Essential residues in the H-NS binding site of Hha, a co-regulator of horizontally acquired genes in Enterobacteria.

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ABSTRACT

Proteins of the Hha/YmoA family co-regulate with H-NS the expression of horizontally acquired genes in Enterobacteria. Systematic mutations of conserved acidic residues in Hha have allowed the identification of D48 as an essential residue for H-NS binding and the involvement of E25. Mutations of these residues resulted in deregulation of sensitive genes *in vivo*. D48 is only partially solvent accessible, yet it defines the functional binding interface between Hha and H-NS confirming that Hha has to undergo a conformational change to bind H-NS. Exposed acidic residues, such as E25, may electrostatically facilitate and direct the approach of Hha to the positively charged region of H-NS enabling the formation of the final complex when D48 becomes accessible by a conformational change of Hha

Keywords

Nucleoid associated proteins; H-NS; Hha; Transcription repression

Abbreviations

HSQC, heteronuclear single-quantum correlation.

HT, horizontal transfer

*Manuscript text (starting from the introduction)
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1. Introduction

The nucleoid-associated protein H-NS is a global transcriptional repressor that controls the expression of many environmentally regulated genes. H-NS homooligomers are able of bridging distant DNA sequences forming nucleoprotein structures that are repressive for the transcription of a wide variety of genes. These include housekeeping genes as well as those acquired by horizontal transfer (HT genes) [1-3]. Tight control of HT genes by H-NS allows their incorporation with minimal fitness cost and facilitates the acquisition of new traits such as those contributing to virulence or antibiotic resistance. Recent studies in *Salmonella* Typhimurium have shown that formation of heterocomplexes between H-NS and Hha, a nucleoid associated protein that does not interact with DNA by itself, is required for the efficient regulation of HT genes [4].

H-NS/Hha heterocomplexes modulate, among others, the transcription of *hly* and *htrA* in *E. coli* [5,6], and *hilA* [7] or the *tra* operon from the conjugative plasmid R27 [8] in *S. enterica* serovar Typhimurium. YmoA, a member of the Hha family, is involved in the regulation of transcription of Yop proteins and YadA adhesin in *Yersinia enterocolitica* [9,10] and the type III secretion system in *Y. pestis* [11].

Although H-NS proteins are widely distributed within Gram-negative bacteria, Hha-like proteins are present only in *Enterobacteriaceae* [12], encoded either in the main chromosome or in transmissible elements such as plasmids.

No structural information is available for full length H-NS homooligomers or H-NS/Hha heterocomplexes. However, the structure of truncated H-NS fragments forming defined homodimers (*Salmonella*, residues 1-58 [13]); *E. coli*, residues 1-47 [14] and *Vibrio cholerae*, residues 1-49 [15]) have been reported. Recently, the structure of the first 83 residues of H-NS from *Salmonella* in an oligomerized state has been solved by

X-ray crystallography [16]. In addition, the structure of the monomeric H-NS C-terminal domain responsible for DNA binding is also known [17]. The three dimensional structure of Hha was solved by NMR. It consists of four α-helical segments separated by flexible loops [18]. Similar structures were subsequently observed for YmoA [19] and the Hha paralogue in *E. coli*, YdgT [20].

In previous studies using NMR, we identified the Hha binding site in H-NS. It is well defined and involves residues from helices 1 and 2 of the N-terminal domain of H-NS. In particular, arginine 12 is essential and its substitution by histidine abolished the interaction with Hha without affecting the domain structure [21]. In contrast, the H-NS binding site in Hha could not be defined by NMR since perturbations caused by the addition of H-NS in the ^{1}H-^{15}N HSQC spectra of Hha affected a large number of residues, some of which are not exposed on the Hha surface. This fact indicated that Hha changed its conformation upon binding to H-NS [22] and it was not possible to distinguish the residues responsible for the intermolecular interaction from those affected by the conformational rearrangement.

However, the essential role of residue R12 of H-NS and the fact that the Hha-H-NS interaction is suppressed by moderate increases in ionic strength led us to hypothesize that negatively charged residue(s) may be essential elements of the H-NS binding site in Hha.

In this work we report a systematic mutational study of the conserved acidic residues of Hha that has provided evidence for the participation of the side chains of E25 and D48 in H-NS binding. In particular, D48 is essential and even conservative mutations of this residue abolished the interaction although they did not have any effect on Hha structure, as seen by NMR. *In vivo* studies of the activity of these mutants confirmed the role of these residues in gene regulation.

2. Materials and Methods

2.1. Bacterial strains, plasmids and protein expression

E. coli strains MG1655 [23], AAG1 [24] and AAG1 Δ*hha* [M. Gibert and A. Juárez, unpublished] were used in this work. The *hha* gene and its putative promoter from *E. coli* MG1655 was amplified by PCR and cloned into pACYC184, generating plasmid pACYC184-hha.

Mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene).

For pull-down experiments, plasmids pET15bHisHha (pET15b + 6xhis-hha) [25], pET15bHisHhaE25Q (pET15b + 6xhis-hha E25Q) and pET15bHisHhaD48N (pET15b + 6xhis-hha D48N) were used.

Wild type and Hha mutants [22], H-NS_{47} [21] and H-NS_{64} [22] were obtained and purified as described elsewhere.

2.2. NMR spectroscopy

NMR experiments to detect the interaction of Hha proteins with H-NS_{47} or H-NS_{64} were carried out and analyzed as previously described [21]. All ^{1}H-^{15}N-HSQC spectra were obtained at 25 ºC at 70-75 μM ^{15}N-labeled protein concentration in 20 mM sodium phosphate (pH 7.0) containing 150 mM NaCl, 1 mM tris(2-carboxyethyl)-phosphine (TCEP), 0.2 mM ethylene diamine tetraacetic acid (EDTA), 0.01 % (w/v) NaN_3. The dissociation constant of wild type Hha with H-NS64 is 19.8 μM [25].

2.3. Pull-down experiments

Isopropyl β-D-thiogalactoside (IPTG)-induced BL21(DE3) cells containing plasmids pET15bHisHha, pET15bHisHhaE25Q or pET15bHisHhaD48N were lysed in buffer A (20 mM HEPES pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 50 mM imidazole and 10% glycerol). His-tagged Hha proteins were purified with Ni^{2+}-NTA beads (Qiagen). Beads were washed five times with buffer A and His-tagged Hha variants were eluted with the same buffer supplemented with 200 mM Imidazole as described previously [26]. Eluted proteins were analysed by SDS-PAGE and stained with Coomassie blue or immunoblotted by western blot. Polyclonal antibodies raised against *E. coli* H-NS were used [26]. The intensity of the bands was quantified using QuantityOne® (Bio-Rad).

2.4. Measurement of hemolysin expression

Hemolysin expression from the reporter gene fusion *hlyA*::*lacZ* from plasmid pHly152 *hlyA*::*lacZ* was evaluated by β-galactosidase activity as previously described [27].

3. Results

3.1. Mutagenesis of negatively charged Hha residues

Hha contains ten negatively charged residues, out of eleven, that are conserved within the Hha family of proteins: D10, D22, E25, E29, E34, D37, E39, D48, E53 and D61 (Fig. 1). To evaluate their potential role in H-NS binding we mutated, one at a time, aspartate and glutamate residues to asparagine and glutamine, respectively to preserve the size and hydrophilic nature of the size chain. All of the mutants were overexpressed with high yields as soluble proteins with the exception of Hha E53Q, which resulted in the formation of insoluble aggregates that prevented further studies. The remaining nine mutations did not cause any significant perturbation of the protein structure as judged

by the similarities of their 1D ^{1}H-NMR spectra to that of the wild type protein (data not shown).

Insert Figure 1 here

The effects of the mutations in H-NS binding was determined by comparing ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled H-NS$_{47}$ in the absence and in the presence of 0.5 equivalents of each unlabeled Hha variant (1 Hha molecule per H-NS$_{47}$ dimer). We had previously demonstrated that Hha binding occurs exclusively in the N-terminal domain of H-NS [21]. The effect of the D10N, D22N, E29Q, E34Q, D37N, E39Q and D61N Hha variants on H-NS$_{47}$ was very similar to that reported for wild type Hha [21]. Complexation induces selective broadening of NMR signals from residues located in helices 1 and 2 and those residues of helix 3 that are making contact to the other two helices in the structure of H-NS$_{47}$. Broadening, resulting in a reduction of signal intensity, is caused by different chemical shifts in the complex and free form being modulated by dynamic processes and by the slower tumbling of the complex. Figure 2A shows the normalized intensity of the HSQC signals for each H-NS$_{47}$ residue in the presence of different variants of Hha with respect to that observed for free protein. These results indicate that the H-NS binding ability of Hha was unaffected by the D10N, D22N, E29Q, E34Q, D37N, E39Q and D61N mutations. In contrast, the decrease in the H-NS$_{47}$ NMR signal intensities induced by addition of Hha E25Q is much smaller than that of wild type Hha indicating less complex formation, although the interaction was not suppressed and the most affected H-NS residues are the same (Fig. 2B).

The D48N mutation suppressed the interaction with H-NS. Addition of equimolar amounts of the D48N mutant did not induce any appreciable intensity reduction in the ^{1}H-^{15}N HSQC spectra of H-NS_{47} (intensity ratio ~1 in Fig. 2B) indicating that D48 is essential for binding to H-NS.

Insert Figure 2 here

In order to rule out an effect of the D48N mutation on the structure of Hha we compared the ^{1}H-^{15}N HSQC spectra of D48N and wild type Hha. Both spectra were almost identical demonstrating that Hha folding was not affected by the mutation. However, addition of equimolar amounts of H-NS_{64} (fragment containing residues 1-64) had no effect on the HSQC spectra (Fig. 3), confirming that the D48N mutation prevents binding to the N-terminal domain of H-NS. Analogous results were obtained for the *E. coli* Hha paralogue YdgT. In YdgT, mutation of aspartic acid 44 (positionally equivalent to D48 in Hha) to asparagine abolished heterocomplex formation (Supplementary Fig. S1), confirming that this position plays an essential role in the interaction with the N-terminal domain of H-NS in different members of the Hha family of proteins.

Insert Figure 3 here

The requirement of aspartate at position 48 of Hha is very strict. The conservative mutation D48E, which preserves the negative charge but increases the length of the side chain by one methylene group, is sufficient to prevent complex formation as judged by

the absence of perturbations in the H-NS_{47} spectra upon addition of equimolar amounts of Hha D48E (Fig. 2B).

Attempts to rescue the effect of the mutations at position 48 of Hha by incorporating compensating mutations in position 12 of H-NS were unsuccessful. We tried to compensate the effect of the D48E mutation in Hha that increases the length of the side chain, by mutating R12 to lysine in H-NS, which also has positive charge but has a shorter side chain. H-NS R12K failed to bind to wild type Hha (Supplementary Fig. S2E) and we could not detect binding either to Hha D48E (Supplementary Fig. S2F). A possible alternative interaction between arginine and aspartic acid was assayed by swapping the essential residues in Hha and H-NS. However, the Hha D48R mutant did not bind wild type H-NS nor H-NS R12D (results not shown).

Next, we assayed the effect of the E25Q and D48N mutations in the formation of heterocomplexes with full length H-NS within the bacterial cell. His-tagged wild type, E25Q and D48N Hha variants were expressed in *E. coli* BL21(DE3) cells and the resulting bacterial soluble fraction was incubated with Ni^{2+}-NTA beads. The beads were washed with buffer and finally with imidazole to detach the Hha variants from the resin. The eluates after each washing step were analysed for the presence of Hha and H-NS by SDS-PAGE and Coomassie staining or western blot analysis, respectively (Fig. 4A). Quantification of the band intensities showed that, under the tested conditions, the E25Q mutation reduced 1.5 fold the binding of H-NS with respect to the wild type while the D48N mutation caused a 7.5 fold reduction that corresponds to an almost complete suppression of H-NS binding.

Insert Figure 4 here

3.2. Effect of Hha mutations E25Q and D48N on the regulation of the hemolysin operon

To investigate the *in vivo* effects of the E25Q and D48N mutations we compared the ability of wild type Hha and the E25Q and D48N Hha mutants to repress the hemolysin operon. The hemolysin operon is one of the best characterized systems under the control of the H-NS/Hha heterocomplex [5]. In addition, two mutants that were not defective in H-NS binding, D37N and D61N, were included as a control. To this end, pACYC184-based plasmids encoding the wild type *hha* gene (pACYC184-hha), the D37N (pACYC184-hha D37N), D61N (pACYC184-hha D61N), D48N (pACYC-hha D48N) and E25Q (pACYC-hha E25Q) variants were transformed into an *hha lacZ* null mutant *E. coli* (AAG1 Δ*hha*) that contained the plasmid pHly152 *hlyA*::*lacZ*. We were able to evaluate the functional phenotype of those *hha* variants by measuring their β-galactosidase activity, using as reporter the β-galactosidase gene fused to the *hlyA* gene from plasmid pHly152 *hlyA*::*lacZ* (Fig. 4B). As expected, in the absence of Hha, the *hly* operon was partially derepressed compared to the wild type strain (3.5 fold). Plasmids pACYC184-hha, pACYC184-hha D37N and pACYC184-hha D61N completely restored the wild type phenotype by complementing the *hha* defective phenotype of the strain AAG1 Δ*hha*. In contrast, plasmid pACYC184-hha D48N completely failed to complement the *hha* phenotype. Thus, Hha D48N is unable to repress the expression of the *hly* operon. On the other hand, plasmid pACYC184-hha E25Q partially complemented the *hha* phenotype, showing a derepression slightly less than 2 fold compared to the wild type strain.

These *in vivo* results validate the *in vitro* observations of the effect of the E25Q and D48N Hha mutations on H-NS binding and confirm the key role of D48 and, to a lower extend, E25 for the formation of a regulatory active Hha-H-NS complex.

4. Discussion

While the Hha binding site in H-NS could be precisely identified by a combination of NMR perturbation and mutagenesis studies, the complementary interface, i.e. the H-NS binding site in Hha appears far less defined in the NMR experiments carried out so far. A large number of residues are affected by a conformational change that takes place when Hha binds H-NS and is not possible to distinguish between direct and indirect effects. Prompted by the observation that Arg12 of H-NS is essential for Hha binding, in this study we have screened by site directed mutagenesis, the conserved acidic residues in Hha. Out of eleven acidic residues, ten are highly conserved in the Hha family of proteins and four of them (E25, D48, E53 and D61) are strictly conserved in all the known Hha-like proteins. All residues that are not strictly conserved could be mutated without affecting H-NS binding.

All single residue variants studied were stable and soluble, except E53N. This variant protein could not be isolated, probably because E53 plays a structural role. In the Hha three-dimensional structure, E53 lies close to a cluster of positively charged residues (R16, R50 and K58). Presumably, E53 contributes to local structural stability and removal of the negative charge at this position may hinder proper Hha folding.

A second strictly conserved acidic residue, D61, could be mutated to asparagine without any observable effect in the structure or the *in vivo* activity of Hha. The reasons for its conservation are not clear and are currently being investigated.

The data presented here demonstrates that E25 (helix 2) and D48 (helix 3) are crucial for H-NS binding. Removal of the negative charge at position 25 severely compromises the interaction with H-NS although it does not suppress it. However, aspartic acid at position 48 is strictly required and suppressing the negative charge while retaining the

size of the side chain (D48N) or retaining the charge but increasing the side chain length (D48E) dramatically decreased complex formation. The essential role of this position for H-NS binding is likely to be general for the Hha family of proteins. In YdgT, one of the more distant Hha homologues sharing only moderate amino acid sequence similarity with Hha (38% identity), mutation of the equivalent aspartate (D44) to asparagine also prevents binding to the H-NS N-terminal domain.

Arginine 12 in H-NS is also an essential residue and a conservative mutations to lysine abolished binding to Hha. The strict structural requirements of one residue in each of the interacting proteins indicate a very specific interaction for each of them. Although the interaction between the two essential residues of opposite charge in Hha and H-NS seems a reasonable hypothesis, attempts to rescue the mutations by compensating mutations on the other protein were unsuccessful and at this point we cannot demonstrate a direct interaction between D48 of Hha and R12 of H-NS.

In the Hha NMR structure, D48 is partially buried (average ~15% solvent accessible) and helix 4 is located at a position that may block H-NS binding (Fig. 1B). Consistent with our previous observation that the interaction with H-NS involves a conformational change of Hha, a structural rearrangement to expose the interaction site may be needed for H-NS binding. Residues in helix 4 and the preceding loop were identified by NMR among the most affected by the interaction with H-NS [22]. We also note that although Hha, YmoA and YdgT have a common core formed by helices 1-3, noticeable differences are observed in the C-terminal part of the three reported structures. The orientation of helix 4 in YmoA [19] and Hha [18] is markedly different and the corresponding sequence of YdgT was described as unstructured [20]. These differences may reflect the dynamic flexibility of this C-terminal region.

Insert Figure 5 here

Despite being a moderately basic protein, E25 and D48 are located on an acidic surface of Hha (Fig. 5). Due in part to the abundance of basic residues in helices 1 and 2 (K8, R14, R16, R17, R26, K30 and K32), the opposite side of the molecule is highly positive. Remarkably, the N-terminal domain of H-NS also displays a highly asymmetrical charge distribution. Although this domain is mainly acidic, helices 1 and 2 form a positively charged surface that has been shown to be essential in gene regulation. Single mutation of the highly conserved residues R12 or R15 causes the loss of the repression activity of H-NS *in vivo* [28]. A role in DNA binding has been proposed for the solvent exposed R12 side chain [14, 16]. In the H-NS/Hha heterocomplex, the locus of R12 is probably buried in the intermolecular interface but it may be functionally replaced by Hha basic residues located on the opposite side of the H-NS binding site. A mutation in the Hha region opposite to the H-NS binding site (C18I) preserves H-NS binding but the complex is inactive [25], supporting the functional importance of this Hha region. Geometrical and net charge differences between H-NS homooligomers and H-NS/Hha heterooligomers may account for the selectivity towards HT genes induced by Hha co-regulation.

Acknowledgments

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Figure Legends

Fig. 1. (A) Sequence alignment of Hha homologues. The following proteins are aligned: EC-Hha (*E. coli* Hha), SF-Hha (*Shigella flexneri* Hha), ST-Hha (*Salmonella enterica* serovar Typhimurium Hha), YmoA (*Yersinia spp.* YmoA), PL-Hha (*Photorhabdus luminescens* Hha), EW-Hha (*Erwinia carotovora* Hha), WG-Hha (*Wigglesworthia glossinidia* Hha), SG-Hha (*Sodalis glossinidius* Hha), R100 (plasmid R100 RmoA), p0157 (plasmid p0157 Hha), pR27 (plasmid R27 Hha), pSG1 (plasmid pSG1 Hha), pCTX (plasmid pCTX Hha), EC-YdgT (*E. coli* YdgT), ST-YdgT (*S. enterica* serovar Typhimurium YdgT). Conserved residues have a grey background. Negatively charged residues are highlighted in red and E25 and D48 are indicated by a star. The α-helical segments of Hha are shown above the sequence.
(B and C) Two views of the Hha structure. Mutated residues are indicated.

Fig. 2. H-NS_{47} cross-peaks intensity ratios in the presence and in the absence of Hha variants. The intensities were normalized in each experiment with respect to residue 47. (A) 0.5 equivalents of wild type, D10N, D22N, E29Q, E34Q, D37N, E39Q or D61N Hha. (B) E25Q (0.5 and 1 equivalents), D48N (1 equivalent), D48E (1 equivalent) and wild type (0.5 equivalents) Hha. The α-helical segments of H-NS_{47} are indicated by a black bar.

Fig. 3. ^{1}H-^{15}N-HSQC spectra of ^{15}N-labeled wild type Hha (A) D48N Hha (B and C) in the absence (B) and in the presence of 0.5 equivalents of H-NS_{64} (C).

Fig. 4. (A) Pull-down experiments. His-tagged Hha variants were purified from whole cell extracts using Ni^{2+}-NTA beads. The upper panels show SDS-PAGE and Coomassie staining of the Hha variants in the first two fractions eluted with 200 mM imidazole. The bottom panels show western blots of the co-purified H-NS. The control lanes correspond to BL21(DE3) with no recombinant plasmid. Decrease of H-NS co-eluted with E25Q and D48N variants with respect to wild type Hha is indicated. Values were normalized with respect to the amount of Hha variant in each sample after substracting

the background from the control BL21 (DE3) sample. (B) β-galactosidase activity from a *hly::lacZ* transcriptional fusion in the wild type AAG1 pHly152 *hlyA::lacZ* (pACYC184) strain (black), the *hha* mutant strain AAG1 Δ*hha* pHly152 *hlyA::lacZ* with plasmid pACYC184 (white) and with plasmids pACYC184-hha WT, pACYC184-hha D37N, pACYC184-hha D461N, pACYC184-hha E25Q and pACYC184-hha D48N expressing different Hha variants (grey) at OD_{600} 0.4. Experiments were performed in triplicate.

Fig. 5. Surface electrostatic representation of Hha (A and B) and H-NS_{47} dimers (C and D). The Hha representations in panel (A) and (B) are presented with the same orientation as in Fig. 1B and 1C respectively. Both molecules display two faces with opposite charges. The positively charged face of H-NS_{47} (C) which is essential for H-NS activity in the absence of Hha, is buried by the interaction with Hha, which takes place by its negative face (B). The positively charged face of Hha (A) may replace the lost interactions of the positively charged face of H-NS, although the different location of the positive regions in free H-NS and in the Hha complex may explain the selectivity to HT genes of H-NS in the presence of the co-repressor Hha.

Figure 1

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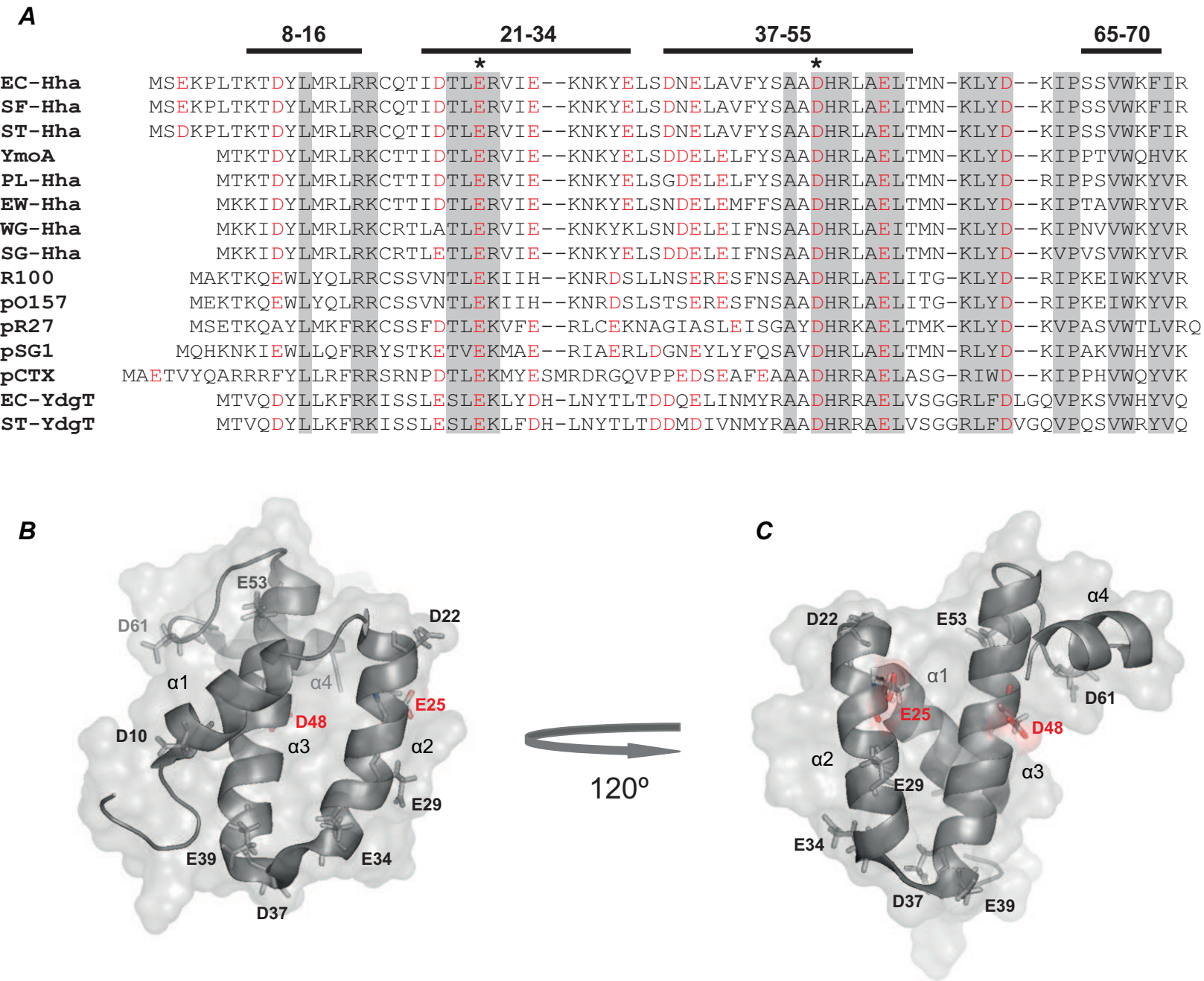

Figure 1

Figure 2

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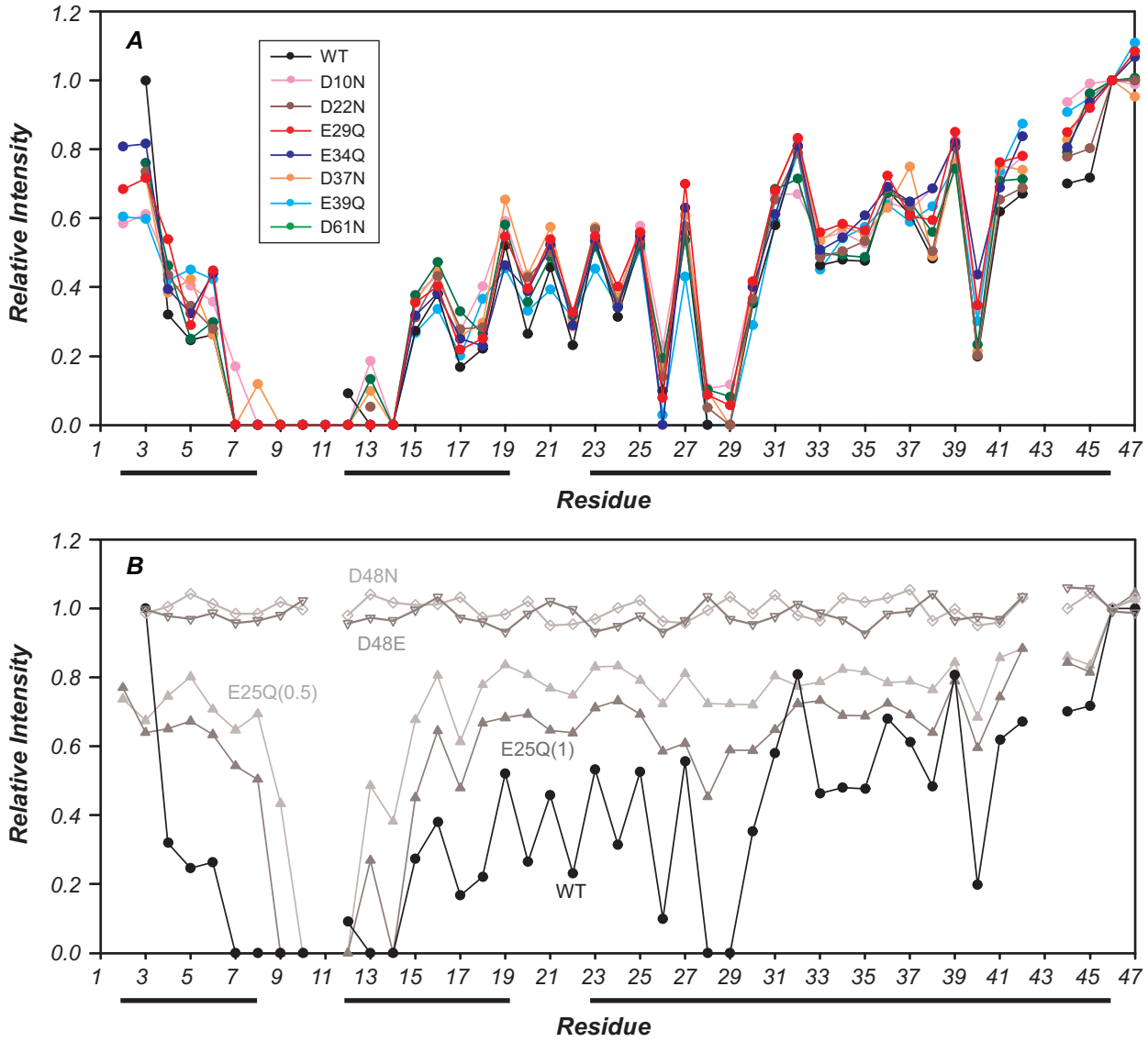

Figure 2

Figure 3
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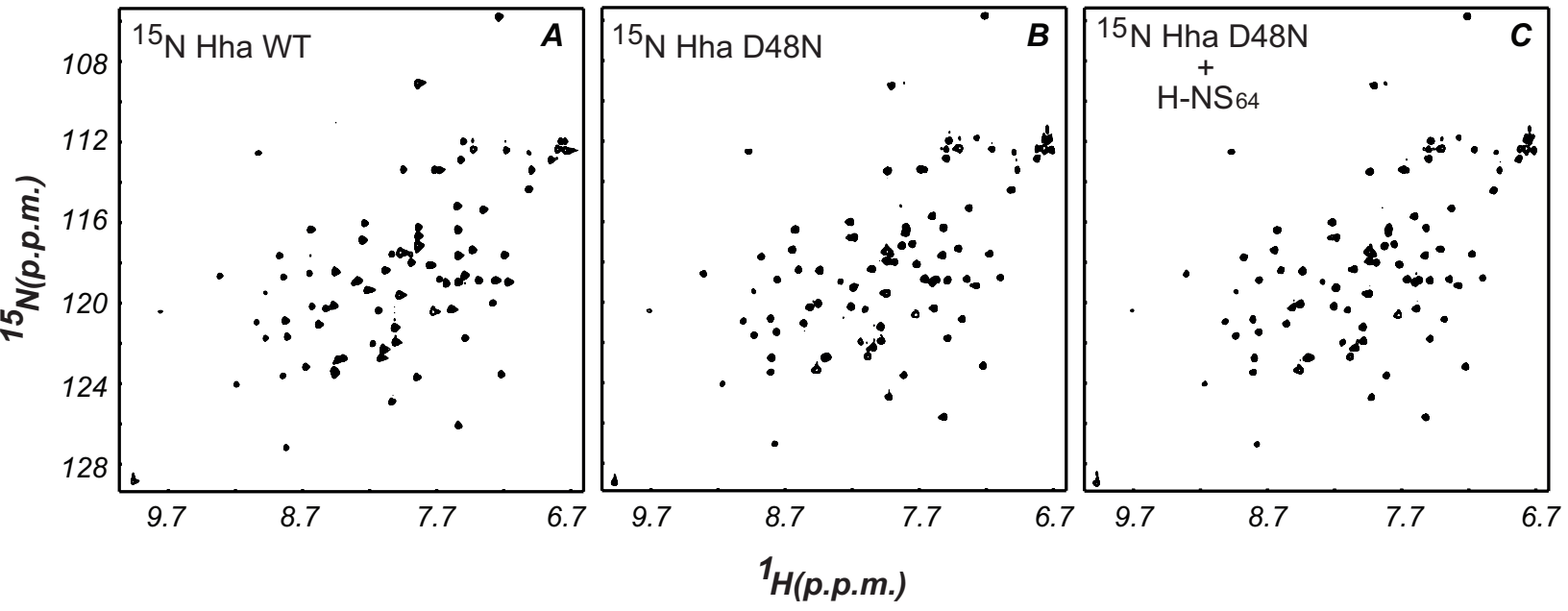

Figure 3

Figure 4
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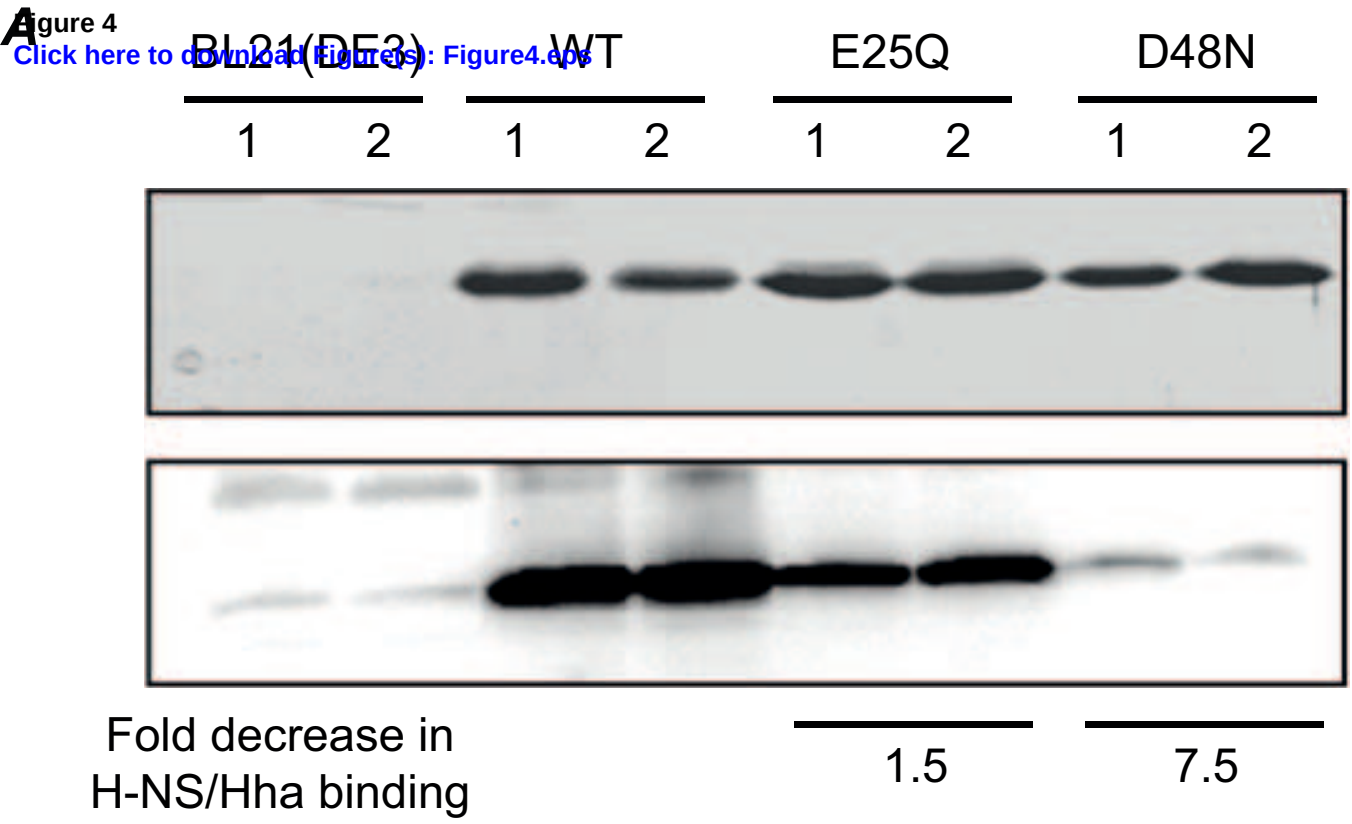

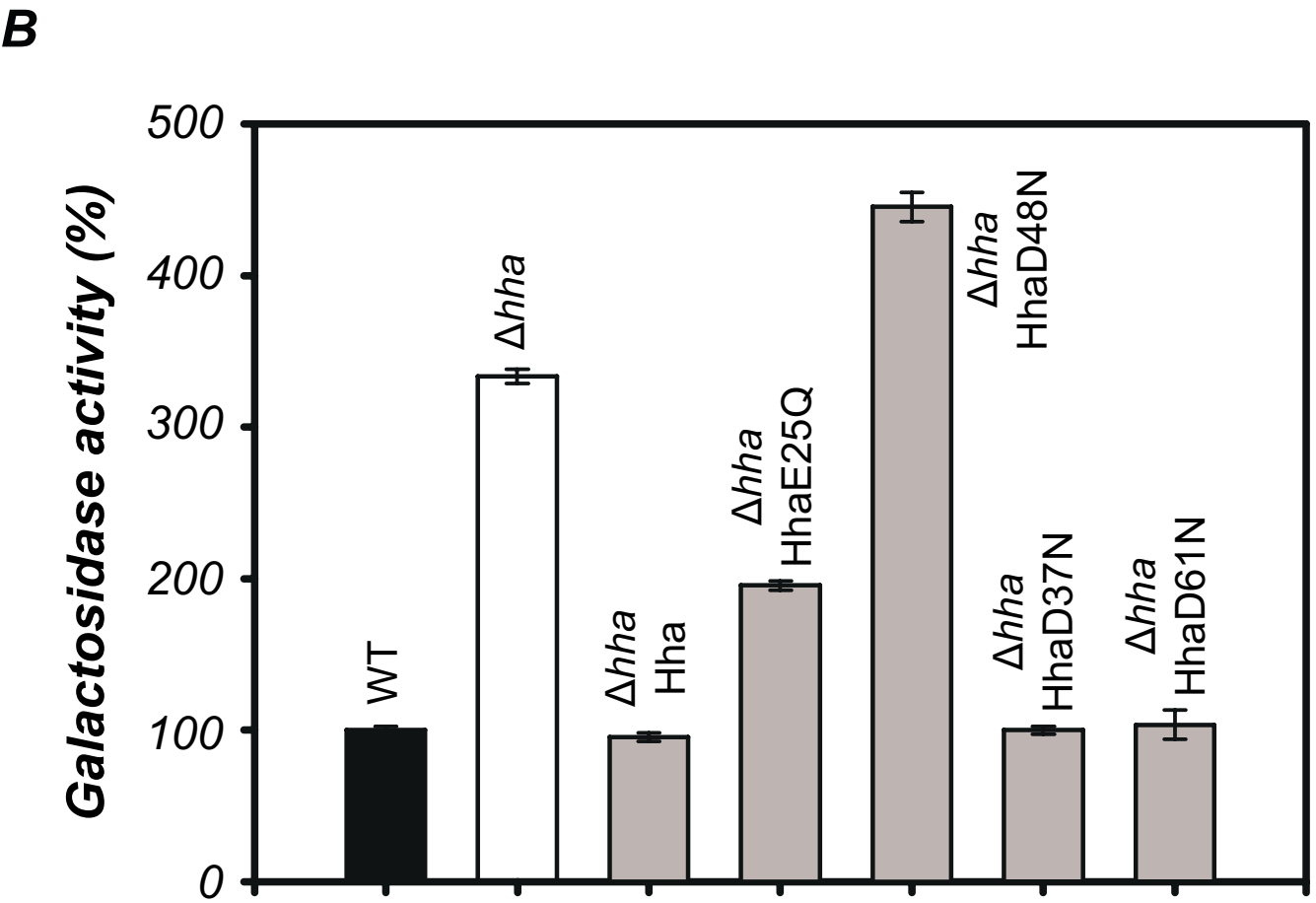

Figure 4

Figure 5

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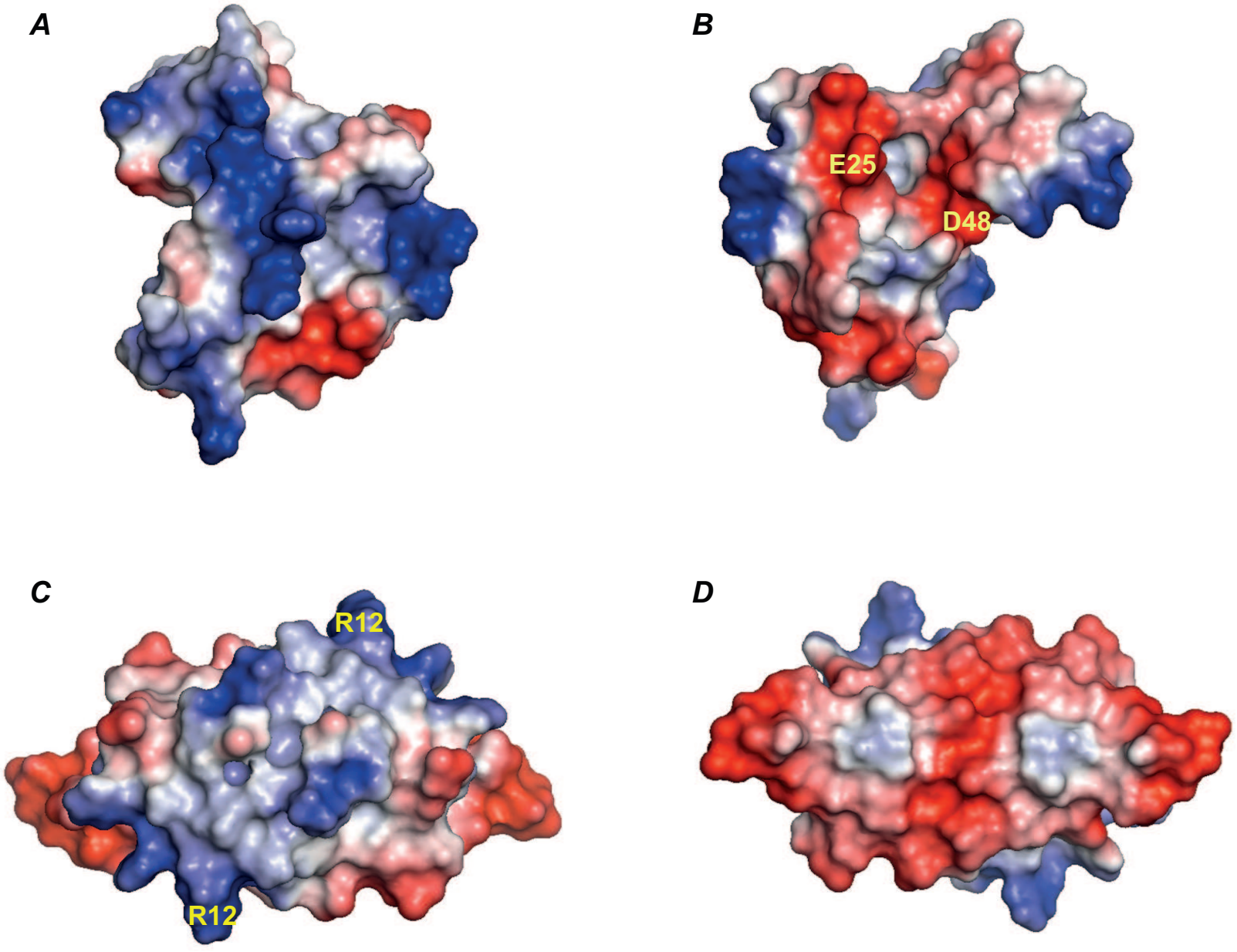

Figure 5

Supplementary Data (for on-line publication only)

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