Heme binding to cold shock protein D, CspD, from *Vibrio cholerae*

Dayeon Nam[a,1], Wataru Motegi[a], Koichiro Ishimori[a,b], and Takeshi Uchida[a,b,*]

[a]Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo 060-8628, Japan

[c]Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

[1]Current address: Department of Creative Research, Exploratory Research Center on Life and Living Systems (ExCELLS), National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan

*Corresponding author: Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

E-mail address: uchida@sci.hokudai.ac.jp (T. Uchida).

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ABSTRACT

Cold shock protein D (CspD) is one of the homologous proteins of cold shock protein A (CspA), inhibiting DNA replication by binding to single-stranded DNA. We found that CspD from *Vibrio cholerae* (*Vc*CspD) possesses one heme regulatory motif (HRM) sequence and specifically binds heme with a stoichiometry of 1:1. The binding of a synthetic single-stranded DNA oligomer (ssDNA) was followed by fluorescence quenching of Trp. The fluorescence quenching associated with the addition of ssDNA was suppressed in the presence of heme, indicating that heme binding to *Vc*CspD inhibited the formation of the *Vc*CspD-ssDNA complex. Such heme-induced inhibition was not observed for the *Vc*CspD mutant with replacement of Cys22 in the HRM with alanine (C22A). Heme binding at Cys22 is, therefore, essential for the inhibition of ssDNA binding for *Vc*CspD. The growth of *Escherichia coli* at 37 °C was slowed when *Vc*CspD was overexpressed, indicating that *Vc*CspD hampers the growth of *E. coli*. When the production of heme in cells was promoted by the addition of a heme precursor, δ-aminolevulinic acid, the growth of *E. coli* expressing *Vc*CspD was decelerated, but the growth of *E. coli* expressing the C22A mutant was not decelerated. These observations allow us to conclude that heme specifically binds to the HRM region in *Vc*CspD and inhibits the binding of target ssDNA, which suggests that heme functions as a regulatory molecule for DNA replication.

1. Introduction

When bacteria encounter a sudden decrease in temperature, the expression of proteins that support adaptation to cold temperature is induced [1,2]. Cold shock proteins (Csps) are proteins that consist of ~70 amino acid residues and function as RNA chaperones by binding to single-stranded nucleic acids [3,4]. *Vibrio cholerae* possesses four Csps: CspA, CspV, CspD and VCA0184 [5,6]. CspA and CspV are cold shock inducible proteins, and CspA is drastically induced by cold shock, which is considered essential for cold adaptation [7], functioning as an RNA chaperone to prevent the formation of secondary structures in RNA molecules at low temperatures [8,9]. In contrast to CspA and CspV, CspD is not induced during cold shock. CspD from *Vibrio cholerae* (*Vc*CspD) is induced during stationary-phase, glucose starvation [10] and oxidative stress [11], inhibiting DNA replication by complex formation with opened and single-stranded DNA regions at replication forks [12]. *Vc*CspD possesses a Cys-Pro (CP) dipeptide motif (Cys22-Pro23) (Fig. S1), which is a short consensus sequence found for heme-regulated proteins using heme as a regulatory or signaling molecule and is well conserved in the heme regulatory motif (HRM) [13–15]. The presence of the CP motif raised the possibility of heme binding to *Vc*CspD, leading to modulation of the function of *Vc*CspD.

In this study, heme binding to *Vc*CspD and its effect on nucleotide binding were examined. The specific heme binding to *Vc*CspD was confirmed by spectrophotometric heme titration, and the heme stoichiometry was determined. Synthetic single strand DNA (ssDNA) was used as a model substrate for the opened and single-stranded DNA regions at replication forks. The binding of the ssDNA substrate to *Vc*CspD was monitored by fluorescence quenching of the tryptophan residue near the supposed substrate binding site of *Vc*CspD. A *Vc*CspD mutant with a mutation at Cys22 in the HRM sequence was also prepared to confirm heme binding to the specific cysteine residue in the HRM region and heme-regulated ssDNA binding of *Vc*CspD. On the basis of these spectroscopic characterizations, the functional significance of heme binding to *Vc*CspD will be discussed.

2. Materials and methods

2.1. Protein expression and purification

The VC1142 gene, corresponding to *Vc*CspD, was purchased from the PlasmID Repository (http://plasmid.med.harvard.edu/PLASMID/Home.xhtml) (clone *Vc*CD00035667) and amplified using polymerase chain reaction (PCR) with the primers 5'-CCA GGG GCC CCA TAT GTA TAG CAT GGC TAC AGG TA-3' and 5'-GGA GCT CGA ATT CTC ATT TTG CTG ATT GTC CCT CG-3'. The amplified fragment was cloned into the pGEX-6P-1 vector (Cytiva, Sweden) using a Gibson Assembly kit (New England Biolabs, Ipswich, MA, UK). Between the glutathione S-transferase (GST) tag and PreScission recognition site, a pentahistidine tag was inserted for purification using a Ni^{2+} affinity column under denatured conditions. The expression plasmid for *Vc*CspD was transformed into the *E. coli* BL21(DE3) strain (Nippon Gene, Japan) and cultured at 37 °C in Luria-Bertani (LB) broth supplemented with 50 μg mL^{-1} ampicillin. After cultures reached an optical density at 600 nm of 0.6–0.8, expression of the GST-His-tagged fusion protein was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were further grown at 37 °C for 4 h and harvested by centrifugation. The pellet was suspended in lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1% Nonidet P-40, and 1 mM dithiothreitol at pH 8.0). The suspension was further incubated for 30 min at 4 °C after adding 1 mg mL^{-1} lysozyme, RNase and DNase. The sample was disrupted by sonication and then centrifuged at 40,000 × g for 30 min. The resulting supernatant was applied to a Ni-NTA agarose column (Qiagen) preequilibrated with buffer A (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0). The column was washed with buffer A containing 8 M urea, and then the urea concentration was decreased stepwise to 0 M. The bound protein was eluted with 50 mM Tris-HCl, 500 mM NaCl, and 250 mM imidazole (pH 8.0). *Vc*CspD was then applied to a HiLoad 16/600 Superdex 75 gel-filtration column (Cytiva) preequilibrated with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH and 100 mM NaCl (pH 7.4).

The protein concentration was estimated from the absorbance at 280 nm with a molar extinction coefficient (ε_{280}) of 13.1 M^{-1} cm^{-1}, calculated in ProtParam (http://web.expasy.org/protparam/). Mutation of Cys22 in *Vc*CspD to alanine was introduced using a PrimeSTAR mutagenesis basal kit from Takara Bio (Japan). The primers used were 5'-TTT ATT GCG CCG GAA GGT GAA GAC GGA-3' and 5'-TTC CGG CGC AAT AAA ACC GAA GCC TTT G-3'. The CspA gene from *V. cholerae* was purchased from Eurofins Genomics (Tokyo, Japan). CspA was expressed and purified as *Vc*CspD.

2.2. ssDNA binding assay

A synthetic ssDNA oligomer consisting of seven thymines, dT_7, was used as a model substrate [16]. The affinity of *Vc*CspD for ssDNA was monitored by tryptophan fluorescence quenching as previously reported [16]. The excitation wavelength was 290 nm (slit width 10 nm), and emission spectra were taken from 310 to 450 nm. *Vc*CspD or *Vc*CspA in 50 mM HEPES and 10 mM KCl (pH 7.5) was placed in a quartz cuvette, after which aliquots of the prepared solution of concentrated ssDNA (200 nM) were added gradually to the *Vc*CspD or *Vc*CspA solution. Fluorescence spectra were recorded at 15 °C after incubation with the ssDNA substrate for 2 min using an FP-8500 spectrofluorometer (JASCO, Tokyo, Japan).

2.3. Absorption spectroscopy

All absorption spectra were obtained using a V-660 UV–vis spectrophotometer (JASCO). Heme titration experiments were carried out by difference absorption spectroscopy. Hemin was dissolved in 0.1 M NaOH, and its concentration was determined on the basis of absorbance at 385 nm using ε_{385} = 58.44 mM^{-1} cm^{-1} [17]. Aliquots of hemin solution (0.5 mM and 0.2 equivalent of the protein) were added to the sample cuvette containing 5 μM *Vc*CspD or *Vc*CspA at 25 °C. Absorption spectra were recorded at 2 min after the addition of hemin. The absorbance difference at 417 nm was plotted as a function of the heme concentration.

3. Results

3.1. Expression and purification of VcCspD

*Vc*CspD was originally expressed as a His-tagged protein using a pET-28b vector. However, the His-tag was not cleaved well, probably because the protease recognition site was buried in the protein. Then, we expressed the protein as a GST-fusion protein using a pGEX-6P-1 vector. GST-tag was cleaved almost completely, but *Vc*CspD was purified as a complex with nucleic acids. The oligonucleotide was not separated from *Vc*CspD after purification with an anion-exchange column due to a strong hydrophobic interaction between *Vc*CspD and nucleic acids [12]. To remove the nucleic acids from *Vc*CspD, urea-induced denaturation was needed. Thus, a pentahistidine tag was inserted between GST and the PreScission recognition site in the pGEX-6P-1 vector for efficient cleavage of the tag and purification under denatured conditions. After cleavage of the GST-His-tag, we obtained purified *Vc*CspD, which provided a single band at approximately 10 kDa on an SDS–PAGE gel (Fig. S2A). The band corresponded to the calculated molecular mass for *Vc*CspD (8.6 kDa). A size-exclusion chromatography analysis indicated that the fraction of purified *Vc*CspD protein was eluted at 80.0 mL on the chromatogram, corresponding to the globular protein of molecular weight ~12 kDa (Fig. S2B). The ratio of the absorbance at 280 nm to that at 260 nm (A_{280}/A_{260}) of purified *Vc*CspD was 1.7, which was identical to the calculated value (1.7), indicating that purified *Vc*CspD does not contain oligonucleotides. These results indicate that *Vc*CspD is probably a monomeric protein, as found for *E. coli* CspA (*Ec*CspA) [18], but different from dimeric *E. coli* CspD (*Ec*CspD) [19], and its structure might be different from a typical globular form.

3.2. Heme-binding properties of CspD

Heme binding of *Vc*CspD was examined by absorption spectroscopy (Fig. 1). The presence of a small peak at 416 nm of purified *Vc*CspD suggests heme binding to *Vc*CspD (Fig. 1A, red). The absorption spectrum of *Vc*CspD with 1 equivalent of heme (Fig. 1A, blue) was compared

with that of heme (Fig. 1A, black). The purified *Vc*CspD spectra contained the Soret peak at 376 nm with a shoulder at 416 nm (blue). The shape of the Soret band was different from that of heme (black) and quite similar to that of CyaY from *V. cholerae* (*Vc*CyaY) [20]. *Vc*CyaY, a frataxin homolog, binds heme with Tyr67 and Cys78 [20]. These results indicate that *Vc*CspD is able to bind heme. To determine the stoichiometry of heme binding to *Vc*CspD, the absorbance difference between the solution containing *Vc*CspD and buffer alone titrated with hemin was calculated (Fig. 1B) and plotted against the concentration of heme (Fig. 1C). The difference at 415 nm increased with an increase in the heme concentration, whose slope changed at ~1.0 equivalents (Fig. 1C). The plot indicates that *Vc*CspD binds to heme in a 1:1 stoichiometry.

3.3. Heme binding site in VcCspD

The amino acid sequence shows that *Vc*CspD possesses a common heme-binding motif at Cyp22-Pro23 (Fig. S1), and thus Cys22 would be a possible heme ligand. To confirm heme binding to Cys22, a mutant with the mutation of Cys22 to Ala (C22A) was constructed, and the heme binding property was examined. The small peak at 416 nm present in the spectrum of the wild-type (WT) (Fig. 1A, red) was not observed in the spectrum of the purified C22A mutant (Fig. 2A, red). After the addition of heme to the purified C22A mutant, a broad Soret band appeared at 378 nm (Fig. 2A, blue), which was similar to that of the WT protein (Fig. 1A). The difference in absorption spectra between the solution of C22A and buffer titrated with hemin was calculated (Fig. 2B) and plotted to examine heme binding (Fig. 2C). The plots of the absorbance difference at 416 nm increased monotonously with increasing heme concentration. Considering these results, the C22A mutant can also bind heme, but its binding is nonspecific.

3.4. ssDNA binding of VcCspD

To confirm the binding of *Vc*CspD to the target ssDNA, we measured tryptophan fluorescence in the titration of ssDNA to *Vc*CspD [16]. Upon the addition of ssDNA, the fluorescence intensity at 349 nm was decreased (Fig. 3A). *Vc*CspD has only one tryptophan residue at position 11 (Fig. S1), and on the basis of the crystal structure of CspB from *Bacillus subtilis* (*Bs*CspB), Trp8 (corresponding residue of Trp11 in *Vc*CspD) is involved in ssDNA binding (Fig. S3) [16]. Therefore, the observed fluorescence decrease was caused by the energy transfer from Trp11 to the nucleotide base of the ssDNA substrate, indicating that ssDNA binds to *Vc*CspD at Trp11.

The same experiment was conducted in the presence of heme (Fig. 3B). Due to quenching by heme, the intensity of the fluorescence was smaller than that in the absence of heme (Fig. 3A). In contrast to the result in the absence of heme, the amount of the fluorescence decrease induced by the ssDNA substrate was repressed by heme. The relative fluorescence changes induced by the addition of ssDNA in the presence of heme were compared to those in the absence of heme, as shown in Fig. 3C. Therefore, heme binding to *Vc*CspD inhibits ssDNA binding.

3.5. Effect of heme binding to Cys22 on ssDNA binding

Cys22 in the CP motif is the putative heme binding site, whereas the heme titration experiment of the C22A mutant indicated nonspecific heme binding (Fig. 2C). We next examined whether heme binding to the C22A mutant affects ssDNA binding. The fluorescence from tryptophan was decreased by the addition of ssDNA to the C22A mutant (Fig. 3D). The behavior was more prominent than that of the WT (Fig. 3A). Although Cys22 is adjacent to the DNA-binding site, the mutation of Cys22 does not inhibit DNA binding but increases the affinity of ssDNA. In the presence of heme, the fluorescence intensity was also diminished by the addition of ssDNA (Fig. 3E), implying that the C22A mutant binds to the ssDNA substrate

in the presence of heme. The relative fluorescence changes of the C22A mutant in the absence and presence of heme are compared in Fig. 3F. The mutation of Cys22, therefore, abolished the heme-induced inhibition of ssDNA binding, indicating that heme binding to the C22A mutant is nonspecific. Heme binding to Cys22 is therefore essential for the inhibition of ssDNA binding in *Vc*CspD.

3.6. Heme and ssDNA binding of VcCspA

Here, we found that heme binds to *Vc*CspD, which inhibits ssDNA binding. As a control experiment, we prepared *Vc*CspA, another Csp from *V. cholerae*. *Vc*CspA and *Vc*CspD share 47% identity and 80% similarity (Fig. S1), but *Vc*CspA does not have the CP motif. The absorption spectrum of *Vc*CspA in the presence of 1 equivalent of heme was quite similar to that in the absence of heme (Fig. S4A). In addition, the absorption differences between *Vc*CspA and buffer with heme (Fig. S4B) were smaller than those in *Vc*CspD (Fig. 1A). These results indicated that *Vc*CspA does not bind to heme. The plot of the absorption difference at 412 nm supports this conclusion (Fig. S4C).

The fluorescence spectra of *Vc*CspA upon incremental addition of ssDNA were recorded. *Vc*CspA also possesses a single tryptophan at the corresponding position of *Vc*CspD (Fig. S1). The fluorescence from tryptophan was decreased by the addition of ssDNA, which indicates that *Vc*CspA also binds to ssDNA (Fig. S4D). The same measurement was conducted in the presence of 2 equivalents of heme. The fluorescence change was compared with that in the absence of heme. The normalized fluorescence intensities overlapped well, indicating that heme does not affect the fluorescence (Fig. S4D). This result clearly showed that heme does not bind to *Vc*CspA and does not affect the ssDNA binding ability, which is in stark contrast to *Vc*CspD.

3.7. The effects of heme binding to VcCspD in E. coli cells

To examine the role of heme binding to *Vc*CspD in bacterial cells, we monitored the growth of *E. coli* expressing *Vc*CspD. For this experiment, the *Vc*CspD gene was inserted into a pUC19 vector. *E. coli* cells containing the *Vc*CspD-pUC19 vector grew slightly slower than those containing an empty pUC19 vector (Fig. 4A). To promote the synthesis of heme in cells, 1.0 mM δ-aminolevulinic acid was added. The growth of *E. coli* with the empty pUC19 vector was not affected by the addition of δ-aminolevulinic acid, whereas that of *E. coli* with the *Vc*CspD-pUC19 vector was slightly decelerated (Fig. 4B).

For comparison, we conducted the same experiment using the C22A mutant. The growth of *E. coli* containing the C22A-pUC19 vector was almost the same as that of *E. coli* with the WT *Vc*CspD-pUC19 vector. The addition of δ-aminolevulinic acid did not affect growth, which is in contrast to WT *Vc*CspD-pUC19 (Fig. 4A, B). We do not know the detailed mechanism, but we can say that *Vc*CspD affects the growth of *E. coli* and that heme is involved in the repression of *E. coli* growth through *Vc*CspD.

4. Discussion

Based on the absorption and fluorescence spectroscopic analyses and mutational experiments, *Vc*CspD specifically binds heme at Cys22 in HRM (Fig. 1). The crystal structure of *Bs*CspB [16], sharing 78% similarity with *Vc*CspD, shows that Glu19, corresponding to Cys22 in *Vc*CspD, is exposed to the solvent (Fig. S3); thus, Cys22 is a possible heme binding site. The circular dichroism (CD) spectrum of *Vc*CspD was not altered by the addition of heme (Fig. S5). This result indicates that heme does not induce any secondary structural changes, which is consistent with the proposal that heme binds to the protein surface. Heme binding inhibits *Vc*CspD from binding to the ssDNA substrate (Fig. 3). Such heme-induced inhibition of the formation of the complex of *Vc*CspD with ssDNA is supported by previous structural studies. All Csps have two nucleic acid-binding motifs, ribonucleoprotein (RNP) 1 and RNP 2,

which are highly condensed regions of basic and aromatic amino acid residues on the protein surface (Fig. S1) [21,22]. Because RNP 1 of *Vc*CspD corresponds to ^{16}KGFGF^{21}I (Fig. S1), Cys22 is adjacent to RNP 1. Phe17 in *Bs*CspB, which corresponds to Phe20 in *Vc*CspD, is one of the residues contributing to nucleotide binding through hydrophobic interactions (Fig. S3) [16,23]. Therefore, Cys22 is not the ssDNA-binding site but is located close to the binding site, and heme binding to Cys22 in *Vc*CspD is expected to induce tertiary structural changes to sterically interfere with ssDNA binding. Inhibition of nucleotide binding by binding to Cys in the HRM has been encountered in some proteins, such as Bach1, Rev-erbα and HrtR [24–26].

Csps are well known as RNA chaperones, which bind to RNA with nonproductive secondary structures and destabilize such structures [8,9]. In addition, CspD is also reported to be a DNA replication inhibitor, presumably by binding to the single-stranded region in the DNA replication fork [12]. Under glucose starvation conditions, CspD is induced and binds to the single-stranded region in the DNA replication fork to inhibit DNA replication, leading to repression of DNA replication. When iron and/or heme is available for *V. cholerae*, biosynthesis of heme increases. Binding of heme to *Vc*CspD interferes with the inhibitory function of *Vc*CspD on DNA replication, resulting in promotion of DNA replication. There is a possibility that the availability of iron and/or heme is related to glucose availability because nutrients contain both iron/heme and glucose. The fact that the expression of CspD is reported to be induced upon nutrient starvation [10] also supports the heme-induced promotion of DNA replication. Thus, we propose that CspD uses heme as a signaling molecule to regulate DNA replication in response to cellular nutrient availability.

5. Conclusion

Based on various spectroscopic analyses and mutational experiments, we found that heme binds to *Vc*CspD and inhibits its ssDNA binding. This property is specific to *Vc*CspD but not observed for *Vc*CspA. As CspD is known to inhibit DNA replication and is a toxic molecule,

the expression of *Vc*CspD in *E. coli* represses the growth of *E. coli*, which is enhanced by heme.

A new function of heme on *Vc*CspD is proposed in this study.

Abbreviation List

Csp cold shock protein
*Vc*Csp Csp from *Vibrio cholerae*
WT wild-type.

Declaration of competing interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.

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Figure legends

Fig. 1. Electronic absorbance spectra of heme titration. (A) Absorption spectra of WT *Vc*CspD as purified (red), heme (black), and heme complex of WT *Vc*CspD (blue). (B) Absorption difference spectra of heme binding to *Vc*CspD following incremental addition of heme (1−3 equivalents) to *Vc*CspD (5 μM) versus buffer alone in 50 mM HEPES and 100 mM NaCl (pH 7.4). (C) Absorbance difference at 415 nm as a function of heme concentration.

Fig. 2. Electronic absorbance spectrum of heme titration. (A) Absorption spectra of the C22A mutant *Vc*CspD as purified (red), heme (black), and heme complex of the C22A mutant (blue). (B) Absorption difference spectra of heme−C22A mutant *Vc*CspD. (C) The difference at 417 nm following incremental addition of heme (1−3 equivalents) to the C22A mutant (5 μM) in 50 mM HEPES and 100 mM NaCl (pH 7.4) against a blank cell containing buffer alone.

Fig. 3. ssDNA titration of *Vc*CspD. Fluorescence spectra of WT *Vc*CspD titrated with 0.2–1.8 equivalents of ssDNA substrate in the (A) absence and (B) presence of heme. The excitation wavelength was 290 nm. (C) Plots of fluorescence at 349 nm against the ssDNA substrate in the absence of heme (black circle) and presence of heme (red circle). (C), (D), (E) of the C22A mutant correspond to (A), (B), and (C) of the WT, respectively.

Fig. 4. Growth curves of *E. coli*. *E. coli* cells were coexpressed with pUC19 vector alone (black) and the vector containing WT (red) and the C22A mutant (blue) *Vc*CspD in the absence (A) and presence (B) of 1.0 mM δ-aminolevulinic acid.

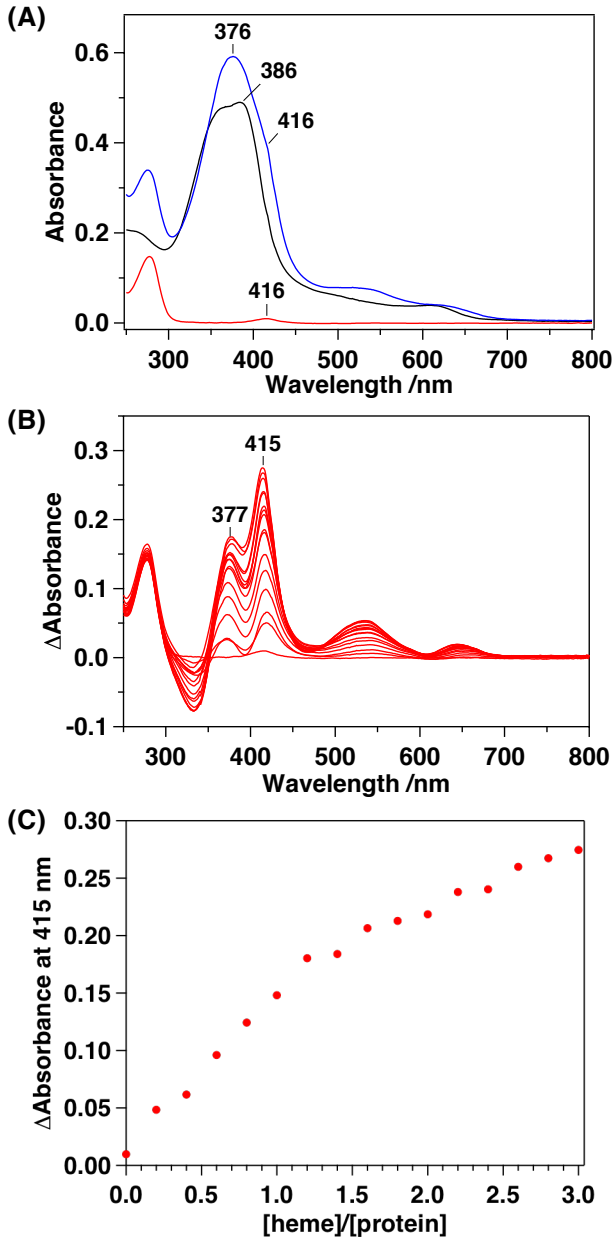
(A)
376
386
416
416
Absorbance
0.0
0.2
0.4
0.6
300
400
500
600
700
800
Wavelength /nm
(B)
415
377
ΔAbsorbance
-0.1
0.0
0.1
0.2
0.3
300
400
500
600
700
800
Wavelength /nm
(C)
ΔAbsorbance at 415 nm
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[heme]/[protein]

Figure 1

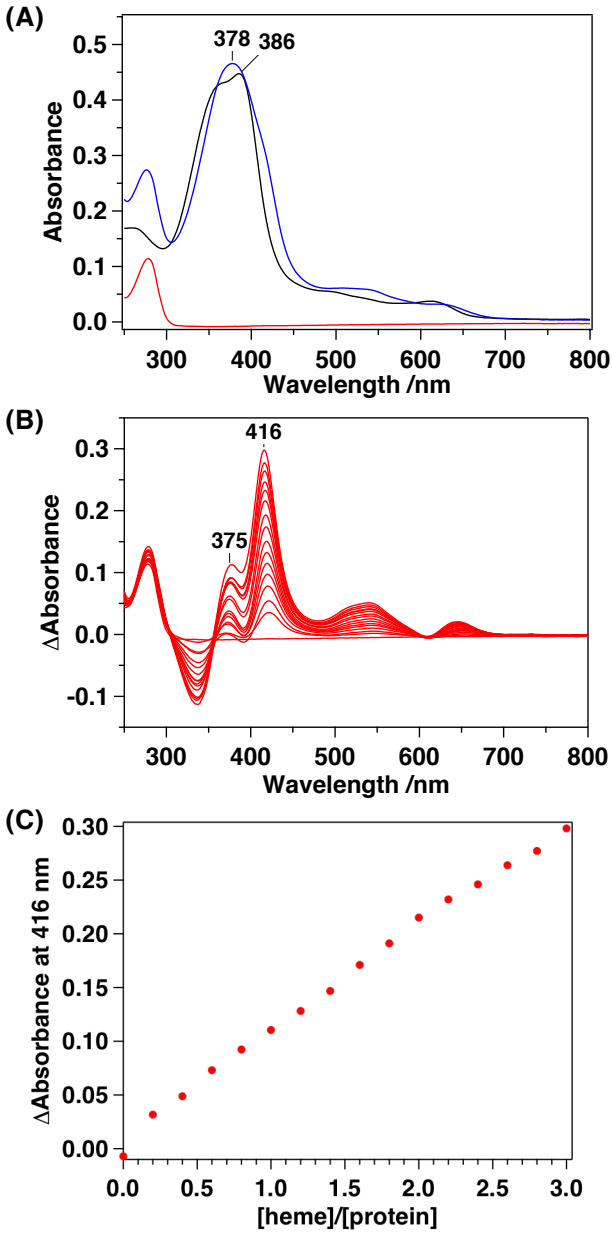
(A)
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
378
386
300
400
500
600
700
800
Wavelength /nm
(B)
ΔAbsorbance
-0.1
0.0
0.1
0.2
0.3
416
375
300
400
500
600
700
800
Wavelength /nm
(C)
ΔAbsorbance at 416 nm
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[heme]/[protein]

Figure 2

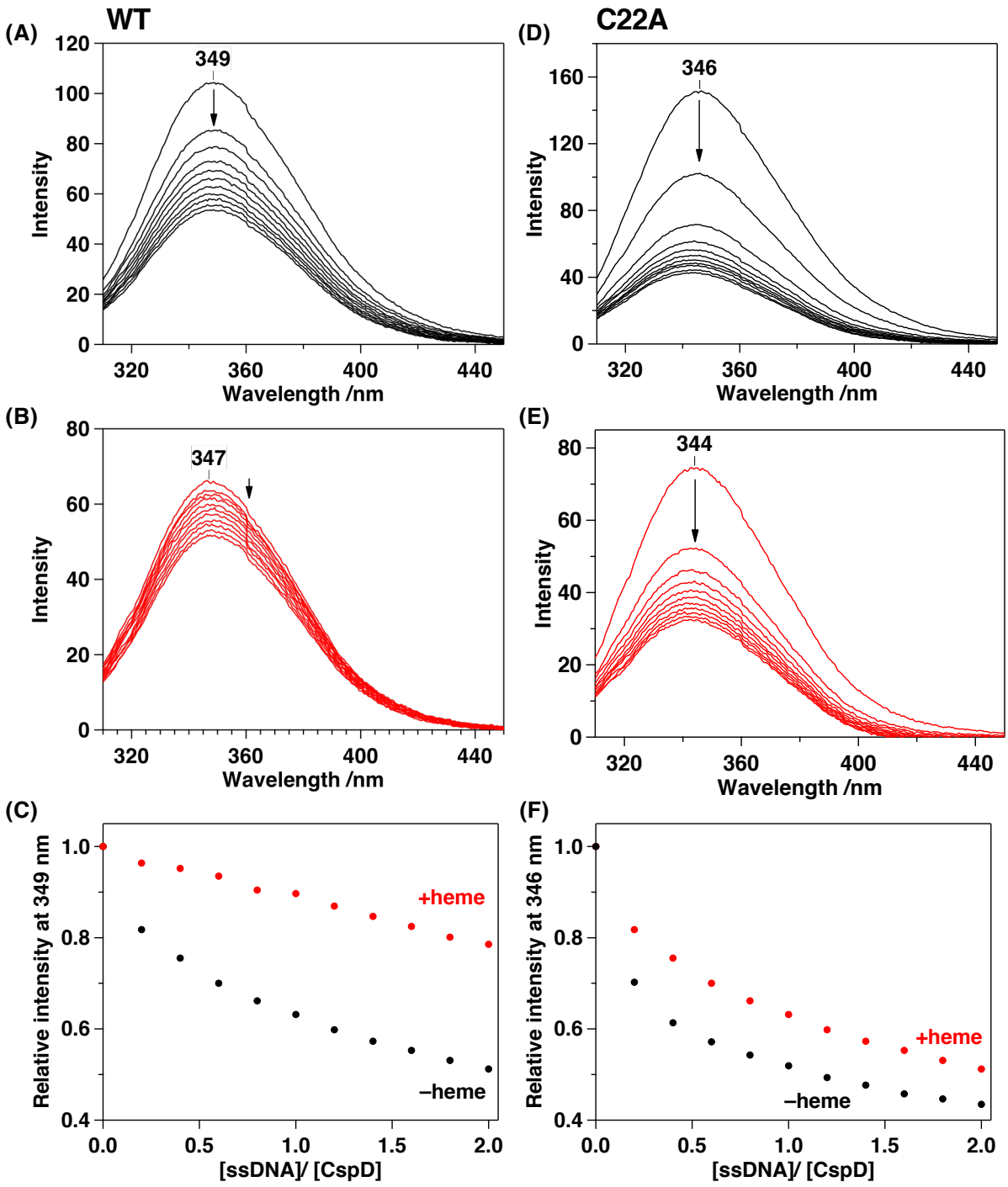
(A)
WT
349
Intensity
Wavelength /nm
(D)
C22A
346
Intensity
Wavelength /nm
(B)
347
Intensity
Wavelength /nm
(E)
344
Intensity
Wavelength /nm
(C)
Relative intensity at 349 nm
+heme
–heme
[ssDNA]/ [CspD]
(F)
Relative intensity at 346 nm
+heme
–heme
[ssDNA]/ [CspD]

Figure 3

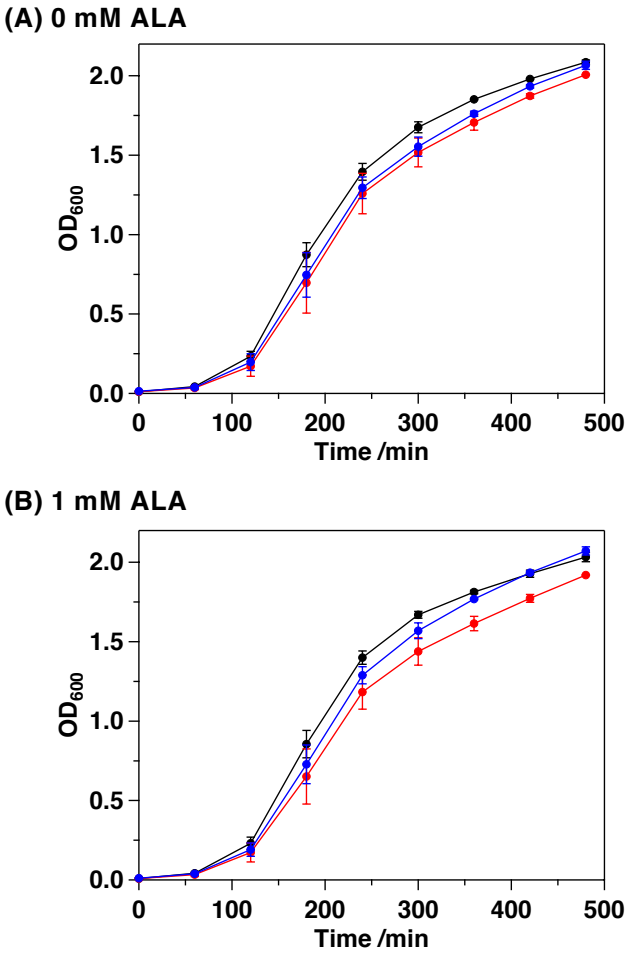

Figure 4