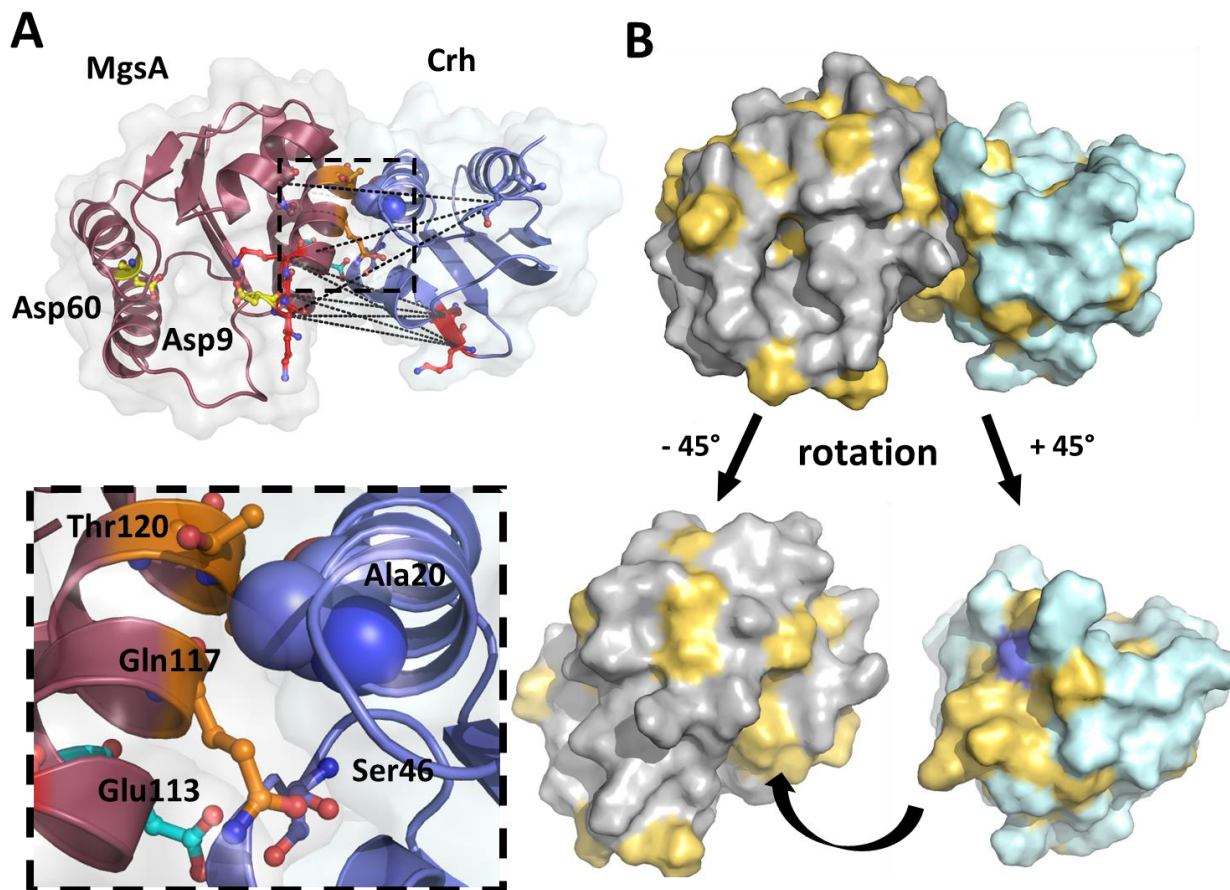


Figure 7. Model of the MgsA-Crh interaction. **A.** The model of MgsA-Crh complex obtained by blind docking experiments fulfills the described criteria. The MgsA molecule (gray surface and molecule colored dark red) is in close contact with Crh (surface in light cyan and the molecule colored in purpleblue) in the vicinity of the MgsA active site. For reference the catalytically active site residues of MgsA, Asp-9 and Asp-60 are depicted in ball and stick mode. The lysines involved in MgsA-Crh crosslinks (11, 12, 13 in MgsA and 40, 41 in Crh) are depicted in ball and stick mode (red) and the distances between the respective C α -atoms are indicated by black dashed lines, which range from 22.8-25.1 Å (from C α to C α). The Ala-20 used as the single constraint in the initial docking step is depicted in purple blue spheres, whereas the phosphorylation sensitive inhibitory site Ser-46 is indicated in ball and stick mode colored with the C-atoms in purple. A nearby positioned negatively charged glutamate (113) interfering with a binding of phosphorylated Crh is indicated in cyan (located in helix 2). **B.** Analysis of the hydrophobic patches on both MgsA and Crh are indicated in light orange on the gray (MgsA) or light cyan (Crh) surfaces. Below the interaction hydrophobic patches of the two molecules are visualized by a rotation of the individual molecules as indicated and omitting the partner molecule. The surface area of Ala-20 used as initial criterion for docking is highlighted in purple blue (see text for details).

Structural basis for the regulatory interaction of the methylglyoxal synthase MgsA with the carbon flux regulator Crh in *Bacillus subtilis*

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