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Heme-dependent autophosphorylation of a heme sensor kinase, ChrS, from *Corynebacterium diphtheriae* reconstituted in proteoliposomes

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ABSTRACT

Corynebacterium diphtheriae **employs the response regulator, ChrA, and the sensor kinase, ChrS, of a two-component signal transduction system to utilize host heme iron. Although ChrS is predicted to encode a heme sensor, the sensing mechanism remains to be characterized. In this report, ChrS expressed in *Eshcherichia coli* membranes was solubilized and purified using decylmaltoside. ChrS protein incorporated into proteoliposomes catalyzed heme-dependent autophosphorylation by ATP. Other metalloporphyrins and iron did not stimulate kinase activity. The UV–Vis spectrum of hemin in the ChrS–proteoliposomes indicated that heme directly interacts with ChrS. This is the first functional reconstitution of a bacterial heme-sensing protein.**

1. Introduction

Iron is one of the most important nutrients for nearly all organisms, because of its essential metabolic roles (reviewed in [1]). Iron functions as the catalytic center of numerous enzymes involved in electron transfer in respiratory cytochromes and DNA synthesis. In the human body, most iron is present in hemoglobin (Hb), which delivers oxygen gas to individual cells (reviewed in [2]). To infect and proliferate in the host, pathogenic bacteria preferentially acquire iron derived from Hb using iron-acquisition systems (virulence toxins, and a siderophore-dependent iron transporter and/or a heme transporter) [3, reviewed in 4].

Corynebacterium diphtheriae, a pathogen of diphtheria, conducts iron/heme-dependent regulatory systems (reviewed in [5]). The transcription of the diphtheria toxin gene is inhibited by the DtxR repressor protein when adding iron to the culture medium [6]. The iron-loaded DtxR protein also represses expression of the *hmuO* gene [7]. The *hmuO* gene encodes heme oxygenase which liberates iron from the heme taken up by an ABC-binding cassette transporter (*hmuTUV*) in the cytoplasm [8,9]. Conversely, expression of the *hmuO* gene is activated by addition of heme or Hb to the culture medium, which is consistent with the physiological function of HmuO [8]. A heme/Hb-dependent, trans-acting element has been discovered and designated *chrA* [10]. The *chrS* gene is adjacent to the *chrA* gene in the bacterial genome. The *chrA* and *chrS* genes are predicted to encode the response regulator (RR) and its cognate sensory histidine kinase (HK), respectively, which comprise a novel two-component signal transduction system.

Two-component signal transduction systems allow bacteria to adapt to changes in environmental conditions by modulating the transcription of specific genes (reviewed in [11,12]). They are usually characterized by a transmembrane HK and a cytoplasmic RR. Once an environmental stimulus is detected by the sensor domain of the HK, a highly conserved histidine residue in the kinase domain is autophosphorylated by transfer of a γ-phosphoryl group from ATP, and the bound phosphoryl group is subsequently transferred to a conserved aspartate residue in the RR. Thousands of HKs

Abbreviations: Hb, hemoglobin; HK, histidine kinase; RR, response regulator; OG, *n*-octyl-β-D-glucoside; DM, *n*-decyl-β-D-maltoside; DMSO, dimethyl sulfoxide; PP, protoporphyrin IX; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane region

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have been discovered in bacterial genomes, showing that the HK domains are highly conserved. By contrast, the amino acid sequences of the N-terminal sensor domains are diverse. The physiological ligands for many HKs are still unknown due to the paucity of direct biochemical investigation concurrent with physiological and genetic studies. Results of a reporter gene assay strongly suggest that ChrS senses extracellular heme, but the molecular mechanism of heme-sensing remains to be elucidated [10].

In the present study, the ChrS protein was solubilized and purified from the membranes of an *Escherichia coli* overproducer, and then incorporated into liposomes. The reconstituted ChrS protein exhibited both direct and specific heme recognition and heme-dependent autophosphorylation.

2. Materials and methods

2.1. Materials

Genomic DNA from *C. diphtheriae* C7(−) was kindly provided by Dr. Iwaki (National Institute of Infectious Diseases, Japan). *n*-Octyl-β-D-glucoside (OG) and *n*-decyl-β-D-maltoside (DM) were purchased from Calbiochem Co. and Dojindo Co. *E. coli* phospholipids (polar extract) were obtained from Avanti Polar Lipids Inc. All other chemicals were analytical grade commercial products.

2.2. Expression of ChrS and preparation of the ChrS-overproducer *E. coli* membranes

The entire coding region of the *chrS* gene was amplified from genomic DNA of *C. diphtheriae* C7(−). The native *chrS* and *chrS*-His tag genes were subcloned into a T7 promoter-carrying vector. The ChrS-His tag expression plasmid, pT7-ChrS-PrSc-Trx-His_{10}, encodes full-length ChrS fused with the PreScission protease cleavage site-thioredoxin-His_{10} at the C-terminus. Details of plasmid construction are described in the Supplementary material. BL21(DE3)/pLysS *E. coli* cells harboring the *chrS* plasmids were grown in a Terrific Broth containing 100 μg/ml ampicillin and 40 μg/ml chloramphenicol at 25 °C overnight without isopropyl-β-D-thiogalactopyranoside induction. Total membrane vesicles were obtained as previously described [13], suspended in 25 mM Tris–HCl (pH 7.8) and 10% glycerol at a concentration of ~20 mg protein/ml, and stored at −80 °C.

2.3. Purification of ChrS

The membrane proteins (200 mg) containing ChrS-PrSc-Trx-His_{10} were solubilized in 25 mM Tris–HCl (pH 7.8), 150 mM NaCl, 5% glycerol and 1.2% DM for 1 h at 4 °C. The supernatants resulting from ultracentrifugation (185 000×*g*, 1 h) were applied to a Ni-NTA agarose affinity column (10 ml bed volume, Qiagen). ChrS-PrSc-Trx-His_{10} was purified by chromatography with 200 ml of an imidazole linear gradient (20–400 mM) in the presence of 0.2% DM. The fusion protein (approximately 15 mg) was digested with 300 units of PreScission protease (GE Healthcare) during dialysis against 1 l × 2 of 25 mM Tris–HCl (pH 7.8), 150 mM NaCl, 5% glycerol and 0.2% DM overnight at 4 °C. After removal of the PreScission protease using a GSTrap HP (1 ml bed, GE Healthcare), the proteins were again subjected to Ni-NTA chromatography with 100 ml of an imidazole linear gradient (20–400 mM). The flow-through and earlier fractions containing the cleaved ChrS protein were collected, concentrated to 2–3 mg/ml using a Centriprep YM10, and stored at −20 °C.

2.4. Reconstitution of purified ChrS into liposomes

Preparation of the ChrS–proteoliposomes using detergent-destabilized liposomes was carried out as previously described [14] with a slight modification. *E. coli* phospholipids were evaporated and dissolved in 50 mM Tris–HCl (pH 7.5) and 3% OG (10 mg/ml). Liposomes were prepared by dialyzing the solubilized lipids against a 100-fold greater volume of 50 mM Tris–HCl (pH 7.5) three times, followed by three freeze/thaw cycles using liquid nitrogen. Purified ChrS was added to the destabilized liposomes in 50 mM Tris–HCl (pH 7.8), 50 mM KCl, 5% glycerol and 0.6% OG (phospholipid:protein = 4.8 mg/ml:0.12 mg/ml) to give 2.5 μM ChrS. The mixture was incubated for 15 min at 25 °C, and OG was removed by dialyzing twice against 1000 volumes of 50 mM Tris–HCl (pH 7.8), 50 mM KCl and 5% glycerol at 25 °C overnight to yield the ChrS proteoliposomes [15].

2.5. Phosphorylation assay

E. coli total membrane vesicles (5 mg/ml) and reconstituted ChrS (1 μM) were suspended in 50 mM Tris–HCl (pH 7.8), 50 mM KCl and 5% glycerol in the presence of 5 and 25 μM $MgCl_2$, respectively. A 1/100 volume of hemin (or other heme analogue)/dimethyl sulfoxide (DMSO) solution was added to give the desired concentrations indicated in the figures. Protoporphyrin IX (PP) was dissolved in 0.1 M Tris base. The reaction was initiated by addition of 3 μl of 0.2 mM [γ-^{32}P] ATP (~0.09 MBq nmol^{-1}) to 27 μl of the protein solution. Samples (6 μl) were removed after 1, 2, 4 and 6 min and were mixed with 9 μl of SDS loading buffer containing 1.5 mM Na-EDTA to terminate the reaction. The phosphorylated proteins were visualized and quantified as previously described [16]. Data were obtained from at least three independent measurements.

2.6. MALDI-TOF mass spectral analysis of ChrS

The native ChrS protein was extracted from the total *E. coli* membrane proteins (200 μg) by electrophoretical elution of the minced SDS–10% polyacrylamide gel electrophoresis (PAGE) gel containing ChrS [17–19], followed by suspension in 20 μl of formic acid:hexafluoro-2-propanol (ratio, 7:3) [20]. The sample was mixed with an equal volume of 50 mM sinapic acid in 70% formic acid. Approximately 0.6 μl of the resulting solution was placed on an MTP AnchorChip var 384 [21] and allowed to air dry. The DM-purified ChrS sample was mixed directly with the sinapic acid/formic acid solution. MALDI mass spectrometry was performed using an Autoflex MALDI-TOF-MS instrument (Bruker). BSA was used as the calibration standard.

2.7. Miscellaneous

The DNA sequence of the *chrS* region was determined using a DNA sequencer ABI310 (Applied Biosystems Japan, Tokyo). Membrane protein was quantified using a BCA protein assay kit (Pierce, Rockford, IL), and detergent-containing purified protein was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA). To determine the N-terminal amino acid sequence of the ChrS candidate protein, SDS–12.5% PAGE was performed on 13 μg of total membrane proteins. The protein band corresponding to ChrS was blotted onto a PVDF sheet and subjected to N-terminal sequence analysis using a Procise 494HT (Applied Biosystems Japan, Tokyo) for five cycles. UV–Vis spectra of the heme–ChrS complex in the proteoliposomes were measured using a spectrophotometer, UV-2500 (Shimadzu, Kyoto) equipped with an integrating sphere (ISR-2200, Shimadzu).

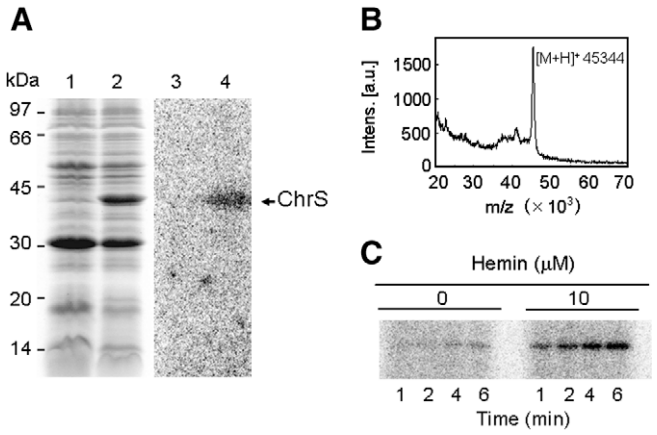

Fig. 1. Expression and phosphorylation of ChrS in *E. coli* membranes. (A) SDS–PAGE analysis of the ChrS-containing *E. coli* membranes. Coomassie-staining: lane 1, membranes of BL21 (DE3) harboring pRSET-A (20 μg); lane 2, ChrS-containing membranes (20 μg). Autoradiograph: lane 3, membranes of BL21 after the phosphorylation reaction; lane 4, ChrS-containing membranes after the phosphorylation reaction in the absence of hemin. (B) MALDI-TOF mass spectrum of ChrS extracted from the SDS–PAGE gel. Sample preparation is described in Section 2. (C) Time course of phosphorylation levels of ChrS in the presence (10 μM) or absence of hemin. Membrane vesicles (5 mg protein/ml) were incubated with 0.2 mM radioactive ATP and 5 μM of $MgCl_2$ for the indicated periods. Thirty micrograms of protein were subjected to SDS–PAGE followed by autoradiography.

3. Results

3.1. Phosphorylation of the native ChrS protein overproduced in E. coli

The *chrS* gene is predicted to encode a 45 238 Da, membrane-bound HK [10]. SDS–PAGE analysis showed that the membranes from BL21(DE3) harboring the *chrS* recombinant plasmid (Fig. 1A, lane 2) contained a large amount of an additional 43 000-Da protein that was absent from cells carrying an empty vector, pRSET-A (lane 1). The five N-terminal amino acids, MLASV, of the corresponding protein extracted from the SDS–PAGE gel were completely identical to the amino acid sequence deduced from the *chrS* gene. The MALDI-TOF mass spectrum of the extracted protein exhibited a molecular mass of 45 300 Da (Fig. 1B). Therefore, it appears that the recombinant ChrS protein was completely translated and then localized in the membranes without proteolytic processing.

The ChrS protein present in membrane vesicles was phosphorylated by ATP (Fig. 1A, lane 4), whereas phosphorylated signals were barely detected in the membranes of cells carrying pRSET-A due to the low level of indigenous *E. coli* HK expression (lane 3). Phosphorylation of ChrS was significantly enhanced in the presence of hemin (Fig. 1C). However, based on these results, we cannot conclude that ChrS was "autophosphorylated" and that elevation of autophosphorylation was due to a "direct" interaction with heme, because approximately 1000 proteins are present in *E. coli* membranes. Thus, to obtain direct evidence that ChrS is a heme-sensing HK, we solubilized and purified ChrS from the *E. coli* membranes.

3.2. Purification of ChrS

The expression plasmid for the ChrS-PrSc-Trx-His_{10} fusion protein was constructed to facilitate protein purification. A 57 000-Da protein detected by SDS–PAGE was solubilized in the presence of 1.2% DM (Fig. 2A, lane 1), and purified using Ni-NTA chromatography (lane 2). Subsequently, PreScission protease treatment resulted in successful purification of the full-length ChrS protein (lane 4). The molecular mass of the purified ChrS protein with an apparent molecular weight of 44 000 based on SDS–PAGE (Fig. 2A) was verified as 46 200 Da by MALDI-TOF mass analysis (Fig. 2B). This result was consistent with the molecular mass predicted from the amino acid sequence of ChrS that contains the additional PreScission cleavage sequence, KLGLEVLFQ, at the C-terminus. Routine solubilization of 200 mg of the *E. coli* membranes gave 15 mg of ChrS-PrSc-Trx-His_{10}, and, ultimately, 5 mg of ChrS.

3.3. Kinase activity of the reconstituted ChrS protein

To characterize the nature of ChrS, the purified ChrS protein was incorporated into liposomes. As shown in Fig. 3A, the reconstituted ChrS protein restored autophosphorylation activity. The autophosphorylation activity of ChrS was significantly stimulated by addition of hemin. The heme-dependent autophosphorylation suggested that the purified ChrS incorporated into liposomes was functional. We then examined the effects of Hb and other heme analogues on ChrS kinase activity. As shown in Fig. 3B, the initial rate of ChrS autophosphorylation was enhanced by hemin, but not by Hb. Neither non-iron metalloporphyrins nor $FeCl_3$ stimulated basal activity (Fig. 3B). DMSO, which was used as a solvent for hemin and the other PPs, did not affect kinase activity in 1% (v/v) concentration (data not shown).

3.4. UV–Vis spectral properties of the heme–ChrS complex

Heme-dependent autophosphorylation of ChrS in the liposomes suggests that heme binds directly to ChrS. To confirm direct inter-

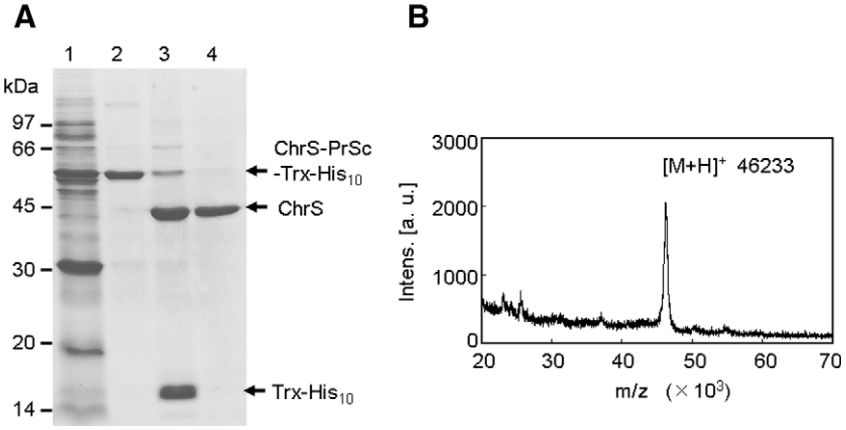

Fig. 2. Purification of the ChrS protein. (A) Coomassie-stained 12.5% SDS–PAGE analysis of purified ChrS. Lane 1, DM-solubilized total membranes; lane 2, Ni-NTA affinity-purified ChrS-PrSc-Trx-His_{10}; lane 3, ChrS-PrSc-Trx-His_{10} cleaved with PreScission protease; lane 4, purified ChrS. (B) MALDI-TOF mass spectrum of the purified ChrS protein.

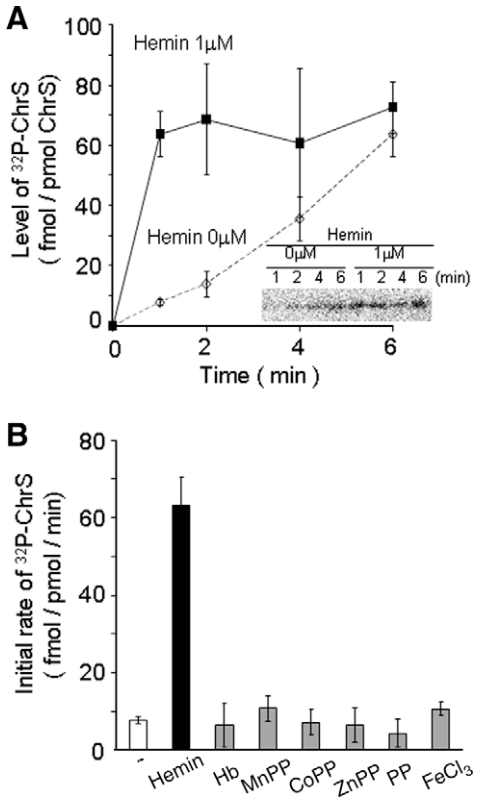

Fig. 3. Autophosphorylation of purified ChrS in proteoliposomes. (A) Time course of autophosphorylation of ChrS in the presence or absence of hemin. (B) Effect of hemin, Hb, and other heme analogues on the autophosphorylation activity of ChrS. Concentration of each ligand was 1 μM. [^{32}P]phospho-ChrS was quantified using a BAS2500 image analyzer. Data are means ± S.E. from at least three independent experiments.

action between heme and ChrS, the UV–Vis spectrum of the heme–ChrS complex was measured using an integrating sphere-spectrophotometer. Free hemin in the Tris buffer exhibited a broad Soret band around 360–400 nm (Fig. 4, dotted line). When liposomes were added to the hemin solution, a narrow Soret band was observed at 400 nm (dashed line). By contrast, addition of ChrS–proteoliposomes resulted in a narrower Soret band at 405 nm with a lowered shoulder at 360 nm (solid line). In addition, a dithionite-reduced ferrous absorption peak was identified at 534 nm in the liposome solution (Fig. 4, inset, dashed line), and that of the ferrous form of ChrS–proteoliposomes exhibited a split at 534 nm and 564 nm (inset, solid line). The overall change in the Soret region and Q-band region provides direct evidence for a new electronic structure in the heme–ChrS complex of the proteoliposomes.

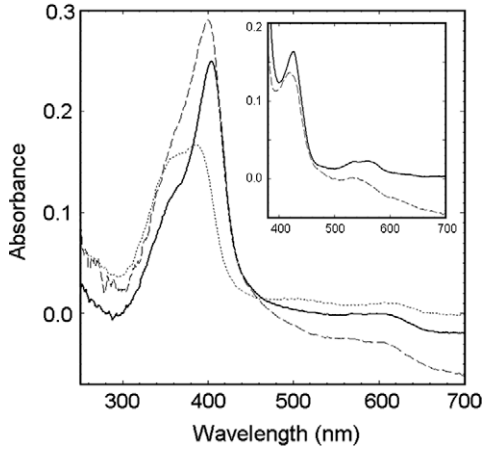

Fig. 4. Absorption spectra of the hemin in the reconstitution dialysis buffer, in the liposome suspension, and in the ChrS–proteoliposomes. The difference spectra were obtained as follows; dotted line, hemin in the buffer *minus* base line; dashed line, hemin in the liposomes *minus* the liposomes; solid line, hemin in the ChrS–proteoliposomes *minus* the ChrS–proteoliposomes. Concentration of phospholipids was 0.48 mg/ml, and 2.5 μM hemin was mixed with the ChrS (2.5 μM)–proteoliposemes. Inset, sodium dithionite-reduced spectra of hemin the in liposomes (dashed line) and hemin in the ChrS–proteoliposomes (solid line).

4. Discussion

The two-component signal transduction system is an area of current biological interest, because it is responsible for adaptation to environmental changes and expression of virulence genes (reviewed in [22,23]). The *chrSA* genes are heme-sensing TCS, which were first discovered in bacteria. Results of an in vivo reporter gene assay using the *hmuO* promoter suggest that exogenous heme and Hb are potential ligands for ChrS [10].

At an earlier stage of this work, we found that purified ChrS protein had negligible autophosphorylation activity in the presence of 0.2% DM, although the heme-purified ChrS complex exhibited a similar spectroscopic profile to that of the heme–ChrS–proteoliposomes (data not shown). The loss of kinase activity observed in the presence of detergent micelles is consistent with previous work on the DcuS protein [14]. These results drove us to study on heme binding and kinase regulation using the ChrS–proteoliposomes.

The present study unambiguously shows that the reconstituted ChrS protein restores heme-dependent autophosphorylation activity by direct interaction with heme. Because Hb did not stimulate the kinase activity of the reconstituted ChrS (Fig. 3B), the stimulatory effect of Hb detected by the reporter assay in previous studies [8,10] may be due to release of heme from degraded Hb into the cell culture medium.

Recently, another heme-sensing TCS, *hssRS*, has been identified in *Staphyloccocus aureus* [24]. Results of a similar reporter assay, which used the *hrtBA* promoter, showed that HssS senses GaPP and MnPP as well as hemin and Hb. These results suggest that HssS senses extracellular heme, although it remains to be determined if signals of metalloporphyrin byproducts are mediated by HssS in vivo [25].

Amino acid sequence analyses predict that ChrS contains six transmembrane regions (TMs) in the N-terminal sensor domain, while HssS has two TMs with a long periplasmic loop (Fig. 5). Because there is no significant homology in the sensor domains of ChrS and HssS, these sensors apparently have distinct mechanisms of heme recognition. Of note, ChrS recognizes heme specifically, not other metalloporphyrins, thereby increasing kinase activity. Although ChrS contains neither a known heme protein sequence nor a heme regulatory motif [26–28], direct interaction between heme and ChrS was examined spectroscopically.

Judged from the accessibility of ATP to the kinase domain leading to the high specific activity of ChrS autophosphorylation, ChrS was incorporated into the destabilized liposomes with inside-out orientation as previously reported [14]. It is currently unknown whether the heme-binding site of ChrS is located in the membrane bilayer or is exposed to the periplasmic space. Since binding assay by ultracentrifugation indicated that hemin is adsorbed to the liposome vesicles (data not shown), nevertheless, exogenous hemin readily binds to the inside-out reconstituted ChrS due to its hydrophobicity. Actually, while hemin in aqueous solution shows a broad visible spectrum (Fig. 5, dotted line), suggesting the formation of either μ-oxo or back-to-back heme dimers [29,30]. The narrow spectrum was observed with addition of liposomes (dashed line), which is due to lipid–hemin interactions that suppress formation of heme dimers [29]. Spectral changes in the Soret region for hemin

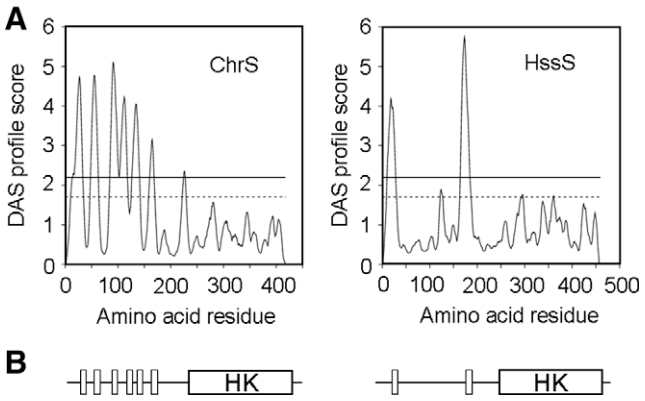

Fig. 5. Comparison of ChrS (*C. diphtheriae*) and HssS (*S. aureus*). (A) Hydropathy profiles of ChrS (AAD 45902.1) and HssS (YP 001576215.1) using the DAS-transmembrane prediction server (http://www.sbc.su.se/~miklos/DAS/). (B) Outline of the primary structures of ChrS and HssS. The predicted sensor domain and kinase domain are shown. The small rectangles indicate the putative TMs, and the large boxes show the HK domains.

in the ChrS–proteoliposomes (solid line) were distinct from those of free hemin and hemin–liposomes. These results indicate that hemin preferentially binds to ChrS. The split peaks of the ferrous heme–ChrS complex, which are similar to those of Soul and HBP23 [31], suggest the presence of hexa-coordinated heme species, although the possibility cannot be excluded that the peak at 534 nm is partly derived from that of hemin in liposomes. At present, the coordination state of heme bound to ChrS is unclear due to both light scattering by liposomes and low resolution. Reconstitution of ChrS into smaller lipid particles, such as bicelles [32] or Nanodiscs [33], is currently underway in our laboratory for spectroscopic determination of the nature of ChrS–heme binding and kinase regulation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.06.001.

References

[1] Dunn, L.L., Rahmanto, Y.S. and Richardson, D.R. (2007) Iron uptake and metabolism in the new millennium. Trends Cell Biol. 17, 93–100.
[2] Conrad, M.E. and Umbreit, J.N. (2002) Pathways of iron absorption. Blood Cell Mol. Dis. 29, 336–355.
[3] Skaar, E.P., Humayun, M., Bae, T., DeBord, K.L. and Schneewind, O. (2004) Iron-source preference of *Staphylococcus aureus* infections. Science 305, 1626–1628.
[4] Wandersman, C. and Delepelaire, P. (2004) Bacterial iron sources: from siderophores to hemophores. Annu. Rev. Microbiol. 58, 611–647.
[5] Litwin, C.M. and Calderwood, S.B. (1993) Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. 6, 137–149.
[6] Schmitt, M.P. and Holmes, R.K. (1991) Iron-dependent regulation of diphtheria toxin and siderophore expression by the cloned *Corynebacterium diphtheriae* repressor gene dtxR in *C. diphtheriae C7* strains. Infect. Immun. 59, 1899–1904.
[7] Schmitt, M.P. (1997) Transcription of the *Corynebacterium diphtheriae hmuO* gene is regulated by iron and heme. Infect. Immun. 65, 4634–4641.
[8] Schmitt, M.P. (1997) Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. J. Bacterial. 179, 838–845.
[9] Kunkle, C.A. and Schmitt, M.P. (2003) Analysis of the *Corynebacterium diphtheriae* DtxR regulon: identification of a putative siderophore synthesis and transport system that is similar to the *Yersinia* high-pathogenicity island-encoded yersiniabactin synthesis and uptake system. J. Bacteriol. 185, 6826–6840.
[10] Schmitt, M.P. (1999) Identification of a two-component signal transduction system from *Corynebacterium diphtheriae* that activates gene expression in response to the presence of heme and hemoglobin. J. Bacteriol. 181, 5330–5340.
[11] Stock, J.B., Ninfa, A.J. and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. 53, 450–490.
[12] Mascher, T., Helmann, J.D. and Unden, G. (2006) Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol. Mol. Biol. Rev. 70, 910–938.
[13] Hanada, K., Yamato, I. and Anraku, Y. (1987) Construction and properties of bifunctionally active membrane-bound fusion proteins. *Escherichia coli* proline carrier linked with beta-galactosidase. J. Biol. Chem. 262, 14100–14104.
[14] Janausch, I.G., Garcia-Moreno, I. and Unden, G. (2002) Function of DcuS from *Escherichia coli* as a fumarate-stimulated histidine protein kinase in vitro. J. Biol. Chem. 277, 39809–39814.
[15] Liu, C.E. and Ames, G.F.-L. (1997) Characterization of transport through the periplasmic histidine permease using proteoliposomes reconstituted by dialysis. J. Biol. Chem. 272, 859–866.
[16] Nakamura, H., Saito, K. and Shiro, Y. (2001) Quantitative measurement of radioactive phosphorylated proteins in wet polyacrylamide gels. Anal. Biochem. 294, 187–188.
[17] Matsui, N.M., Smith-Beckerman, D.M. and Epstein, L.B. (1999) Staining of preparative 2-D gels: coomassie blue and imidazole-zinc negative staining. Method Mol. Biol. 112, 307–311.
[18] Seelert, H. and Krause, F. (2008) Preparative isolation of protein complexes and other bioparticles by elution from polyacrylamide gels. Electrophoresis 29, 2617–2636.
[19] Song, J., Braun, G., Bevis, E. and Doncaster, K. (2006) A simple protocol for protein extraction of recalcitrant fruit tissues suitable for 2-DE and MS analysis. Electrophoresis 27, 3144–3151.
[20] Schey, K.L. (1996) Hydrophobic proteins and peptides analyzed by matrix-assisted laser desorption/ionization. Method Mol. Biol. 61, 227–230.
[21] Schuerenberg, M., Luebbert, C., Eickhoff, H., Kalkum, M., Lehrach, H. and Nordhoff, E. (2000) Prestructured MALDI-MS sample supports. Anal. Chem. 72, 3436–3442.
[22] López-Goñi, I., Guzmán-Verri, C., Manterola, L., Sola-Landa, A., Moriyón, I. and Moreno, E. (2002) Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. Vet. Microbiol. 90, 329–339.
[23] Gooderham, W.J. and Hancock, R.E. (2009) Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. 33, 279–294.
[24] Stauff, D.L., Torres, V.J. and Skaar, E.P. (2007) Signaling and DNA-binding activities of the *Staphylococcus aureus* HssR-HssS two-component system required for heme sensing. J. Biol. Chem. 282, 26111–26121.
[25] Torres, V.J., Stauff, D.L., Pishchany, G., Bezbradica, J.S., Gordy, L.E., Iturregui, J., Anderson, K.L., Dunman, P.M., Joyce, S. and Skaar, E.P. (2007) A *Staphylococcus aureus* regulatory system that responds to host heme and modulates virulence. Cell Host Microbe 1, 109–119.
[26] Zhang, L. and Guarente, L. (1995) Heme binds to a short sequence that serves a regulatory function in diverse proteins. EMBO J. 14, 313–320.
[27] Hira, S., Tomita, T., Matsui, T., Igarashi, K. and Ikeda-Saito, M. (2007) Bach1, a heme-dependent transcription factor, reveals presence of multiple heme binding sites with distinct coordination structure. IUBMB Life 59, 542–551.
[28] Ishikawa, H., Kato, M., Hori, H., Ishimori, K., Kirisako, T., Tokunaga, F. and Iwai, K. (2005) Involvement of heme regulatory motif in heme-mediated ubiquitination and degradation of IRP2. Mol. Cell 19, 171–181.
[29] Kamiya, N., Furusaki, S. and Goto, M. (1997) Peroxidase activity and stability of surfactant-heme complex in nonaqueous media. Biotechnol. Lett. 19, 1015–1018.
[30] de Villiers, K.A., Kaschula, C.H., Egan, T.J. and Marques, H.M. (2007) Speciation and structure of ferriprotoporphyrin IX in aqueous solution: spectroscopic and diffusion measurements demonstrate dimerization, but not μ-oxo dimer formation. J. Biol. Inorg. Chem. 12, 101–117.
[31] Sato, E., Sagami, I., Uchida, T., Sato, A., Kitagawa, T., Igarashi, J. and Shimizu, T. (2004) SOUL in mouse eyes is a new hexameric heme-binding protein with characteristic optical absorption, resonance Raman spectral, and heme-binding properties. Biochemistry 43, 14189–14198.
[32] van Dam, L., Karlsson, G. and Edwards, K. (2004) Direct observation and characterization of DMPC/DHPC aggregates under conditions relevant for biological solution NMR. Biochim. Biophys. Acta 1664, 241–256.
[33] Bayburt, T.H., Grinkova, Y.V. and Sligar, S.G. (2002) Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. Nano Lett. 2, 853–856.