Evidence from Mutational Analysis for a Single Transmembrane Substrate Binding Site in the Histidine ATP-Binding Cassette Transporter of *Salmonella enterica* Serovar Typhimurium

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ABSTRACT The histidine ATP-binding cassette (ABC) transporter of *Salmonella enterica* serovar Typhimurium is among the best-studied type I ABC import systems. The transporter consists of two transmembrane subunits, HisQ and HisM, and a homodimer of the nucleotide-binding subunit, HisP. Substrates are delivered by two periplasmic solute binding proteins, HisJ and LAO, with preferences for histidine and for lysine, arginine, and ornithine, respectively. A homology model was built by using the arginine-bound crystal structure of the closely related Art(QN)₂ transporter of *Thermoanaerobacter tengcongensis* as the template. In the homodimeric Art(QN)₂, one substrate molecule is bound to each of the ArtQ subunits, whereas the structural model and sequence alignments predict only one substrate molecule in contact with HisM. To address the question whether one or two binding sites exist in heterodimeric HisQM, we have studied the functional consequences of mutations by monitoring (i) the complementation of growth on D-histidine of auxotrophic tester strains, (ii) the growth of tester strains on arginine as a nitrogen source, and (iii) ATPase activity of purified variants in a lipid environment. Our results demonstrate that two negatively charged residues, namely, HisM-E166 and HisQ-D61, are indispensable for function. Furthermore, the complete reconstruction of an ArtQ-like binding site in HisQ resulted in an inactive transporter. Likewise, switching the positions of both negatively charged residues between HisQ and HisM caused transport-deficient phenotypes. Thus, we propose that one substrate molecule is primarily liganded by residues of HisM while HisQ-D61 forms a crucial salt bridge with the α -amino group of the substrate.

IMPORTANCE Canonical ATP-binding cassette (ABC) importers are major players in the translocation of numerous nutrients, vitamins, and growth factors to the cytoplasm of prokaryotes. Moreover, some ABC importers have been identified as virulence factors in bacterial pathogenesis. Thus, a full understanding of their mode of action is considered a prerequisite, among others, for the development of novel antibacterial drugs. However, mainly owing to the lack of structural information, the knowledge of the chemical nature and number of substrate binding sites formed by the transmembrane subunits of ABC importers is scarce. Here, we provide evidence from mutational analyses that, in contrast to homologous homodimeric systems, the heterodimeric histidine transporter of *Salmonella enterica* serovar Typhimurium is liganding only one substrate molecule between its transmembrane subunits, HisM and HisQ.

KEYWORDS ATP-binding cassette transporters, *Salmonella enterica* serovar Typhimurium, type I ABC importer, histidine transporter, mutational analysis, transmembrane substrate binding site

Citation Heuveling J, Landmesser H, Schneider E. 2019. Evidence from mutational analysis for a single transmembrane substrate binding site in the histidine ATP-binding cassette transporter of *Salmonella enterica* serovar Typhimurium. J Bacteriol 201:e00521-18. <https://doi.org/10.1128/JB.00521-18>.

Editor Ann M. Stock, Rutgers University-Robert Wood Johnson Medical School

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This article is dedicated to Giovanna Ferro-Luzzi Ames (UC Berkeley) in recognition of her contributions to this field.

Received 28 August 2018

Accepted 14 October 2018

Accepted manuscript posted online 22 October 2018

Published 20 December 2018

Canonical prokaryotic ATP-binding cassette (ABC) import systems mediate the uptake of various nutrients, vitamins, ions, and osmoprotectants at the expense of ATP (1, 2). The translocation unit is built from the standard ABC modules, i.e., two transmembrane subunits/domains (TMDs) that form a translocation path and two nucleotide-binding subunits/domains (NBDs) that bind and hydrolyze ATP (3). As a unique and essential feature, an extracellular substrate-binding protein (SBP) is required for function (4, 5).

SBPs consist of N- and C-terminal lobes, which display α -/ β -folds, connected by a hinge region (6). The binding of a substrate leads to the rotation of these lobes toward each other, resulting in the inaccessibility of the central cleft to the aqueous surrounding ("Venus flytrap" model) (7). While both the open and closed forms of SBPs can dock on their cognate transporters, only the latter productively triggers ATPase activity and, consequently, the transport cycle (2, 8–12). In Gram-negative bacteria, SBPs are soluble proteins which freely diffuse in the periplasm, whereas in Gram-positive bacteria and in some archaea, SBPs are anchored to the outer leaflet of the cytoplasmic membrane via acyl chains covalently linked to an N-terminal cysteine residue (13). In other archaea, SBPs are tethered to the membrane by an N-terminal hydrophobic alpha-helix. Thus far, in a few cases, solute binding domains were found that are fused to the TMDs of their cognate transporters, resulting in one, two, or four substrate binding sites (2, 14–17).

On the basis of their numbers of transmembrane helices and the chemical nature of their substrates, canonical ABC importers have been subdivided into types I and II (18). While type I importers feature transmembrane cores of 10 to 14 helices, type II importers display transmembrane domains with up to 20 helices and restricted specificity for metal chelates, heme, and vitamin B₁₂. Evidence was provided for distinct mechanistic differences between both subtypes (19, 20).

Several crystal structures of the maltose/maltodextrin transporter (MalE-FGK₂) of *Escherichia coli* (21) provided a framework for the proposed mode of action of type I importers, termed the "alternate access model" (22). Accordingly, in the apo state, i.e., in the absence of nucleotides, the transporter resides in an inward-facing conformation. The concomitant binding of liganded SBP (MalE) to the TMDs (MalF and MalG) and ATP to the NBDs (MalK₂), which causes the latter to fully close, results in a switch of the TMDs to an outward-facing conformation. As a consequence, the substrate is delivered to a low-affinity binding site formed by the MalF subunit (23). The subsequent hydrolysis of ATP eventually leads to the release of the ligand to the cytoplasm and the return of the transporter to the apo state (reviewed in reference 9).

For type II transporters, no evidence for a specific transmembrane substrate binding site(s) exists to date (24), whereas the structural data from two type I transporters revealed substrate binding pockets within the transmembrane subunits. In the heterodimeric maltose transporter, as already pointed out above, maltose is exclusively in contact with residues from the MalF subunit (23, 25). In contrast, in the homodimeric transporter for positively charged amino acids of *Thermoanaerobacter tengcongensis* (*Caldanaerobacter subterraneus* subsp. *tengcongensis*) belonging to the phylum *Firmicutes*, Art(QN)₂, each ArtQ subunit was found to bind one molecule of arginine or histidine (Fig. 1A) (26). Although we have recently demonstrated that, mechanistically, one intact binding site is sufficient for function (27), it remains elusive whether this holds true under physiological conditions. Moreover, because of the lack of additional crystal structures, it is unknown to date whether one or two binding sites are representative for hetero- and homodimeric systems, respectively.

To address this question by other means, it would be desirable to compare hetero- and homodimeric transporters sharing the same substrate specificity. In case of the *E. coli* maltose transporter, to our knowledge, no homodimeric homolog has thus far been identified. However, transporters for positively charged amino acids exist in both homo- and heterodimeric compositions. The best-studied heterodimeric system is the histidine transporter, HisQMP₂, of *Salmonella enterica* serovar Typhimurium (2, 28–31).

Here, we report on a comprehensive mutational analysis of the putative substrate binding pockets in HisM and HisQ. Our results led us to propose that arginine/histidine

FIG 1 Comparison of arginine binding sites in the crystal structures of Art(QN)₂ and the homology model of HisQM. (A, left) Overall structure of Art(QN)₂ (PDB access code 4YMU). Two transmembrane subunits (ArtQ) are colored red and purple, and two (Continued on next page)

is primarily interacting with HisM, but a negatively charged residue in HisQ (D61) is indispensable for transporter function.

RESULTS

Structural features and experimental setup. Two negatively charged residues—E152 and E159—characterize the substrate binding pocket in each of the ArtQ subunits of the Art(QN)₂ transporter of *T. tengcongensis* (26) (Fig. 1A). While E152 forms salt bridges with the guanidine group of arginine via its δ -carboxyl group, E159, as well as residues L67, N98, and M156, is involved in hydrogen bonds and van der Waals interactions with the substrate. In addition, the benzene ring of Y102 pairs with the guanidine group (Fig. 1A). The binding of histidine involves the same residues (26). Interestingly, the crystal structures revealed that the α -carbon atom of P66 forms a hydrogen bond with the carboxyl group of the substrate in one ArtQ subunit only. However, a mutational analysis using the closely related Art(MP)₂ transporter of *Geobacillus stearothermophilus* as a model system revealed that only negatively charged residues are essential for function (27).

The well-characterized histidine transporter of *S. Typhimurium*, HisQMP₂, used here as model system to investigate the putative transmembrane binding site(s) of a heterodimeric system, is composed of one copy each of the membrane-integral subunits, HisQ and HisM, and a homodimer of the nucleotide-binding subunit, HisP. As a special feature, the substrates are delivered by two periplasmic substrate-binding proteins, HisJ and LAO, which display the highest affinities in the nanomolar range for histidine and for lysine, arginine, and ornithine, respectively (32, 33). The crystal structures of HisJ (32) and LAO in complex with different ligands (34) and of HisP with bound ATP (35) have been solved. Furthermore, biochemical and biophysical data revealed that the histidine transporter basically undergoes the same conformational changes during the transport cycle as demonstrated for the maltose transporter (36, 37). Although structural information on the full transporter is elusive, a homology model of the HisQM dimer was built by the Swiss Model homology server (<http://swissmodel.expasy.org>) using Arg-bound ArtQ as the template (PDB access code 4YMU), which guided our study (Fig. 1B and C). (Structural models of HisM and HisQ using the His-bound ArtQ as the template are shown in Fig. S1 in the supplemental material.)

HisQ and HisM display 33% and 31% sequence identity, respectively, with ArtQ (26), which is significant considering the rather distant taxonomic relationship of *S. Typhimurium* and *T. tengcongensis*. For comparison, HisQ and HisM exhibit only 17% and 24% sequence identity, respectively, with the MetI subunit of the methionine ABC transporter of *Escherichia coli*, Met(IN)₂ (GenBank accession no. [NP_414740](#)). The latter is the only other amino acid ABC transporter of which structural information (without bound substrate) is available (38, 39).

All but one residue from the binding pocket of ArtQ are conserved in HisM (Fig. 1B and D). The only exception is E159, which is replaced by S173 in HisM, thereby eliminating one negatively charged residue from the binding site (Fig. 1D). In contrast,

FIG 1 Legend (Continued)

nucleotide-binding subunits (ArtN) are colored cyan and light green. Arginine (in ArtQ) and ATP molecules (in ArtN) are shown as ball-and-stick models. (Right) Magnification of one substrate binding site in ArtQ (indicated by the black square on the left). Residues of ArtQ interacting with one arginine molecule and neighboring residues are indicated. Hydrogen bonds are represented as dashed lines. (B, left) Structural alignment of ArtQ₂ (colored as in panel A) with the modeled HisQM heterodimer (colored purple and green, respectively). Homology model of HisQM was built by the Swiss model homology server using Arg-bound ArtQ (PDB access code 4YMU) as the template. (Right) Magnification of proposed substrate binding site in HisM (indicated by the black square). Residues proposed to form hydrogen bonds with an arginine molecule (represented by dashed lines) and neighboring residues are indicated. (C, left) As in panel B, left. (Right) Magnification of proposed substrate binding site in HisQ (indicated by the black square). Residues proposed to form hydrogen bonds with an arginine molecule (represented by dashed lines) and neighboring residues are indicated. The figures were drawn with Biovia Discovery Studio Visualizer v4.5. (D) Sequence alignment of peptide regions from ArtQ, ArtM, HisM, and HisQ encompassing the key residues from the binding pocket interacting with arginine (in bold). Residues in HisM and HisQ that differ from those in ArtQ are red. Numbers indicate the relative positions of the mutated residues within the polypeptide chains of the respective proteins.

FIG 2 Cartoon illustrating the complementation assay used to monitor transport activity of histidine transporter variants. The tester strains BL21-5 and BL21-6 (see Table S1 in the supplemental material and Materials and Methods for construction) carry chromosomal deletions of *hisM* and *hisQ*, respectively, in addition to a deletion of *hisF*. The latter encodes an essential enzyme (imidazole glycerol phosphate synthase, catalytic subunit with HisH) of the histidine biosynthesis pathway. As a consequence, the tester strains display a histidine-auxotrophic transport-deficient phenotype. While L-histidine can be internalized by the bacteria via several transport systems (43), D-histidine uptake is totally dependent on the HisQMP₂ transporter (40). Therefore, in the presence of plasmids carrying wild-type or mutant alleles of the transporter genes *hisQM*, the growth of the respective strain is assayed in minimal glucose medium supplemented with D-histidine as source for L-histidine. Intact transporter variants mediate uptake of D-histidine followed by racemase-catalyzed conversion to L-histidine (40), which eventually results in cell growth monitored either on agar plates or in liquid medium.

in HisQ, only two residues from the ArtQ binding site are conserved (P60 and Y107) (Fig. 1D). Moreover, unlike in HisM, the model predicts only one interaction of the carboxyl group of arginine with the α -amino group of D61 (Fig. 1C). Thus, these observations give rise to the question whether a (second) substrate binding site within HisQ actually exists.

To address this question, we performed mutational analyses of each of the residues from the putative binding site(s) in HisM and HisQ to unravel their role in liganding a substrate molecule. N112 and Y116 of HisM and Y107 of HisQ were not considered, because the homologous residues in ArtM of *G. stearothermophilus* were recently found to be dispensable for function (27). To study the effects of mutations in HisQM *in vivo*, we took advantage of the earlier finding that the growth of an *S. Typhimurium* strain defective in histidine biosynthesis on D-histidine as a source for L-histidine is dependent on the overproduction of the histidine transporter (40). Consequently, we constructed two tester strains (BL21-5 and BL21-6) (see Table S1) lacking the *hisF* gene (required for histidine biosynthesis) and the *hisM* (BL21-5) and *hisQ* genes (BL21-6) (see Fig. 2 and Materials and Methods for details). An *in vitro* analysis of the mutants was performed by monitoring the ATPase activity of purified variants in a lipid environment stimulated by HisJ/histidine and LAO/arginine, respectively.

Mutational analysis of HisM. As shown in Fig. 3, when transformed with a plasmid carrying the wild-type alleles of *hisQMP*, both tester strains exhibited growth on minimal glucose medium supplemented with D-histidine (0.3 μ M) after 24 h and even

HisM variants	Growth after 24h 48h	HisQ variants	Growth after 24h 48h
wildtype		wildtype	
P70A		P60A	
L71D		D61L	
E166Q		D61E	
E166A		D61N	
E166D		I103N	
S173E		N157E	
S173A		A164E	
		D61L,A164E	

FIG 3 Growth on D-histidine as the sole source of L-histidine of *E. coli* tester strains producing wild-type or variants of the histidine transporter. Plasmids carrying wild-type or mutant alleles of *hisQMP* (Table S2) encoding HisM and HisQ variants as indicated were introduced into *E. coli* strains BL21-5 ($\Delta hisF \Delta hisM::kan$) and BL21-6 ($\Delta hisF \Delta hisQ::kan$), respectively (Table S1). Cells were grown overnight in LB-amp-kan medium, washed twice with minimal medium, and (10 μ l of resuspended cells) spotted on an agar plate containing M9 medium supplemented with antibiotics, 0.4% glucose, 1 mM IPTG, and 0.3 μ M D-histidine. Growth was monitored after 24 h and 48 h. See Materials and Methods for details.

stronger growth after 48 h. (Please note that an additional plasmid for the overproduction of HisJ proved to be unnecessary, likely because sufficient amounts of HisJ were produced from the chromosomal copy of the *hisJ* gene.) In case of the HisM variants P70L and L71D, no growth defect was observed, suggesting that both residues are dispensable for function. According to the homology model, the α -carbon atom of P70 forms a hydrogen bond with the carboxyl group of the substrate (Fig. 1B), which was also found in the crystal structure of one of the two ArtQ subunits of *T. tengcongensis* (26). Thus, any residue should be able to replace P70 for interacting with the substrate, which will be confirmed by the mutation. Consistently, a mutation affecting the corresponding proline residue in the ArtM subunits of *G. stearothermophilus* (P66) had no phenotype (41). In contrast, replacing L67 of ArtM with aspartate caused a severe transport deficiency (27) which could be explained by electrostatic repulsion between L67D and the nearby D160 (Fig. 1D) (E159 in ArtQ [Fig. 1A]). Thus, the lack of a negatively charged residue at the corresponding position in HisM (S173) might account for the observed functional tolerance of an aspartate residue in place of L71.

Notably, introducing a glutamate instead of S173 and thereby fully reconstructing the binding pocket of ArtQ in HisM led to substantially reduced growth of the transformed tester strain, while the exchange for an alanine residue produced no phenotype (Fig. 3). Additional growth experiments in liquid cultures basically confirmed these results but revealed no growth of the HisM-S173E-containing strain at limiting D-histidine concentrations (Fig. 4A and B).

Like E152 in ArtQ (Fig. 1A) (26) and E153 in ArtM (27), the corresponding E166 of HisM is proposed to form salt bridges to the guanidinium group of arginine (Fig. 1B). The assumed importance of this residue in liganding the substrate is corroborated by our mutational analysis. The results from growth experiments (Fig. 3) clearly demonstrate that not even a conservative replacement by aspartate is tolerated, indicating that a glutamate residue at this position is indispensable for function.

Mutational analysis of HisQ. A similar set of growth experiments with variants carrying mutations at homologous positions in HisQ confirmed that P60 is dispensable for activity (Fig. 3). Furthermore, mutations of I103 ($\rightarrow$ N), N157 ($\rightarrow$ E), and A164 ($\rightarrow$ E) resulted in no phenotype. These data suggest that residues found in ArtQ or HisM (Fig. 1D), when introduced at the corresponding positions in HisQ, do not interfere with transport activity. In contrast, a negative charge at position 61 (aspartate is clearly

FIG 4 Growth in liquid minimal/D-histidine medium of tester strains producing plasmid-encoded wild-type or variants of the histidine transporter. Strains BL21-5 (Δ hisF Δ hisM::kan) (A and B) or BL21-6 (Δ hisF Δ hisQ::kan) (C and D) were transformed with plasmids encoding the indicated transporter variants, and cell growth in M9 medium supplemented with 0.4% glucose, antibiotics, 1 mM IPTG, and either 0.3 μ M D-histidine (A and C) or 0.075 μ M D-histidine (B and D) was monitored spectroscopically at 650 nm. (Insets) Immunoblots developed with antibodies raised against the His₆ tag fused to the C terminus of HisP demonstrating protein production 5 h after inoculation. From left to right: wild type, M-S173E, and M-S713A (A and B); wild type, Q-A164E, Q-D61L, and A164E (C and D).

avored over glutamate) is required for function (Fig. 3). Notably, the HisQ-D61L,A164E double mutant, in which the position of the negatively charged residue is switched, displayed some growth after 48 h, which was confirmed by growth experiments in liquid cultures at saturating concentrations of D-histidine only (Fig. 4C and D).

Altogether, these results suggest that, as in HisM, the only negatively charged residue from the putative substrate binding site(s) is indispensable for a functional transporter.

ATPase activities of purified transporter variants. The observed defects in growth might be due to either a loss of ATPase activity of the variants or the uncoupling of ATPase and transport activities. To explore this further, we purified all variants (except HisM-P70A and HisQ-P60A variants) and monitored their ATPase activities in proteoliposomes stimulated by substrate-loaded binding proteins, HisJ/histidine and LAO/arginine, respectively. As shown in Fig. 5, wild type HisQMP₂ displayed low basal activity that was stimulated approximately 6-fold (His) and 4-fold (Arg) by the substrates, which is in line with previous findings (39).

Consistent with its wild-type-like growth behavior, the HisM-L71D variant exhibited approximately the same rate of ATP hydrolysis (Fig. 5A). Strikingly, all three variants of E166 displayed only slightly lower ATPase activities than the wild type, which is in clear

FIG 5 ATPase activities of HisQMP₂ variants with replacements in the substrate binding site. Purified variants of HisQMP₂ were incorporated into liposomes formed from *E. coli* total lipids and assayed for ATPase activity in the absence or presence of HisJ/histidine and LAO/arginine, as described in Materials and Methods. (A) Variants containing mutations in HisM. (B) Variants containing mutations in HisQ. The addition of HisJ or LAO in the absence of substrate did not change the basal activities of the transporter variants. Data are means and standard errors ($n \geq 3$).

contrast to their inability to complement the growth of the tester strain on D-histidine (Fig. 3). Thus, these mutations had no effect on ATP hydrolysis. Rather, since the observed activities are still substrate dependent, histidine and arginine have access to the binding pocket and thereby trigger ATP hydrolysis. However, likely because of improper interactions with the HisM(HisQ) binding site(s), the translocation of the substrates across the membrane was hampered.

The ATPase activities of both HisM-S173 variants differed from that of the wild type. While the growth-impaired S173E mutant displayed normal activity with histidine but reduced activity with arginine (see also below), the S173A mutant exhibited increases (approximately 2-fold) in ATPase activity with both substrates (Fig. 5A). The observation that the growth of the tester strain in liquid culture at a limiting concentration of D-histidine was delayed in the presence of this variant (Fig. 4B) suggests that the increased ATPase activity was not coupled to a corresponding increase in the transport rate. An uncoupled upshift in ATPase activity was previously observed for mutations affecting residues from or close to the binding site in ArtQ (26) and ArtM (25, 41).

In the case of HisQ variants, the mutations (I103N, N157E, and A164E) that enabled the growth of the tester strains to be similar to that of the wild type did not substantially affect ATPase activity, although somewhat lowered values were observed (Fig. 5B). This holds true also for HisQ-D61E, which enabled only low growth of the tester strain on D-histidine (Fig. 3) and thus, once again, uncoupled ATP hydrolysis from transport. Strikingly, the transporter variant containing HisQ-D61L was fully inactive in the presence of HisJ/histidine and LAO/arginine, strongly indicating that ATPase activity at the HisP subunits is no longer initiated. Either the mutation completely blocks the access of the substrate to the binding pocket(s) or no or unproductive interactions with HisQM occur. Interestingly, the loss in HisJ/histidine-stimulated ATPase activity was cured (partially for LAO/arginine) when D61L was combined with the A164E mutation (Fig. 5), indicating that the access of the substrate to the binding site(s) might have been restored. Nonetheless, ATP hydrolysis of the double mutant was uncoupled from transport (Fig. 3 and 4C and D).

Some mutations affect growth of tester strains on arginine as the sole nitrogen source. As mentioned above, some mutations affected ATPase activities initiated by HisJ/histidine or LAO/arginine differently. Compared to that of the wild type, the measured rates of ATP hydrolysis were reversed between histidine and arginine in the HisM-E166A variant, increased in the HisM-S173A variant, and significantly lower for arginine in HisM-S173E, HisQ-A164E, and HisQ-D61L/A164E variants (Fig. 5A and B). To

FIG 6 Growth on arginine as the sole nitrogen source of *E. coli* tester strains expressing wild-type or mutant variants of the histidine transporter. Plasmids carrying wild-type and mutant alleles of *hisQMP* encoding the indicated transporter variants were introduced into *E. coli* strains BL21-3 (Δ *hisM::kan*) (A) and BL21-4 (Δ *hisQ::kan*) (B). Cells were grown overnight in M9 medium (without NH_4Cl) supplemented with 0.4% glucose, antibiotics, 1 mM IPTG, and 3 mM arginine. Subsequently, the cells were washed twice with M9 salts (without NH_4Cl) and resuspended in M9 medium (without NH_4Cl) supplemented with 0.4% glucose, antibiotics, 1 mM IPTG, and 1 mM arginine. (Insets) Immunoblots developed with antibodies raised against the His₆ tag fused to the C terminus of HisP demonstrating protein production after overnight growth in the presence of 3 mM arginine. From left to right: wild type, M-E166A, M-S173E, and M-S173A (A); wild type, Q-D61L, Q-A164E, and Q-D61L, A164E (B).

test whether these differences have effects on arginine transport, we took advantage of the earlier observation that the growth of *S. Typhimurium* on arginine as the sole nitrogen source is dependent on an intact histidine transporter (42). To this end, tester strains containing chromosomal deletions of either *hisM* (BL21-3) or *hisQ* (BL21-4) (Table S1) were transformed with the respective plasmids and grown in minimal glucose medium supplemented with 3 mM arginine. Under these conditions, all strains displayed comparable growth behaviors, likely due to the uptake of arginine by various transport systems (43). The expression of the transporters in the variants was verified by immunoblotting equal amounts of cells with an antibody directed against the His tag that was fused to the carboxy-terminal ends of the HisP subunits (see Fig. 6, insets). Then, the cells were collected by centrifugation, washed with minimal salt medium, and transferred to the same medium supplemented with 1 mM arginine. As shown in Fig. 6, wild-type cells grew after a lag phase of approximately 7 h, while no growth was observed of transformants containing the transport-inactive variant HisQ-D61L (Fig. 6B). The latter result can be taken as evidence for the reliability of the approach. In the case of the HisM variants, E166A and S173E strains failed to grow with 1 mM arginine, while the S173A variant behaved like the wild type (Fig. 6A). Likewise, the HisQ A164E variant grew comparably to the wild type, whereas the D61L-A164E double mutant exhibited intermediate growth. Altogether, these results are consistent with those of the growth experiments on D-histidine (Fig. 3), indicating that the transport of both substrates was equally affected by the mutations. Furthermore, with the exception of HisQ-D61L, the observed differences in ATPase activities of the transport-deficient mutants when stimulated by either histidine or arginine did not reflect a discrimination between the substrates.

Partial or full reconstruction of an ArtQ-like binding site in HisM and HisQ resulted in inactive transporter variants. The results shown in Fig. 3 that single mutations of both negatively charged residues in the HisQM heterodimer (HisQ-D61 and HisM-E166) caused transport-deficient phenotypes do not exclude the possibility that replacing all residues in question in HisQM with those found in the binding pocket of ArtQ would restore transporter function. This notion holds true, despite the observation that the exchange of HisM-S173, the only residue differing from those in ArtQ, for glutamate resulted in a less active transporter (Fig. 3). Here, the lack of compensating mutations in HisQ might be the reason for this phenotype. Consequently, we fully reconstructed step-by-step an ArtQ-like binding site in HisQ in combination with either wild-type

HisM or HisM-S173E and analyzed the growth on D-histidine of the tester strain BL21-6 (*ΔhisF ΔhisQ*) transformed with the respective expression plasmids. (Please note that the presence of the chromosomal copy of *hisM* was insufficient to enable growth. Thus, the construction of a tester strain carrying deletions of both *hisM* and *hisQ* proved to be unnecessary). As shown in Fig. S2A, none of the intermediate or final mutants complemented the tester strain to enable growth on D-histidine, suggesting that two autonomous substrate binding sites, as seen in the ArtQ homodimer, are unlikely present in HisQM. Monitoring of the ATPase activity in the purified variants once again demonstrated that, except for those containing HisQ-D61, transport was uncoupled from ATP hydrolysis (Fig. S2B).

As outlined above, each ArtQ subunit contains two negatively charged residues (Fig. 1A and D) that in the close homolog ArtM were demonstrated to be crucial for productive interaction with a substrate molecule (27). Consistently, both negatively charged residues in HisQ (D61) and HisM (E166) were also found to be indispensable for transport (Fig. 3); however, they were positioned differently in the primary structures compared to in ArtQ and ArtM (Fig. 1D). Thus, we constructed a second set of mutants in which the positions of the negatively charged residues were varied between HisQ and HisM. Again, no active transport complex was obtained (Fig. S2).

DISCUSSION

Here, we report on a comprehensive mutational analysis of the putative transmembrane substrate binding site(s) of a canonical heterodimeric type I ATP-binding cassette import system, the histidine transporter of *S. Typhimurium*. We addressed the question of whether one or two substrate molecules are bound in the HisQMP₂ complex. On the basis of a structural model (Fig. 1B) and on sequence alignment (Fig. 1D), a major contribution of the HisM subunit in liganding a substrate molecule was predicted. In contrast, an autonomous second binding site in HisQ was much less obvious (Fig. 1C and D).

Our results clearly suggest that, consistent with the structural data of the Art(QN)₂ transporter (reference 26 and Fig. 1A), two negatively charged residues are indispensable for function (Fig. 3 and 4). However, these residues are distributed between HisM (E166) and HisQ (D61). Moreover, a serine residue (S173) in place of the second negatively charged residue in the otherwise fully conserved ArtQ-like binding site in HisM indicates that two and only two negatively charged residues in HisQM are required and sufficient for substrate binding. This notion is underlined by the substantially reduced growth of a tester strain when transformed with the HisQM(S173E)P₂ variant (Fig. 3). In addition, the observed complete loss of transporter function when (i) an ArtQ-like binding site was constructed in HisQ and (ii) negatively charged residues were switched between HisQ and HisM (see Fig. S2 in the supplemental material) led us to conclude that the relative positioning of both negatively charged residues as seen in wild-type HisQM is functionally required. The only exception is the HisQ-A164E variant, which displayed transport activity similar to that of the wild type (Fig. 3 and 4), suggesting that a second negatively charged residue in HisQ when combined with wild-type HisM (but not HisM-S173E) (Fig. S2) is functionally tolerated. The observation that the transport activity of the variant containing HisQ-D61L was partially restored when combined with A164E (Fig. 3 and 4) underlines the prominent positioning of this residue.

Further evidence against a (second) binding site in HisQ is provided by the finding that single replacements of HisQ-D61 or HisM-E166 resulted in a transport-negative phenotype (Fig. 3 and 4), while the homodimeric Art(MP)₂ transporter displayed activity with one intact substrate binding site only (27).

To further explore the apparent differences between the homodimeric transporters Art(QN)₂ (26) and Art(MP)₂ (27) and the heterodimeric histidine transporter, we extended the sequence alignment as shown in Fig. 1D by including both homodimeric and heterodimeric transporters which have been studied experimentally (Fig. 7, showing N- and C-terminal peptide regions of the putative binding sites only). A total of

FIG 7 Extended sequence alignment of N- and C-terminal binding regions of HisM and HisQ homologs from experimentally studied homodimeric and heterodimeric transporters. Sequences were grouped in the order of HisQ homologs (I), homodimeric HisM homologs (II), and heterodimeric HisM homologs (III). Conserved residues are shown in bold at the bottom. The proteins considered are as follows: HisQ_ST and HisM_ST, membrane-spanning subunits of the *S. Typhimurium* histidine transporter (GenBank accession no. [NP_461295](#) and [NP_461294](#)); AotM_PA and AotQ_PA, membrane-spanning subunits of the *Pseudomonas aeruginosa* arginine/ornithine transporter (accession no. [NC_002516.2](#) and [NC_002516.2](#)); NocM_AT and NocQ_AT, membrane-spanning subunits of the *Agrobacterium tumefaciens* nopaline transporter (GenBank accession no. [NP_396542.1](#) and [NP_396543](#)); OccM_AT and OccQ_AT, membrane-spanning subunits of the *Agrobacterium tumefaciens* octopine transporter (GenBank accession no. [AAA98379.1](#) and [AAF77142.1](#)); ArtQ_EC and ArtM_EC, membrane-spanning subunits of the *E. coli* arginine transporter (GenBank accession no. [CAA60103](#) and [CAA60104](#)); WP_064638084_MC and WP_064638080_MC, membrane-spanning subunits of a putative lysine transporter of *Moraxella catarrhalis*; ArtQ_TT, membrane spanning subunit of the *T. tengcongensis* transporter for positively charged amino acids (accession no. [WP_011024939](#)); ArtM_GST, membrane-spanning subunit of the arginine transporter of *G. stearothermophilus* (the protein is 100% identical with the sequence ZP_03556510 of the sequenced genome from *Geobacillus* sp. strain Y412MC61); YqiY_BS, membrane-spanning subunit of the *Bacillus subtilis* arginine transporter (GenBank accession no. [BAA12605.1](#)). Alignment was created by the webPRANK server (<https://www.ebi.ac.uk/goldman-srv/webprank/>).

fifteen sequences were grouped in (heterodimeric) HisQ homologs (I) and homodimeric (II) and heterodimeric (III) HisM homologs. From this analysis, it is obvious that heterodimeric systems pair a negatively charged residue equivalent to HisQ-D61 (group I in Fig. 7) with a glutamate in their HisM-like counterpart (aligning with HisM-E166) (group III in Fig. 7). In contrast, and like in ArtQ, homodimeric systems (group II in Fig. 7) have the second negatively charged residue placed seven residues from the carboxy terminus of the invariant glutamate (position 152 in ArtQ) (Fig. 1A and D). Consequently, the positions equivalent to HisQ-D61 are replaced with an uncharged residue (L/M). Consistently, an aspartate residue in place of L67 in ArtM caused a transport-negative phenotype (25).

On the other hand, in the heterodimeric HisM homologs, a serine, threonine, or glycine residue is in place of the second negatively charged residue of homodimeric subunits. Interestingly, in HisQ homologs, the residue at this position can either be an alanine (as in HisQ and AotQ) or a negatively charged residue. This observation is thus consistent with our result discussed above that a variant containing HisQ-A164E has no phenotype (Fig. 3 and 4). (An even more comprehensive alignment of HisM and HisQ homologs by using the evfold server at <http://evfold.org/evfold-web/evfold.do> fully confirmed our analysis.)

In summary, we propose that, in contrast to the homodimeric Art(QN)₂ and Art(MP)₂ systems, in the heterodimeric histidine transporter of *S. Typhimurium*, only one substrate molecule is bound between HisM and HisQ, which provide the essential negatively charged residues E166 and D61, respectively. Because of the

number of conserved residues compared to ArtQ, a substrate molecule is thought to be primarily liganded by residues of HisM, while HisQ-D61 forms a crucial salt bridge with the α -amino group of the substrate (see Fig. S3 for an illustration). (Please note that in the absence of structural information on the native transporter, a role of D61 different from that considered here cannot yet be fully excluded.) Strong support for this hypothesis was provided by electron spin echo envelope modulation (^2H -ESEEM) spectroscopy combined with molecular modeling, which identified only one histidine molecule in the transporter (N. Isaev, J. Heuveling, E. Schneider, and H.-J. Steinhoff, submitted for publication).

The extended sequence alignment shown in Fig. 7 further indicates that this hypothesis might hold true for other closely related heterodimeric transporters. In contrast, in the heterodimeric maltose transporter, even longer substrates such as maltohexaose are bound in a transmembrane pocket exclusively formed by residues from the MalF subunit (25).

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are listed in Tables S1 and S2 in the supplemental material, respectively. Strain XL10-Gold was used for mutagenesis, BL21(DE3) T1^R was used for the overproduction and its derivatives as the background strain for growth experiments. Strain NEB 5 α was used for plasmid storage and production.

Site-directed mutagenesis and strain design. Mutagenesis of *hisQMP* was carried out using a QuikChange Lightning site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Oligonucleotides were purchased from Sigma-Aldrich (Germany). All constructs were verified by commercial sequencing carried out by LGC (Berlin, Germany).

Strains carrying deletions of *hisF::kan*, *hisM::kan*, and *hisQ::kan* were purchased from the Keio Collection (<https://cgsc2.biology.yale.edu/KeioList.php>), and the deletions were P1-transduced to strain BL21(DE3) T1^R according to standard procedures (44). The kanamycin cassette of the *hisF* deletion was flipped out according to the protocol of Datsenko and Wanner (45). The resulting strains were verified by PCR analysis using cassette-specific primers k1, k2, and kt and gene-specific primers according to reference 45.

Growth experiments. The growth tests, i.e., the utilization of D-histidine of a histidine-auxotrophic *E. coli* mutant and growth on arginine as the sole source of nitrogen, were developed on the basis of the protocols of the G. Ames group who worked with *Salmonella* strains (40, 42). To test the ability of the deletion strains BL21-5 (Δ *hisF* Δ *hisM::kan*) and BL21-6 (Δ *hisF* Δ *hisQ::kan*) carrying plasmids coding for variants of HisQMP₂ to grow on D-histidine, the transformants were grown overnight in liquid LB (44) supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin and 50 $\mu\text{g}/\text{ml}$ kanamycin. The next day, the cells were washed twice in M9 minimal medium (44), and 10 μl of cells resuspended at equal densities (optical density at 650 nm [OD₆₅₀]) was spotted on M9 minimal agar plates supplemented with 0.4% glucose, 0.3 μM D-histidine, antibiotics, and 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside). The cells were incubated at 37°C, and the growth was measured after 24 and 48 h.

For growth in liquid medium, the cells were washed, inoculated in the same medium with 0.3 or 0.075 μM D-histidine at an OD₆₅₀ of 0.1, and incubated at 37°C with shaking. Growth was monitored over 48 h.

The ability to grow on arginine as the sole nitrogen source, i.e., being able to transport arginine, was tested with M9 medium lacking ammonium chloride but supplemented with 3 mM or 1 mM arginine, as well as 0.4% glucose, antibiotics, and 1 mM IPTG. The strains BL21-3 (Δ *hisM::kan*) and BL21-4 (Δ *hisQ::kan*) were transformed with plasmids coding for selected variants of HisQMP₂ and incubated in medium with 3 mM arginine, which enables all mutants to grow to stationary phase overnight. The next day, the cells were washed twice in medium containing 1 mM arginine and inoculated to an OD₆₅₀ of 0.1. Growth was monitored for 24 h at 37°C.

To prove overproduction of wild-type and variants of HisQMP₂ under the growth test conditions, immunoblots with anti-His antibodies were performed to detect the His₆ tag fused to the C terminus of HisP. Cell samples (1.5 ml) with equal densities (e.g., OD₆₅₀ of 1) were centrifuged, and the pellets were dissolved in 100 μl SDS sample buffer and boiled prior to SDS gel electrophoresis and blotting. Samples were taken after 5 h of growth or, for arginine, after growth overnight in 3 mM arginine.

Protein preparations. The overproduction of wild-type and variants of HisQMP₂ was performed by growing *E. coli* strain BL21(DE3) T1^R harboring pLysS, pVE26, or its derivatives (providing a His₆ tag fused to the C terminus of HisP) in TP_i medium (46) supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin and 15 $\mu\text{g}/\text{ml}$ chloramphenicol at 30°C to an OD₆₅₀ of 0.8 to 1.0 prior to the addition of 0.1 mM IPTG. Growth was continued at 22°C until stationary phase was reached (after ~3 to 4 h), and the cells were then collected by centrifugation at 6,000 $\times$ g for 10 min. Subsequent purifications of HisQMP₂ (wild type and variants) as well as the overproduction and purification of HisJ and LAO were carried out essentially as described in reference 36.

Preparation of proteoliposomes. Proteoliposomes containing HisQMP₂ (wild type and variants) were essentially prepared as described elsewhere (36, 47).

ATPase assay. ATPase activity of reconstituted transporter complexes was assayed in the presence or absence of 0.4 mg/ml HisJ with 1 mM L-histidine or LAO with L-arginine, as described previously (30, 47).

Analytical procedures. Protein determination, SDS-polyacrylamide gel electrophoresis, and immunoblotting were performed as described in reference 47.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00521-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

ACKNOWLEDGMENTS

We thank M. Brose and S. Braetz (both from Humboldt Universität zu Berlin) for their contributions during the initial stage of the project.

This work was supported by the Deutsche Forschungsgemeinschaft by grants SCHN 274/15-2 and SCHN 274/16-1 (to E.S.).

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