

Stability and Conformation of a Chemoreceptor HAMP Domain Chimera Correlates with Signaling Properties

Nattakan Sukomon,¹ Joanne Widom,¹ Peter P. Borbat,^{1,2} Jack H. Freed,^{1,2} and Brian R. Crane^{1,*}

¹Department of Chemistry and Chemical Biology and ²National Biomedical Center for Advanced ESR Technologies, Cornell University, Ithaca, New York

ABSTRACT HAMP domains are dimeric, four-helix bundles that transduce conformational signals in bacterial receptors. Genetic studies of the *Escherichia coli* serine receptor (Tsr) provide an opportunity to understand HAMP conformational behavior in terms of functional output. To increase its stability, the Tsr HAMP domain was spliced into a poly-HAMP unit from the *Pseudomonas aeruginosa* Aer2 receptor. Within the chimera, the Tsr HAMP undergoes a thermal melting transition at a temperature much lower than that of the Aer2 HAMP domains. Pulse-dipolar electron spin resonance spectroscopy and site-specific spin-labeling confirm that the Tsr HAMP maintains a four-helix bundle. Pulse-dipolar electron spin resonance spectroscopy was also used to study three well-characterized HAMP mutational phenotypes: those that cause flagella rotation that is counterclockwise (CCW) A and kinase-off; CCW B and also kinase-off; and, clockwise (CW) and kinase-on. Conformational properties of the three HAMP variants support a biphasic model of dynamic bundle stability, but also indicate distinct conformational changes within the helix bundle. Functional kinase-on (CW) and kinase-off (CCW A) states differ by concerted changes in the positions of spin-label sites at the base of the bundle. Opposite shifts in the subunit separation distances of neighboring residues at the C-termini of the $\alpha 1$ and $\alpha 2$ helices are consistent with a helix scissors motion or a gearbox rotational model of HAMP activation. In the drastic kinase-off lesion of CCW B, the $\alpha 1$ helices unfold and the $\alpha 2$ helices form a tight two-helix coiled-coil. The substitution of a critical residue in the Tsr N-terminal linker or control cable reduces conformational heterogeneity at the N-terminus of $\alpha 1$ but does not affect structure at the C-terminus of $\alpha 2$. Overall, the data suggest that transitions from on- to off-states involve decreased motional amplitudes of the Tsr HAMP coupled with helix rotations and movements toward a two-helix packing mode.

INTRODUCTION

Bacteria employ transmembrane receptors to sense and respond to their changing environment (1–3). Essential components of these receptors are so-called HAMP domains (comprising histidine kinase, adenylate cyclase, and methyl-accepting chemotaxis proteins (MCPs) and some phosphatases) (4–7). Found in more than 26,000 receptors (8), they often lie proximal to the cytoplasmic leaflet of the membrane and act to couple extracellular signals to intracellular responses (9).

The HAMP domain is a small, homodimeric protein with ~50 residues in each subunit (9,10). HAMP structures are parallel four-helix bundles with each subunit supplying two α -helices (AS1 and AS2 for amphipathic sequence 1

and 2) that each contain typical heptad sequence repeats reflecting internal hydrophobic packing and the helix periodicity (residue positions, a - g , where a , d , and to a lesser extent e , and g are held by hydrophobic residues) (10,11). A nonhelical linker (CTR) connects the $\alpha 1$ and $\alpha 2$ helices and contains two conserved hydrophobic residues (HR1 and HR2) that are important for stability and function (9,12,13). Subsequent biochemical and structural studies of HAMP domains in both MCPs and histidine kinases show high conservation of these general properties (14–16). However, among the known structures, HAMP domains display a range of conformations that differ in terms of helix rotation, translation, and crossing angles (10,12,17–21).

HAMP domains have been extensively studied in the context of MCPs, which regulate chemotaxis in eubacteria and archaea (2,22). MCPs are modular receptors comprising a ligand-sensing domain, a transmembrane domain, HAMP domain(s), and a kinase control module (KCM) (23,24). In general, the periplasmic ligand-binding domain monitors

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*Correspondence: bc69@cornell.edu

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chemoeffector levels, and the KCM interacts with the histidine kinase CheA and the adaptor protein CheW. CheA transfers phosphoryl groups from ATP to the response regulator CheY depending on the ligand occupancy of the receptor. Phosphorylated CheY (CheY-P) interacts directly with the flagellar motor to change the rotational bias from counterclockwise (CCW) to clockwise (CW) (23–25). In *Escherichia coli*, CheA is activated when attractants dissociate or repellants bind (kinase-on). These effects cause higher levels of CheY-P, CW flagella rotation, and cell tumbling. Attractant binding, or repellant dissociation deactivates CheA (kinase-off) and lowers the level of CheY-P, which causes CCW flagella rotation and straight swimming (23–25). In MCPs, the effects of ligand binding are countered by covalent modification through the activities of the CheR methyl transferase and CheB methyl-esterase, which together provide a feedback system that adapts receptor output to current conditions. CheR methylates conserved Glu residues to shift the receptors toward kinase-on states. Methylation can be generally mimicked by Glu to Gln substitutions (24,26,27). So-called QQQQQ states favor kinase-on whereas EEEEE states favor kinase-off. Partially converted states (QEQUE) produce intermediate activity phenotypes. Functional studies of *E. coli* serine receptor (Tsr) have shown that the HAMP domain is critical for switching CheA activity state upon receptor stimulation (9,24,27–29).

Several models have been proposed to elucidate how conformational signals propagate through HAMP domains. These models range from those that emphasize more static two-state behavior to those that emphasize the dynamics (stabilities) of on- and off-conformations (9,21). The two-state models focus on discrete kinase-on and kinase-off states, which invoke the opposite physiological outputs. A well-known example is the gearbox model, which arose from the discovery of an unusual *x-da* hydrophobic packing arrangement of the Af1503 helical heptad repeat positions (*a-g*) and the subsequent study of a key residue A291 in $\alpha 1$ of Af1503 (10,17,30,31). Increase in the volume of this core residue promotes HAMP packing to switch from the complementary *x-da* packing mode to the more conventional, knobs-into-hole (*a-d*) arrangement (17). The packing change is proposed to induce a gearbox-type counterrotation of the $\alpha 1$ and $\alpha 2$ helices (10,17,30,31). Another proposal for a two-state conformational switching involves scissor-type motions of the $\alpha 1$ and $\alpha 2$ helices that explain changes in cross linking of cysteines engineered into the *E. coli* aspartate receptor Tar (18). Although the initial gearbox model was phrased in terms of helix rotations (10), it subsequently became apparent that changes in helix rotation state would be coupled to compensatory changes in helix winding periodicities, crossing angles, and overall bundle shape (17,30,31). Thus, both rotational and scissor motions of HAMP helices may well manifest in conformational switching.

The functional characterization of a large number of Tsr mutant receptors has led to the biphasic dynamic bundle

model of HAMP stability (27–29,32). This model proposes that HAMP domains operate in regimes of conformational dynamics. The signaling outputs correspond to the ensembles of HAMP structures with similar stabilities rather than discrete conformations. HAMP domains at both dynamic extremes (highly unstable and highly stable) promote the kinase-off state. Of these, the more stable state [CCW(A)] is most likely the functional kinase-off configuration, as the highly unstable form [CCW(B)] results from lesions that distort the domain outside of its physiological range. HAMP bundles with stabilities intermediate to these two extremes cause the kinase-on (CW) output. Over the physiological regime then, the kinase-on states have HAMP domains that are more dynamic than those of the kinase-off states (27,28,32,33).

Structural and biochemical studies of the concatenated HAMP domains from *Pseudomonas aeruginosa* Aer2 support an intermediate view (12,33). The Aer2 poly-HAMP structure contains three N-terminal HAMP domains, of which two (HAMP1 and HAMP3) have similar conformations, whereas a second (HAMP2), is quite different (12). The HAMP1/3 and HAMP2 structures were distinguished by complex differences in helical register, rotation, and tilting, which include a rotation at the end of $\alpha 2$ (12,33). In vitro and in vivo experiments showed that when the HAMP1 and HAMP2 conformers are fused to the Tar KCMs, they elicit opposite effects on kinase activities and cell swimming responses (34). The two conformers and their functional consequences are convertible by mutation of conserved hydrophobic residue HR2, which plays an important role in stabilizing the HAMP2 conformer. Nonetheless, pulse-ESR experiments of spin-labeled proteins also indicate that the HAMP1 and HAMP2 conformers have very different dynamic properties, with the HAMP1 kinase-on form more conformationally heterogeneous than the HAMP2 kinase-off form, as would be predicted by the dynamic bundle model (12,33).

HAMP domains from other classes of bacterial transmembrane receptors, including sensor histidine kinases and sensory rhodopsin transducers, also suggest a range of activation mechanisms (9,16,35). For example, cysteine cross-linking analyses on variants of PhoQ, a sensor histidine kinase in *Salmonella enterica*, indicate a change from *a-d* to *x-da* helix packing in the HAMP domain along with a tilt of the $\alpha 1$ helices (16,36). Helix tilt angles do vary among known HAMP structures with tilts of $\alpha 1$ compensated by opposite tilts of $\alpha 2$ (21). In contrast, studies of the sensory rhodopsin transducer HtrII indicate that signal propagation is associated with helix displacements along the long axis (piston motions) and again there are compensating shifts between $\alpha 1$ and $\alpha 2$ (37,38). Computational studies of isolated HAMP domains emphasize such piston motions over rotations, but the simulated HAMP domains are not coupled to input and output modules that may otherwise dampen such motions (39,40). In line with the

dynamic bundle model, several other studies have also suggested changes in HAMP dynamics, compactness, and folding upon activation, but the signs of the changes do not always correlate among different systems (9,37,41,42). While signal transduction by different HAMP domains need not involve the same conformational transitions, the conservation of residues involved in interactions among $\alpha 1$ and $\alpha 2$ at the dimer interface and involving the connector, suggests commonality of mechanism.

Despite the rich genetic and functional data available for the Tsr HAMP, there is limited structural and biophysical data on this system due to the instability of the isolated domain, and the difficulty in producing the functional intact membrane protein for biophysical studies. The effects of the residue substitutions on domain stability have mainly been inferred from the likely consequences of similar substitutions on known HAMP structures (13,28,29). To provide access to structural and biophysical studies of Tsr HAMP, we created a chimeric protein containing Tsr HAMP spliced into the poly-HAMP domains from *P. aeruginosa* Aer2. This work reports its initial characterization. Furthermore, we demonstrate that residue substitutions in the Tsr HAMP domain indeed alter its stability, as suggested by the bistability model. However, changes in stability are also accompanied by conformational changes consistent with helical rotations. Additionally, we show that the substitution of a critical residue in the so-called Tsr control cable that joins HAMP to the transmembrane helices produces complex effects, but in this system does not appear to alter structure near the C-terminal output of $\alpha 2$. Although we investigate the Tsr HAMP outside of its normal environment, our findings do demonstrate how single residue sub-

stitutions can affect HAMP structure, dynamics, and stability, and thus provide a general rationale for the types of changes expected by HAMP mutational alternations. Moreover, the properties of the variants correlate reasonably well with prevailing models.

MATERIALS AND METHODS

Cloning and mutation

A chimera was designed to splice the Tsr HAMP domain between the H1 and H2 HAMP domains of Aer2H1–3. Construction of the chimera maintains helical phase within all four HAMP domains by replacing the Aer H2 sequence with the corresponding residues of Tsr HAMP and then appending the H1–H2 linker to the Tsr HAMP C-terminus so that the now downstream H2 has the same coupling to the upstream HAMP (in this case, Tsr) as found in Aer2H1–3 (Fig. 1). Specifically, the coding region for the Tsr HAMP domain (residues 212–264) was PCR-amplified from *E. coli* genomic DNA. The coding region for the Aer2 poly-HAMP (residues 1–172) was previously cloned in pET28a (12). The chimeric Tsr-Aer2H1–3 was cloned into pET28a by the following steps. First, the Tsr HAMP coding region was fused upstream of the Aer2 linker-Aer2H2–3 coding region (residues 57–172) using PCR overlap extension. Then, the Aer2H1–Aer2 linker coding region (residues 1–62) was introduced upstream of the former construct by also using overlap extension, resulting in a chimeric Tsr-Aer2H1–3 construct that contains the Tsr HAMP domain (residue 216–264) embedded between Aer2H1 and Aer2H2–3 with the Aer2 H1–H2 linker sequence repeated at both ends of the Tsr HAMP. During this step, the primers for overlap extension were designed to generate two sets of substitutions. In the first, silent mutations were introduced at the E61 and L62 positions in the first Aer2H1–2 linker to create unique restriction sites. In the second, the Aer2 residue 1–4 (Met Gly Leu Phe), which is located before the N-terminus of the Aer2 H1, was modified to His Met Ala Ser to aid expression and stability. Point mutations of the recombinant HAMP domains were introduced by using either the QuikChange PCR protocol (Agilent Technologies, Santa Clara, CA) or overlap extension methodology (43).

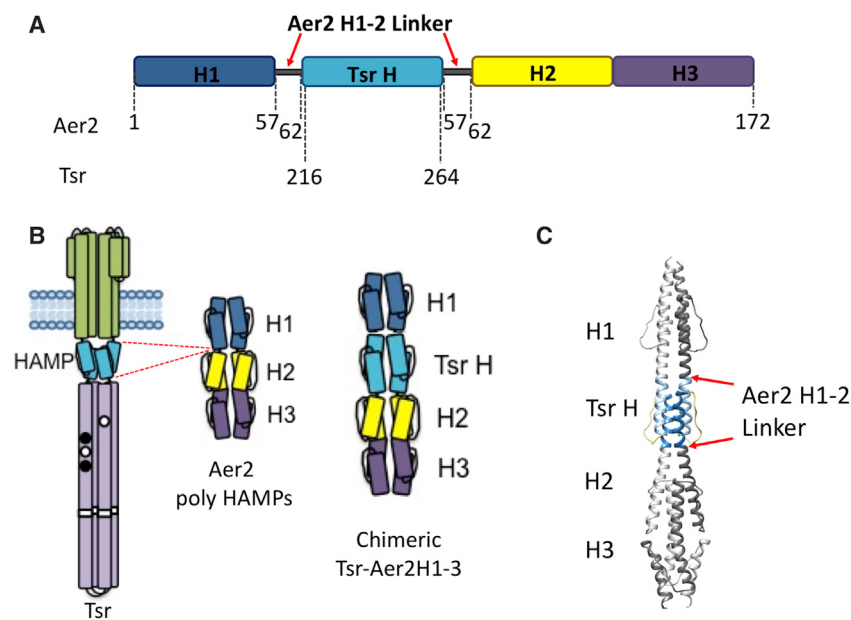


FIGURE 1 Schematic representations of the recombinant Tsr-Aer2 HAMP domains. (A) Domain organization of the chimeric Tsr-Aer2H1–3 protein that is composed of the Tsr HAMP (Tsr H) and the PaAer2 poly-HAMP domains (H1, H2, and H3). Residue numbers for each domain are: Aer2H1, 8–56; Aer2H2–H3, 62–172; Aer2H1–2 linker, 57–62; Tsr HAMP, 216–264. The chimera maintains the helical registers of all four HAMP domains, based on the alignment of Tsr HAMP to Aer2 H2. (B) Schematic diagram of the domain architecture of the recombinant Tsr-Aer2 HAMP domains. The Tsr HAMP domain is fused between the PaAer2H1–2 domains with the Aer2H1–2 linkers at both ends. The α -helices are depicted as cylinders. (C) Ribbon diagram showing the α -helical structure of the chimeric Tsr-Aer2H1–3 protein.

Expression and purification of proteins

All of the proteins were expressed in *E. coli* BL21(DE3) under 0.4 mM isopropyl β -D-1-thiogalactopyranoside induction at 4°C for 16 h. The proteins were purified by Ni-NTA affinity chromatography under the manufacturers' protocols. After overnight digestion with thrombin (0.7 μ g/mL), the tag-free proteins were subjected to a Superdex 75 26–60 size exclusion column (GE Healthcare Life Sciences, Marlborough, MA) and eluted in 10 mM Tris (pH 8.0), 150 mM NaCl, and 10% (v/v) glycerol.

Circular dichroism spectroscopy

Circular dichroism (CD) experiments on the recombinant HAMP domains were carried out with a model No. 202-01 spectropolarimeter (Aviv Biomedical, Lakewood, NJ). The proteins were prepared at a concentration of 10 μ M (250 mg/mL) in 10 mM sodium phosphate buffer (pH 7.5), 150 mM NaCl, and 10% (v/v) glycerol. Wavelength scan experiments were performed at 4°C. For the temperature scan experiments, the protein samples were heated at the rate of 1°C per min, and allowed to reach equilibrium for 1 min. Ellipticity was then measured as an average over 1 min.

Site-directed spin labeling

Cysteine was substituted for selected residues in the recombinant HAMP domain with QuikChange (Agilent Genomics, Santa Clara, CA) mutagenesis or overlap extension. The purified proteins were reacted with cysteine-specific nitroxide S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonylthioate spin-label (MTSL; Toronto Research Chemicals, North York, Ontario, Canada) on a Ni-NTA column for 4 h at room temperature, and subsequently at 4°C for 8 h. The proteins were eluted with 20 mM Tris (pH 8.0), 250 mM imidazole (pH 8.0), 500 mM NaCl, and 10% (v/v) glycerol. The samples were subjected to size-exclusion chromatography (Superdex 75 26–60) for further purification and removal of unreacted MTSL.

Pulse-dipolar electron spin resonance spectroscopy measurements

The spin-labeled samples (100 μ M) were prepared in 10 mM Tris (pH 8.0), 150 mM NaCl, and 35% (v/v) glycerol. The distance distributions between the two spins were detected with double electron-electron resonance (DEER) on a homebuilt two-dimensional Fourier-transform-ESR instrument (44–46). The dipolar evolution was measured at 17.35 GHz with four-pulse DEER with a 16-ns π -pump pulse centered on the nitroxide spectrum in a 16/32/32-ns pulse sequence. The pulse-dipolar electron spin resonance spectroscopy (PDS) signals were analyzed after correcting the baseline of the time domain data with a log-linear polynomial function. Subsequently, the DEER signals were converted to distance distributions with Tikhonov regularization, and further refined by maximum entropy refinement (44,47,48).

RESULTS

Expression of a Tsr HAMP within the Aer2 poly-HAMP

The Tsr HAMP domain was expressed with the *P. aeruginosa* Aer2 poly-HAMP 1-2-3 domains in the context of a chimeric protein (Aer2H1–3; Fig. 1). The N-terminal fusion point was chosen by aligning the Tsr HAMP sequence with that of Aer2 HAMP2, whereas the C-terminal fusion point and linker was

determined by aligning Tsr HAMP sequence with that of Aer2 HAMP1. Thus, the Tsr sequence A216–M264 was inserted after residue 62 of Aer2 and the H1–H2 linker (residues 57–62 of Aer2) was then repeated at the C-terminus of Tsr to provide the same spacing for the connection to the downstream Aer2 HAMP2 domain as found in Aer2H1–3 (Fig. 1). The CD spectrum of the chimeric poly-HAMP protein (Tsr-Aer2H1–3) showed typical α -helical structure, with roughly 3/4 the helical signal of the parent Aer2H1–3 (Fig. 2 A). Aer2H1–3 thermally unfolded with a single transition of $T_m = 53^\circ\text{C}$ (Fig. 2 B; (33)). In contrast, Tsr-Aer2H1–3 unfolds in two stages with a lower transition temperature of 26°C in addition to a higher temperature of 57°C. The lower temperature transition accounts for roughly 1/4 of the helical content loss (Fig. 2 B) and we assign it to the Tsr HAMP domain, which, as expected, would be very unstable if expressed separately. Fusion into Aer2H1–3 appears

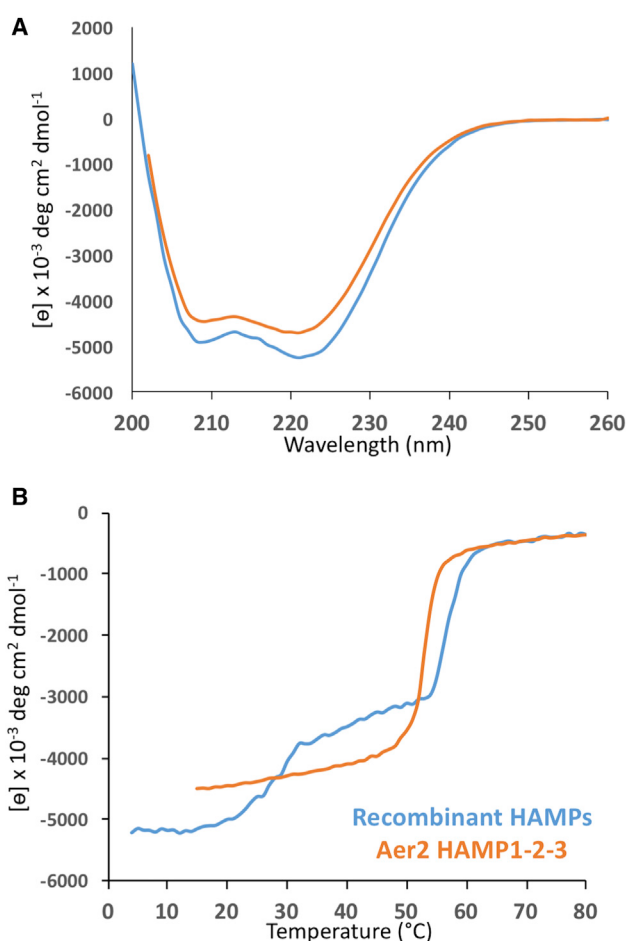


FIGURE 2 Helical content and thermostability of the chimeric Tsr-Aer2H1–3 protein compared to that of Aer2H1–3. (A) The CD wavelength scans show the overall α -helical structure of Tsr-Aer2H1–3 and Aer2H1–3 alone. (B) The CD melting curves show the two-step unfolding character of the chimeric protein. An additional transition in the chimera at 26°C is not present in the Aer2H1–3 domain, which has a melting temperature of 53°C.

to protect the domain from aggregation and allows it to assume helical structure at low temperature. We note that the melting curves of the HAMP chimeras and their variants (discussed below) are at best only partially reversible. This behavior may relate to the dissociation of subunits that accompanies unfolding. Thus, although thermodynamic quantities cannot be derived from the melting curves, the curves report on the relative helical stabilities of different variants. Comparative measurements were taken with the same concentrations of proteins to limit subunit dissociation effects on the T_m values. Fortunately, as shown below, substantial changes in the melting curve behavior of the HAMP variants support general inferences about the consequences of the amino-acid replacements.

Site-specific spin-labeling and PDS were used to verify the dimeric coiled-coil structure of the Tsr domain within the context of Tsr-Aer2H1–3. Nine engineered cysteine res-

idues were introduced along the $\alpha 1$ and $\alpha 2$ helices of the Tsr HAMP domain and subsequently labeled with the nitroxide spin-probe MTSL. Owing to HAMP dimerization, one spin-probe site will produce a dipolar interaction with its symmetric position on the adjacent subunit. The chosen residues were mainly located at the exposed *b*, *c*, and *f* positions of the helical heptad repeats (Fig. 3). The distance distributions between spin pairs generated by the HAMP dimers were measured by DEER (44,49,50). Notably, the derived distance distributions [$P(r)$] report on conformational heterogeneity in the sample and do not measure dynamics directly (i.e., there are no timescales indicated by these data). However, we assume that $P(r)$ distributions with larger breadths reflect dynamical exchange among states that differ more greatly in conformation and hence undergo larger amplitude motions. In previous studies that measure both DEER distance distributions and dynamical parameters

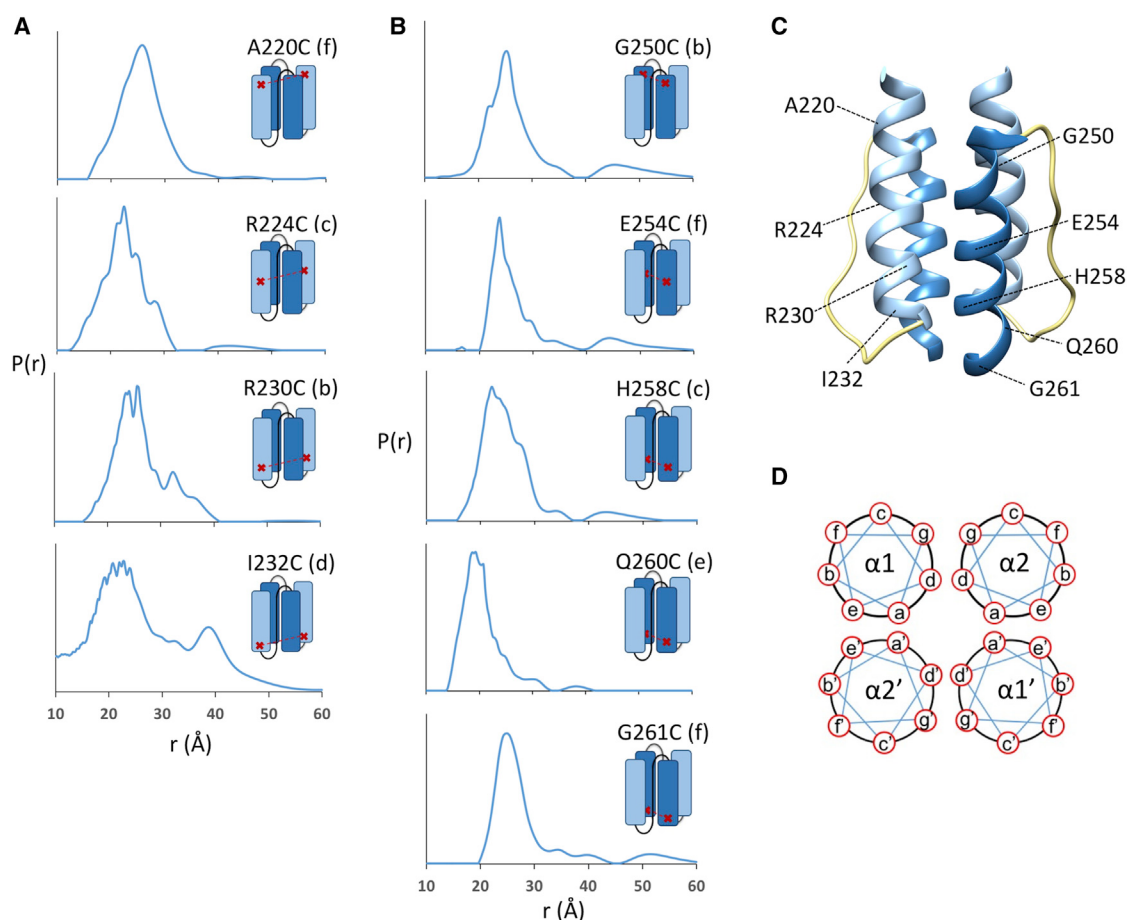


FIGURE 3 Structural properties of the Tsr HAMP domain as revealed by PDS. (A) Intersubunit spin-probe distances of nitroxide-labeled proteins measured by PDS. Chosen sites are marked on the $\alpha 1$ (light blue) and $\alpha 2$ (dark blue) helices of the Tsr HAMP domain. Pairwise distance distributions [$P(r)$] were generated for each pair of spins on adjacent subunits. Based on sequence alignments (12,28), the heptad positions of the spin-label sites are defined as follows— $\alpha 1$: 220, *f*; 224, *c*; 230, *b*; 232, *d*; and $\alpha 2$: 250, *b*; 254, *f*; 258, *c*; 260, *e*; 261, *f*. (B) Homology model of the Tsr HAMP domain. The atomic coordinates for the model were obtained by threading the Tsr HAMP sequence onto the Af1503 A291F HAMP using SWISS-MODEL. (C) Ribbon diagram. The Tsr HAMP domain is depicted as a dimeric four-helix bundle with the $\alpha 1$ helices in light blue, the $\alpha 2$ helices in dark blue, and the CTR in gold. The spin-label positions are shown on only one subunit; $\alpha 1$ on the left and $\alpha 2$ on the right. (D) Helical wheel representation of the heptad repeat positions of a parallel four-helix bundle with *a-d*, knobs-into-holes, packing.

we have found that broader distributions do correlate with greater dynamic rates (34).

DEER modulation depths were monitored to evaluate oligomeric states and aggregation properties of the engineered HAMP domains. The modulation depth of the time-domain DEER spectrum (Δ) is defined as $\Delta(p) = (\text{DEER}(t=0) - \text{DEER}(t=\infty))/(\text{DEER}(t=0))$, where p is the fraction of spins flipped by the pump π -pulse (51–54); Δ depends on the number of interacting spins, N , as $\Delta(p, N) = 1 - (1 - p)^{N-1}$ (51–53,55). For a dimer, $\Delta(p, 2) = p$ and for an oligomer, $\Delta > p$. Under our experimental conditions (16 ns π -pulse pumped at the center of the nitroxide spectrum) Δ is ~ 0.35 for a dimer, and ~ 0.73 for a tetramer with all spins interacting. Most of the samples examined in this study have Δ in the range of 0.35, with some values slightly larger, perhaps owing to modest oligomerization (Figs. S1 and S2 in the Supporting Material). Exceptions are the wild-type (WT) protein spin-labeled at the 220 and 230 positions (Figs. S3 and S4). For 220, the protein eluted on size-exclusion chromatography with a bimodal peak (Fig. S3). Protein from the larger, fast eluting fractions gave $\alpha \sim 0.6$, whereas the lighter slow-eluting fractions gave values consistent with a dimer $\Delta \sim 0.4$ (Fig. S3). Diluting samples from the larger fraction also lowered the modulation depth, as would be expected on dissociation of a weakly associating tetramer. The resulting distance distributions [$P(r)$] reflect the oligomerization in the larger species, and thus, only the [$P(r)$] from the dimeric fraction was used in further analysis. In the case of position 230, the size-exclusion chromatography profile was more uniform, but the modulation depth remained high (~ 0.7) for all fractions across the elution peak, except for the remote shoulder, where it began to diminish only slightly (Fig. S4). Thus, WT Tsr-Aer2H1–3 labeled at the 230 site forms a tight, likely tetrameric state, whose overall structure is uncertain, but may contain the typical HAMP dimer (see below).

The derived distance distributions from all spin-labeled residues showed similar separations of ~ 21 – 26 Å (Figs. 3, A and B and S1; Table S1), which are generally expected based on structurally characterized HAMP domains (Fig. 3 B). In general the (f) positions give the longest distances followed by the (b) and (c) positions and then the (e) and (d) positions, as would be expected from their placement in the four-helix bundle (Fig. 3, C and D). For example, the 232 (d) position, which is predicted to orient toward the interior of the helix bundle, produces the shortest distance of the $\alpha 1$ sites. As mentioned above, labeling at the 230 site favors formation of a higher-order oligomer, probably a tetramer. Nevertheless, the resulting 230 $P(r)$ is indicative of (b) position and is very similar to the 250 (b) site (Fig. 3 A). Thus, the average spin-separation within the 230 tetramer may be close to the intersubunit spacing in the dimer. Three neighboring sites at the C-terminus of $\alpha 2$ agree well with predictions of canonical coiled-coil structure: the 260 (e) position gives the shortest distance, fol-

lowed by the 258 (c) position and then the 261 (f) position. Broader spatial $P(r)$ distributions correlate with larger spin-label amplitude motions (34), and the overall pattern of $P(r)$ breadth indicate that $\alpha 1$ of the Tsr HAMP may be more dynamic than $\alpha 2$ (Table S1). In summary, the Tsr HAMP structure in the context of Tsr-Aer2H1–3 meets the expectations of a parallel four-helix bundle, with the $\alpha 1$ helices slightly more destabilized compared to the $\alpha 2$ helices.

Effects of amino-acid replacements on the stability of Tsr HAMP domain

Single amino-acid replacements in the Tsr HAMP domain dramatically alter signaling responses of chemoreceptors (28,32,56). We employed the recombinant Tsr-Aer2H1–3 to investigate the types of physical changes in Tsr HAMP that such residue substitutions may generate. In this initial study we focused on replacements that give three types of phenotypes: kinase-off CCW(A), caused by Tsr E248L; kinase-off CCW(B), caused by Tsr M222P; and kinase-on CW-biased, caused by Tsr A233P (28).

The Tsr E248L substitution was chosen from the group of replacement mutants that give an attractant-mimic [kinase-off; CCW(A)] phenotype. E248 resides with the highly conserved DEXG motif (5) at the very N-terminus of the $\alpha 2$ helix in a predicted f position. Structural studies of a solubilized receptor variant (57) indicate that the Glu residue within DEXG may be positioned to interact with cytoplasmic extensions of N-terminal transmembrane helices; however, the extension of Tsr transmembrane helix 1 is likely too short to permit contact and thus, local interactions of E248 may instead determine its role in Tsr HAMP signaling. E248L increased helical content of Tsr-Aer2H1–3 and also substantially raised the T_m of the lower transition to 31°C (Fig. 4 B). Thus, E248L stabilizes the Tsr HAMP bundle. The higher melting transition showed a slightly decreased T_m compared to WT, reflecting a small compensating destabilization of the Aer2 HAMP domains. The primary effect of this substitution on the lower transition temperature supports the assignment of this transition to the Tsr HAMP.

Tsr M222P was chosen to represent those mutations that cause a kinase-off CCW(B) phenotype (28); i.e., a HAMP off-state that results from major destabilization of the helix bundle. M222 resides in $\alpha 1$ at an (a) position within the hydrophobic core of the four-helix coiled-coil. Replacements of this residue to proline (28) or polar amino acids generally give nonfunctional receptors; i.e., CheA is not active and there is no cell tumbling [CCW(B)]. Not surprisingly, this variant partially lost α -helical content (Fig. 4 A) and showed helical melting in a single step with a low T_m of 37°C (Fig. 4 B). Thus, M222P appears to destabilize Tsr-Aer2H1–3 in entirety and causes the protein to unfold as a single unit.

Tsr A233P was studied as a mutation that produces high CheA activity [kinase-on; CW] when receptors are in their

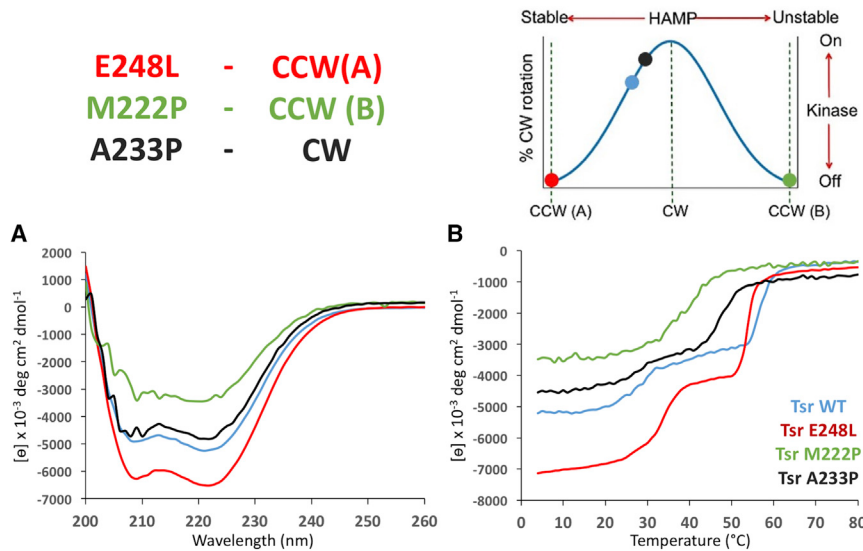


FIGURE 4 Effects of single residue substitutions on the secondary structure and thermal stability of the Tsr HAMP domain. (A) The CD spectra of the recombinant HAMP domains containing the WT Tsr HAMP (blue) compared to the E248L (red), M222P (green), and A233P (black) variants. The recombinant proteins with the WT Tsr, E248L, and A233P variants maintain their overall α -helical structure. The M222P variant shows the most reduced helical content. (B) CD thermal melts of the variant Tsr-Aer2H1–3 proteins. The E248L and A233P mutations do not affect the two-step unfolding of the recombinant protein, but have altered transition temperatures. The E248L variant has melting transitions at 33 and 54°C, and the A233P variant has transitions at 27 and 48°C. In contrast, the M222P variant unfolds in a single step at 39°C. (Inset) Upper-right panel shows a schematic representation of the dynamic bundle model of HAMP domain activity (adapted from the literature (9,28,32)). The measured relative stabilities against thermal unfolding for the WT and variant HAMP domains are mapped onto a hypothetical bell-shaped curve relating helical bundle stabilities to physiological output.

default QEQEE modification state (CheRB⁺). The A233P substitution resides on $\alpha 1$ in an (e) position and slightly increases the CW bias of Tsr receptors from 75 to 81% in *E. coli* cells that lack the adaptation system (28). The A233P variant also responds to the adaptation system, producing 15% CW rotation in CheRB⁺ cells, which is a lower level than that of WT Tsr ($25 \pm 4\%$) (9,28). Based on the bistability model (9), residue replacements that yield CW phenotypes are expected to be less destabilizing to the HAMP bundle compared to those that generate CCW(B) phenotypes, but more destabilizing compared to those that generate CCW(A) phenotypes. In the context of Tsr-Aer2H1–3, A233P had reduced α -helical content and a less well-defined lower transition temperature compared to WT (Fig. 4, A and B). Furthermore, the upper transition associated with Aer2H1–3 also had a lowered melting temperature compared to WT (Fig. 4 B). Although the stability effects of Aer2H1–3 cannot be easily separated into Tsr and Aer2 effects, the net result of the substitution is destabilization of the protein. In summary, the stability behavior of these three Tsr mutations largely follows the expectations of the bistability model.

Effects of mutations on the structure and dynamics of the Tsr HAMP domain

The structural variation of HAMP domains from different proteins suggest that the HAMP fold can assume different conformational states that could relate to signal transduction. We applied PDS and site-specific spin-labeling to characterize changes in structure and dynamics associated with the CCW(A), CCW(B), and CW phenotype-causing mutants studied above. Additional cysteine residues were

introduced into the Tsr unit of Tsr-Aer2H1–3 for nitroxide labeling. Residues R230 and I232 at the C-terminal end of $\alpha 1$, and H258 and Q260 at the C-terminal end of $\alpha 2$ were chosen as the spin-label positions. To simplify subsequent discussion, the proteins with reporter sites will still be referred to as “WT”. The conformation of the $\alpha 2$ C-terminus was of interest because it normally couples to the KCM of the receptor. The two residues on each helix reside at different heptad positions but are close in sequence and should report on rotational as well as translational motions.

The changes in spin-label distance distributions for the four probe sites are not dramatic but show some clear trends (Figs. 5, 6, and S2; Table S2). Foremost, the $\alpha 1$ sites give quite different $P(r)$ widths, indicative of changes in conformational heterogeneity. The CCW(B) M222P variant is the most unstable, showing a very broad $\alpha 1$ signal for both the 230 (b) and the 232 (d) sites (Fig. 5). Furthermore, the 230 site now gives a modulation depth below 0.4 (Fig. S2). These signals suggest that $\alpha 1$ is essentially unfolded in and that higher order oligomerization has diminished. The next most dynamic $\alpha 1$ is found in the CW state, followed by WT and then by E248L CCW(A) (Fig. 6); very much in keeping with predictions of the bistability model (28). However, the same trend is not observed for $\alpha 2$, where the two probe positions show similar $P(r)$ widths for the four variants (Figs. 5 and 6). That said, E248L tends to give the sharpest distributions (Fig. 6 A). Changes in the mean positions of the distributions are also evident among the variants. Comparisons are perhaps most instructive between the sites that give opposing functional phenotypes: CCW(A) E248L and CW A233P. Of the two $\alpha 1$ reporter sites, the 232 (d) separation increases from CCW(A) to CW, whereas

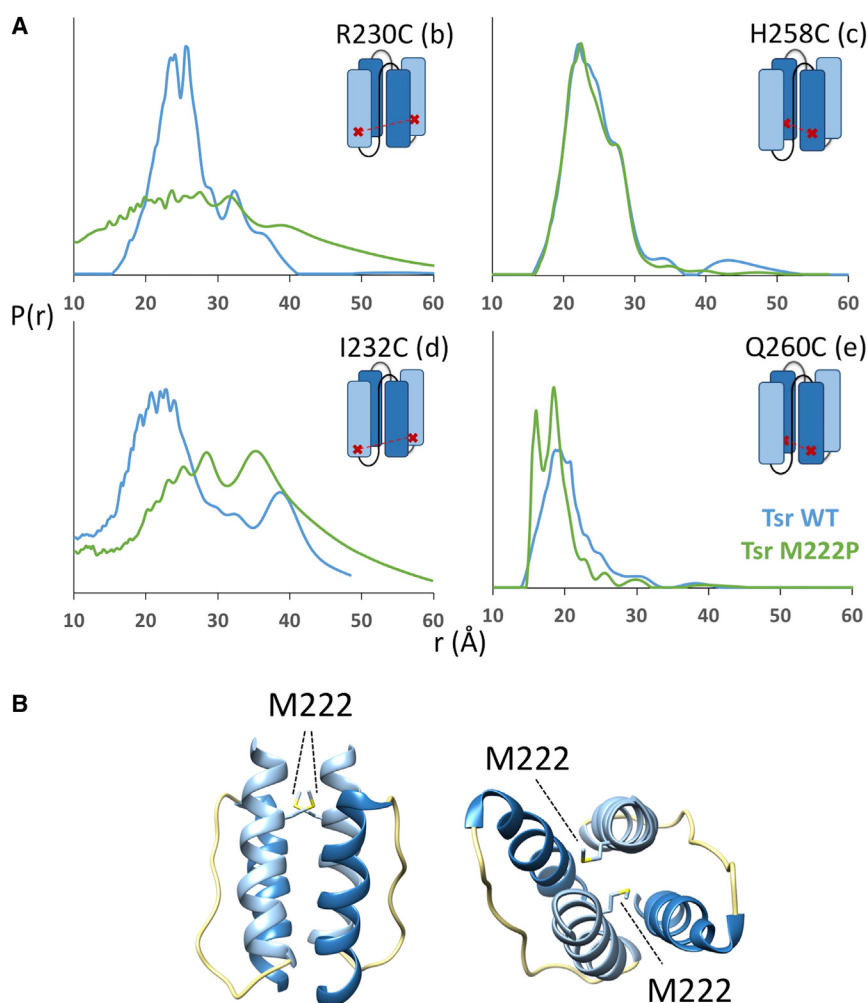


FIGURE 5 Effects of the nonfunctional kinase-off [CCW(B)] substitution on the structural properties of the Tsr-Aer2H1-3 chimeric protein. (A) PDS distance distributions for the Tsr WT (blue) and M222P – CCW(B) (green). Schematic diagrams for the Tsr HAMP are marked with the spin-label sites. (B) Ribbon diagrams showing the position of M222P on $\alpha 1$ in the hydrophobic core of the Tsr.

230 (b) decreases (Fig. 6 A). For the 230 site, oligomerization may again complicate this interpretation, but, assuming the dimeric bundle structures are largely the same for 230 and 232, opposite changes in distance are consistent with a left-handed counterclockwise rotation at the C-terminal end of the $\alpha 1$ helices (Fig. 6 B). A gearbox-type rotation of the bundle would necessitate a compensating right-handed clockwise rotation of the $\alpha 2$ helices. Consistent with an $\alpha 2$ right-handed rotation, the 260 (e) sites change to a shorter separation on transition from CCW(A) to CW and the 258 positions undergo a slight shift in the distribution mean to longer distances (Fig. 6 B). The broad distribution of the 258 positions (which could be due to spin-label conformational heterogeneity) may mask a more definitive shift in separation distance. The WT distribution lies in between the two extremes of CW and CCW(A) for position 232, resembles CCW(A) for position 230, and resembles CW for 260, which in sum are consistent with an intermediate phenotype (Fig. 6 A). These patterns thus suggest that the CCW(A) and CW mutations are distinguished by conformational changes in both the $\alpha 1$ and $\alpha 2$ helices, which can be interpreted as rotations. Nonetheless, other motions, such as

helix translation and tilting, could also contribute to the altered PDS distance distributions. Notably, both the $\alpha 1$ and $\alpha 2$ helices appear more dynamic in the CW state. For CCW(B), the $\alpha 1$ helix is essentially unfolded but the $\alpha 2$ helices maintain close interactions characteristic of a two-helix coiled-coil. Interestingly, for CCW(B), the most C-terminal 260 site shows a sharp, short, bimodal distribution very similar to that of the Aer2 HAMP2 domain fused to the KCM of the Tar receptor (34). Like the HAMP2-Tar chimera (33), the CCW(B) variant in Tsr also gives an exclusively CCW phenotype and a kinase-off state.

Effects of the N-terminal linker sequence on Tsr structure and dynamics

The five-residue control cable of Tsr links transmembrane TM2 to the HAMP domain and transmits the piston displacement of TM2 that downregulates kinase activity (1,56,58,59). The linker sequence is similar to that of Aer2 used in the chimera, but differs in one important way. Functional studies of Tsr have shown that I214 on the control cable is critical for triggering responses, possibly

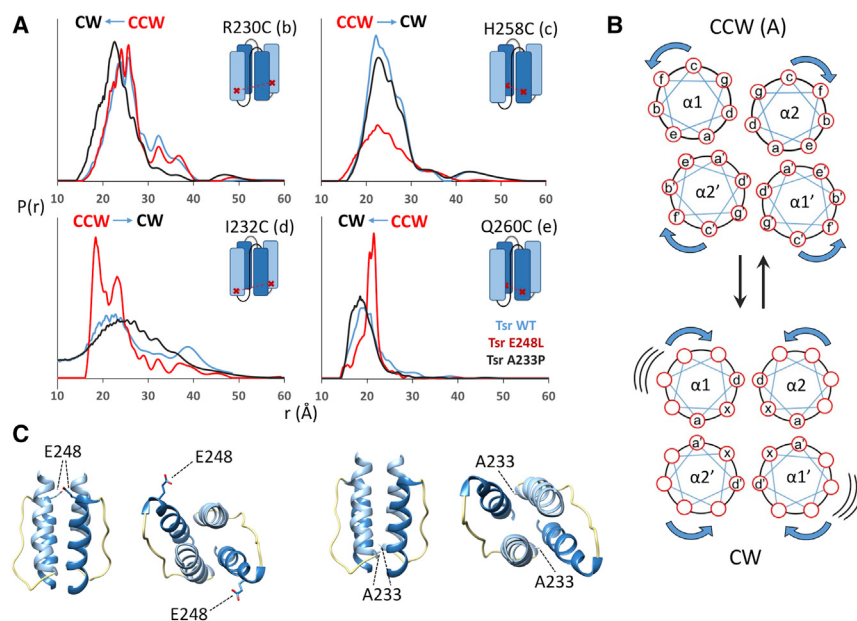


FIGURE 6 Effects of the functional kinase-off [CCW(A)] and kinase-on (CW) substitutions on the structural properties of the Tsr-Aer2H1-3 chimeric protein. (A) PDS distance distributions. Schematic diagrams for the Tsr HAMP are marked with the spin-label sites. Distance distributions [$P(r)$] measured by PDS are shown for the Tsr WT (blue) and variants E248L – CCW(A) (red), and A233P – CW (black). (B) Schematic diagram summarizing the possible conformational changes relating CCW(A) and CW as inferred by the four spin-label positions at the base of $\alpha 1$ and $\alpha 2$. For $\alpha 1$, increase in the d - d separation and decrease in the b - b separation suggest a counterclockwise rotation in switching from the CCW(A)-kinase-off state to the CW kinase-on state. For $\alpha 2$, decrease in the e - e separation indicates a corresponding clockwise rotation. The c - c position of $\alpha 2$ shows little change, probably due to breadth of the distribution. (C) Ribbon diagrams showing the positions of E248 at the very N-terminus of $\alpha 2$ in a predicted f position and A233 at an e position on the N-terminus of $\alpha 1$.

through the interaction of its side chain with the aromatic residues at TM2 C-terminus (56,60–63). Glu substitution at this position (61), as found in Aer2, has impaired function (56). Thus, we altered Glu61 to Ile to better reflect the control cable sequence, and investigated the impact on stability and conformation (Fig. S5 A).

E61I Tsr-Aer2H1-3 showed substantially higher thermal stability of both Tsr HAMP (36°C) and Aer2 HAMP1-2-3 domains (67°C) than the WT counterparts (26 and 57°C, respectively) (Fig. S5, B and C). In PDS studies (Figs. S5 D and S6; Table S3), the substitution substantially ordered a probe site at the N-terminus of $\alpha 1$, showing that increased hydrophobicity in this position of the control cable stabilizes the otherwise variable $\alpha 1$. However, the substitution does not appreciably affect the 258 site at the C-terminus of $\alpha 2$. Thus, in the context of the chimera, the control cable conformation does not greatly impact $\alpha 2$ (Fig. S5 D). This result further confirms the stable nature of Tsr $\alpha 2$.

DISCUSSION

To permit expression of the otherwise unstable Tsr HAMP, we generated a chimeric protein containing the Tsr HAMP domain spliced into the Aer2 poly HAMP. Under these circumstances, the Tsr HAMP maintained its α -helical, four-helix coiled-coil structure. Compared to Aer2 poly-HAMP alone, the thermal denaturation profile of the chimeric protein revealed an additional lower temperature transition that we attribute to Tsr HAMP. The chimeric protein thus provided an opportunity to evaluate the effects of residue substitutions of known functional consequence on the stability and conformational properties of the Tsr HAMP.

A common signaling mechanism of HAMP domains is supported by the ability to swap HAMP domains among various proteins while maintaining function (64,65). The structural studies of several HAMP domains have led to a two-state model for the HAMP signaling mechanism. The gearbox model proposes conversion between discrete conformations that differ by a - d and x - da packing arrangements of HAMP bundles (10,15,17,31). Chemoreceptors in off-states are proposed to be associated with the a - d packing mode and on-states with the x - da mode (17,29,66). In contrast, the extensive genetic studies of Tsr HAMP mutations have led to the dynamic bundle model, which accounts for the observations that multiple amino-acid substitutions of similar character invoke the same signaling outputs. This model proposes that, for each signaling state, HAMP domains operate across a range of related conformations that differ by helix-packing stabilities and dynamics (28,29,32). Both highly stable and highly unstable domains produce kinase-off (CCW) states, whereas domains of mid-range stability favor kinase-on (CW) states. The output response to domain stability has been conceptualized as a bell-shaped curve, with intermediately stable states producing the highest CW rotational bias (see inset to Fig. 4). HAMP domains with residue substitutions lie at various positions on the curve, but can be shifted depending on the modification state of the receptor. In the context of the Tsr receptor, the packing stabilities of HAMP helical bundles are expected to oppose those of the KCM helical bundles owing to a mismatched phase of hydrophobic packing between $\alpha 2$ and the methylation helices of the kinase control module; i.e., one of the two bundles can pack in stable fashion, but not both simultaneously (19,29,34). It should be emphasized that to alter the dynamics of the HAMP bundle (which can be viewed as the amplitudes of

motions as reflected by the variance of the conformational ensemble), interactions within the domain must change, and hence the mean conformations of the activating and inactivating states must differ. Thus, a discrete conformational switching model and the dynamic bundle model emphasize different aspects of altering the energy landscape of the HAMP domain. The results of this study underscore this perspective.

This initial evaluation of three residue substitutions representative of the CCW(A), CCW(B), and CW-biased states defined in the dynamic bundle model provides some insight into how such changes alter Tsr HAMP stability and structure. The CCW(B) mutation, M222P, which replaces an internal hydrophobic residue with a helix-breaking proline residue, greatly destabilizes the $\alpha 1$ helix and produces an extremely broad PDS distribution. However, the $\alpha 2$ helices remain folded and associated with a comparatively tight PDS distribution at the 260 position; thus, the domain has effectively become a two-helix coiled-coil and hydrophobic packing of $\alpha 2$ can occur independently from that of $\alpha 1$. Indeed, deletion of the Tsr $\alpha 1$ helices causes CCW-locked behavior (27) (which depends on the C-terminal packing residues of $\alpha 2$). The two substitutions that produce CCW(A) and CW phenotypes represent differences in functional states. The CCW(A) substitution, E248L, resides in the DExG motif (NEMG in the Tsr HAMP) at the connector to $\alpha 2$. The DExG motif is highly conserved in HAMP domains that lie proximal to the membrane, and is critical for coupling transmembrane signals (5,33). The CW phenotype of A223P in QEQUEE cells, which increases only slightly over WT, tolerates the substitution of a larger residue in the (e) position near the end of $\alpha 1$ (28). As evaluated by thermal melts, the CCW(A) mutation produces a domain more stable than the WT, whereas the CW mutation produces a domain less stable than the WT. The breadth of the PDS distributions reflects these trends, with conformational heterogeneity in $\alpha 1$ following the trend CW > WT > CCW(A) and a more rigid association of $\alpha 2$ in CCW(A) (Fig. 6 A).

When comparing E248L (CCW(A) and A233P (CW), the centers of the PDS distributions at the C-terminal ends of $\alpha 1$ and $\alpha 2$ also indicate a conformational change consistent with concerted helical rotation. Under this premise, the internal hydrophobic packing converts between the *x-da* arrangement to a more canonical *a-d* (knobs-into-holes) packing (Fig. 3). In transition from *a-d* to *x-da* packing, the $\alpha 1$ helices rotate counterclockwise and the $\alpha 2$ helices rotate clockwise. Opposite shifts in separation of neighboring residues can be explained by such motions but are not consistent with a helical tilt away from the dimer axis or a piston motion, wherein the spacing of two neighboring positions would change in the same direction or stay the same. However, helical translations in a plane perpendicular to the dimer axis or a scissors motion of symmetric helical pairs could also generate the observed distance changes.

These types of distortions would produce a more rhombic structure in the CCW state (Fig. 6). Ambiguities arising from flexibility in the spin-label prevent a further discrimination of these models.

Interestingly, the residue substitutions that cause CCW(A) and CCW(B) states have very different adaptational modification properties, which predicted that their $\alpha 2$ structures should be different (28). Indeed, the CCW(A) and CCW(B) representative variants do not produce the same conformations at the C-terminus of $\alpha 2$, as reported by the spin-probe at position 260, although they share the feature of less conformational heterogeneity compared to the CW and WT variants. It is possible that the rotational phase of the $\alpha 2$ helix must be within a specific range for the downstream helices of the KCM to be stable, as has been seen in other helical signaling systems (67,68). The distance distributions for both the WT and CW states lie between those of CCW(A) and CCW(B). CCW output may result if HAMP $\alpha 2$ enforces the downstream helices to be either over- or underwound. Fusion of the Aer2 HAMP1 or HAMP2 to the Tar KCM causes opposite effects on KCM dynamics, kinase activity and cell swimming behavior (33,34). At their C-termini the Aer2 HAMP1 and more rhombic HAMP2 domains also differ by $\alpha 2$ rotation and positioning, with the off-state involving closer two-helix knobs-into-holes (*a-d*) packing of HAMP2 (33,34).

Thus, the transition between HAMP activation states likely involves restructuring of the domain in a manner that alters not only the mean conformation, but also the conformational variance. States with greater variance will not influence downstream modules with the dominance of those that closely cluster about the actual signaling conformation. Because chemoreceptor signaling domains without HAMP domains are kinase-on (69–71) and the Tsr HAMP domain appears to have low intrinsic stability (melting at room temperature), for the HAMP domain to send a kinase-off signal, it must change conformation, but to enforce the effects of this change on downstream modules, the new state must also be less distributed. From the dynamic bundle model perspective, the HAMP domain stabilizes to influence the signaling domain, but, such stabilization requires a change in atomic interactions and position. Viewed from either side, changes in conformation mean and variance are both consistent with the helical melting and PDS data.

In the context of transmembrane chemoreceptors, the five-residue control cable connects and transmits piston displacements of the ligand-bound TM2 to the $\alpha 1$ helix of the HAMP domain (9,56,60). The genetic studies in the Tsr receptor have shown that the control cable helicity enables Tsr to adopt proper signaling states by modulating the structural mismatches between the TM2 and the $\alpha 1$ helix. However, only isoleucine 214 on the control cable is critical for this transmission mechanism (56,59,60,63). *E. coli* cells expressing the Tsr receptor with the I214E substitution showed

higher CW flagella rotation (88%) compared to cells expressing the WT receptor (75%) (56). Changing Aer2 Glu⁶¹ to Ile increased stability of the Tsr HAMP as judged by thermal melts and reduced conformational heterogeneity in $\alpha 1$ (Fig. S5). However, there appeared to be little change at the $\alpha 2$ C-terminus. Hence, the Tsr I214 control cable residue may have less impact on the $\alpha 2$ conformation in this chimeric system than in the native receptors where I214 resides close to the membrane.

In summary, the Tsr-Aer2H1–3 chimera has allowed study of mutational effects on Tsr HAMP conformation and stability. Although the Tsr HAMP domain is not harbored in a full-length chemoreceptor, it is still coupled to dimeric helical bundles at both the N- and C-termini. The results of the residue substitutions on biophysical properties of Tsr HAMP match expectations of prevailing models for HAMP activation. Mutations favoring kinase-on (CW) or kinase-off (CCW) differ by changes that can be interpreted in terms of helix rotation, juxtaposition, and altered stability. The activating states of HAMP appear to be associated with more conformational variability (larger amplitude motions), less stability versus an unfolded state, and movement of the $\alpha 2$ helices at their C-termini that bring (*e*) sites closer together and (*c*) sites further apart. A right-handed clockwise rotation toward *x-da* packing is consistent with such a change. Functional kinase-off states (CCW(A)) are associated with a stable association of the $\alpha 2$ helices that favors a more *a-d* arrangement. The more drastic CCW(B) kinase-off state differs from that of CCW(A) in $\alpha 2$ positioning, despite also forming a tight two-helix association at the C-terminus of $\alpha 2$. Although the CCW(B) state is the most unstable, the loss of stability largely manifests by unfolding the $\alpha 1$ helices. For kinase-on (CW) states, an overall conformationally variable (dynamic) HAMP domain allows the KCM signaling domain to assume its favored activating conformation. In contrast, for kinase-off states, a stable two-helix coil in HAMP $\alpha 2$ may either over- or underwind the helices of the KCM to force its conformation outside of the range that promotes kinase activation.

SUPPORTING MATERIAL

Six figures and three tables are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(17\)30252-7](http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)30252-7).

AUTHOR CONTRIBUTIONS

N.S., P.P.B., and B.R.C. designed research; N.S., J.W., and P.P.B. performed research; N.S., P.P.B., J.W., J.H.F., and B.R.C. analyzed data; and N.S. and B.R.C. wrote the article with contributions from all authors.

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