Conservation of function with diversification of higher-order structure within sensor histidine kinases

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Abstract

Integral to the protein structure/function paradigm, oligomeric state is typically conserved along with function across evolution. However, notable exceptions – such as the hemoglobins – show how evolution can enable new regulatory mechanisms by oligomerization changes. We examine this linkage in the histidine kinases (HKs), widely-distributed prokaryotic environmental sensors. While most HKs are transmembrane homodimers, our finding of a monomeric soluble HK (EL346, a photosensing Light-Oxygen-Voltage (LOV)-HK) raises questions about the relationship between oligomerization and regulation within this family. Here we characterize multiple EL346 homologs and find a diverse range of HK oligomeric states. Three LOV-HK homologs are dimers with differing structural responses to light, while two are Per-ARNT-Sim (PAS)-HKs that interconvert between differentially-active monomers and dimers, suggesting dimerization as a regulatory control. Finally, we examine putative interfaces in a dimeric LOV-HK, finding that multiple domains contribute to dimerization and function. Our findings suggest potential for novel regulation in this important family of environmental sensors.

Introduction

Sensor histidine kinases (HKs) are signal transduction receptors that are widespread in prokaryotic organisms, enabling them to sense and respond to diverse environmental stimuli (Galperin, 2018). HKs are typically found in two-component systems (TCS), which are most simply composed of a sensor HK and a downstream response regulator (RR) collocated in the same operon. The RR responds to a signal from the HK by acting as a phosphorylation-dependent transcription regulator, usually binding to DNA and controlling gene expression in various bacterial processes, including virulence (Kenney & Anand, 2020; Mechaly et al., 2017; Wang, 2012; West & Stock, 2001). The majority of sensor HKs characterized to date are obligate homodimeric transmembrane receptors, facilitating transmission of signals across the membrane from periplasmic sensor domains to cytoplasmic kinase domains via intermonomer, symmetry-breaking conformational changes (Bhate et al., 2015; Gushchin & Gordeliy, 2018; Szurmant et al., 2007). In contrast, some sensor HKs adopt varying oligomeric states, ranging from large multimeric assemblies observed for bacterial chemosensors (Muok et al., 2020) to the monomeric soluble HK EL346 (Rivera-Cancel et al., 2014). Soluble sensor HKs are relieved of the constraints of transmitting a signal across a lipid bilayer, which typically necessitates signal transmission via motions of one monomer with respect to the other (Bhate et al., 2015; Herrou et al., 2017). This raises the open question: Are such soluble HKs more likely to adopt varied oligomeric states including non-dimeric architectures? We view the relatively understudied HWE/HisKA2 family of HKs (Herrou et al., 2017), which is enriched in soluble cytoplasmic proteins that participate in atypical signaling pathways, to be a tempting testbed for this hypothesis.

Typical sensor HKs contain an N-terminal sensor domain and a C-terminal kinase domain consisting of a two-helix dimerization histidine phosphotransfer (DHp) domain and a catalytic ATP-binding (CA) domain (Bhate et al., 2015). Stimulus detection at the sensor domain, or in some cases at other domains (Kenney & Anand, 2020), modulates the CA domain autophosphorylation of a conserved His residue in the DHp domain, a signal which is subsequently transferred to a conserved Asp in the RR receiver domain (West & Stock, 2001). The homodimeric nature of canonical transmembrane HKs is largely believed to be required for both the structural changes between the “off” and “on” state and determining whether autophosphorylation occurs between monomers (*in trans*) or *in cis* within a monomer (Ashenberg et al., 2013; Bhate et al., 2015). Dimerization is mediated via the DHp domains of two monomers, forming a four-helix bundle and facilitating pivot-, piston-, or scissoring-type

movements as part of the activation process (Bhate et al., 2015; Herrou et al., 2017), echoing similar themes in integrins (Luo et al., 2007) and certain other transmembrane receptors.

This traditional view of sensor HK structure has been built up from extensive structural and biochemical studies (Bhate et al., 2015), most of which have focused on HKs in only one of several subfamilies of histidine kinases (Pfam (El-Gebali et al., 2019) family HisKA). In contrast, the related HWE-HK and HisKA_2 families (referred to here as the HWE/HisKA2 family) of HKs have not been as extensively studied from a structural perspective (Herrou et al., 2017). This family, which differs from the canonical HisKA family in the DHp and CA domains, appears to be enriched in HKs lacking transmembrane segments and correspondingly likely localized to the cytosol. These HKs also tend to signal in an atypical manner, utilizing phosphorylation to control protein-protein interactions of RRs, rather than directly impacting them as transcription factors *per se* (Herrou et al., 2017). HWE/HisKA2 HKs are also enriched in the *Alphaproteobacteria* (Karniol & Vierstra, 2004; Staroń & Mascher, 2010).

Our prior work on the HWE/HisKA2 family has focused on three soluble HKs involved in the general stress response of the marine alphaproteobacterium *Erythrobacter litoralis* HTCC2594 (EL346, EL362, and EL368) (Correa et al., 2013; Rivera-Cancel et al., 2014; Swartz et al., 2007). Each of these proteins senses blue light through a light oxygen voltage (LOV) sensor domain (Crosson et al., 2003; Pudasaini et al., 2015), a sub-class of the versatile Per-ARNT-Sim (PAS) domains that are present in over 30% of HKs (Gao & Stock, 2009). The response to blue light provides an experimentally tractable way to explore the inactive and active states of histidine kinases, typically corresponding to dark and light states of LOV-HKs (Möglich, 2019; Swartz et al., 2007; Tseng et al., 2010). These states can be conveniently distinguished spectrophotometrically: while the dark state spectra of LOV-HKs exhibit a triplet peak at around 450 nm, characteristic of protein-bound flavin chromophores, the light state spectrum has a broad, singlet absorption peak at 390 nm (Zoltowski & Gardner, 2011). This spectroscopic change is due to the formation of a photochemically-generated covalent adduct between the C(4a) of the isoalloxazine ring and a conserved cysteine residue, a bond that is thermally reversible in the dark on a timescale varying from seconds to hours among LOV domains (Pudasaini et al., 2015).

While our prior studies indicate that EL368 and EL362 (when reconstituted with flavin) are dimeric, EL346 is a soluble and functional monomer (Correa et al., 2013; Rivera-Cancel et al., 2014). The EL346 crystal structure reveals that it is held in a monomeric state by an intramolecular association between its DHp-like domain and the LOV sensory domain, blocking the face typically used for DHp domains to dimerize (Rivera-Cancel et al., 2014). Despite this

substantial deviation from current HK signaling models, the monomeric EL346 fully responds to stimulus with a light-induced increase in autophosphorylation (Rivera-Cancel et al., 2014). More broadly, EL346 suggests that soluble HKs may not require dimerization as a key element in signal transduction, unlike conventional transmembrane HKs. Thus, we set out to characterize the oligomeric and functional states of several novel HWE/HisKA2 HKs.

To do so, we used sequence homology searches to identify multiple soluble LOV- and PAS-HKs with similarity to EL346 and used a combination of approaches to test if these proteins were similarly functional monomers. We report here our characterization of three novel LOV-HKs, each of which properly photocycle by UV-visible absorbance spectroscopy and are dimeric with a range of light-triggered conformational changes, as detected by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). In contrast, we also detail results from two light-insensitive PAS-HKs which exist in monomeric and dimeric states with differing autophosphorylation activity, suggesting dimerization as a possible regulatory switch in this family of HKs. Finally, we combine structural and sequence information to engineer a “monomerized dimer” by deleting putative dimerization interfaces in a dimeric LOV-HK, finding that a distributed interface of interactions encodes both dimerization and autokinase activity. Taken together, our data indicate that a wider range of oligomerization states are accessible to HKs, raising exciting questions about regulation and evolution potentially analogous to other protein families which exhibit differences in quaternary structure and control, such as the hemoglobins (Pillai et al., 2020).

Methods

Bioinformatics

EL346 homologs were identified by searching for non-redundant protein sequences in the GenBank database using BLAST with EL346 amino acids 1-346 as query. EL346 and the best 100 sequences were aligned using Clustal Omega (Sievers et al., 2011) and a distance tree was calculated from this alignment using the same software. Graphic display of this tree was accomplished using iTOL (Interactive Tree of Life, <http://itol.embl.de/>) (Letunic & Bork, 2019) and Adobe Illustrator CS6. This tree was used to select HKs for cloning from the strains available at DSMZ (<http://www.dsmz.de>).

Cloning, protein expression and purification

The HKs selected from each strain are shown in **Table 1**. The proteins were cloned into a pHis-Gβ1 vector (Sheffield et al., 1999). Constructs were verified by DNA sequencing. The

proteins were overexpressed in *Escherichia coli* BL21(DE3) (Stratagene) in LB as previously described (Dikiy et al., 2019). Cells were harvested, resuspended in Buffer A (50 mM Tris pH 8.0, 100 mM NaCl, and 10 mM MgCl₂), and lysed by sonication. The lysate was centrifuged at 48,000 x g and 4 °C for 45 min. The supernatant was loaded into a Ni²⁺ Sepharose affinity column (GE Healthcare) and the His6-Gβ1 tagged protein was washed with 4 column volumes of Buffer A supplemented with 15 mM imidazole and eluted with 4 column volumes of Buffer A supplemented with 250 mM imidazole. Eluted proteins were exchanged into Buffer A by dialysis. The fusion tag was cleaved by incubation with His6-TEV protease overnight at 4 °C. The tag-less proteins were separated from the tags and His6-TEV protease by Ni²⁺ affinity chromatography and were further purified by size-exclusion chromatography on a HiLoad 16/600 Superdex 200 column (GE Healthcare) equilibrated with HSEC buffer containing 50 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, and 0.5 mM DTT. For light-sensitive proteins, all purification steps were performed under dim red light. Concentrations were determined from the theoretical absorption coefficient, ϵ_{280} for PAS-HKs, calculated from the sequence using the ExPASy ProtParam server (Gasteiger et al., 2005), and $\epsilon_{446} = 11,800 \text{ M}^{-1} \text{ cm}^{-1}$ for flavin-containing proteins.

RH376 deletion mutants were generated as follows: RH2 and RH3 were ordered as synthetic constructs from Genewiz (South Plainfield, NJ) and inserted into the same pHis-Gβ1 vector as the WT protein. The RH1 deletion was generated by PCR using an N-terminal primer lacking the sequence to be deleted using WT and RH3 plasmids as templates, to generate RH1 and RH1+3, respectively.

Absorbance and dark state reversion kinetics

UV-visible absorbance spectroscopy measurements were acquired using a Varian Cary 50 spectrophotometer at 24 °C with a 1 cm path length quartz cuvette. First, a UV-visible spectrum in the range 250-550 nm was acquired in light and dark conditions for each LOV-HK. For dark measurements, all steps were performed under dim red light. For light measurements, the samples were illuminated for 1 minute using a blue LED panel (225 LED bulbs in 30.5 × 30.5-cm panel, 13.8 W, 465 nm maximum illumination wavelength; LEDwholesalers [Hayward, CA]) prior to each scan. To measure dark state recovery, each LOV-HK sample was illuminated under a blue LED panel for 1 minute and then transferred to the spectrophotometer. A_{446} of the samples in HSEC buffer with 1 mM ATP was monitored at 5-min intervals for 400-800 min at 24 °C. Time constants τ were determined by fitting measurements to a monoexponential decay using the following equation:

Abs = $A_{inf} - (A_{inf} - A_0)e^{-t/\tau}$, where $A_{inf} = A_{446}$ at infinity, $A_0 = A_{446}$ initial, and t = time

Raw data were fit and plotted using RStudio and plots were edited in Adobe Illustrator CS6.

SEC-MALS

All samples and buffers were filtered with a 0.1-um pore size filter before use. ATP was added to each HK sample (~15 μ M, 400 μ L) to a final concentration of 1 mM. Samples were then injected at a 0.4 mL/min flow rate onto a Superdex 200 GL 10/300 SEC column (GE Healthcare) using HSEC buffer with 1 mM ATP. For lit experiments, samples were illuminated with a blue LED panel for 1 min prior to injection and the blue LED panel was faced toward the column during the run. For dark experiments, all steps were performed under dim red light. Samples were detected post-elution by the inline miniDAWN TREOS light scattering and Optilab rEX refractive index detectors (Wyatt Technology [Santa Barbara, CA]). FPLC runs were performed at 4 °C, while MALS and refractive index measurements were at 25 °C. Data analysis and molecular weight calculations were performed using the ASTRA V software (Wyatt Technology). Raw data were plotted using RStudio and plots were edited in Adobe Illustrator CS6 and overlaid in Adobe Photoshop CS6.

Autophosphorylation assays

Autophosphorylation was measured as described previously (Dikiy et al., 2019; Rivera-Cancel et al., 2014). Two trials were conducted for each protein under each condition. Proteins were added to a reaction buffer containing 50 mM Tris, 100 mM NaCl, 5 mM $MnCl_2$, and 1 mM DTT with pH 8.2. Protein concentrations were confirmed by analysis of dilution series on Coomassie-stained SDS-PAGE. Concentrations typically ranged from 4.7 to 19.8 μ M, with EL346 and RH2 able to be more concentrated at 34.1 and 67.1 μ M. A mixture of unlabeled ATP and 10 μ Ci [γ - 32 P] ATP was added to each mixture to initiate the reaction (final ATP concentration of 3.6 μ M). Aliquots were removed at time points of 0.5, 1, 2, 4, 8, 6, and 32 min and placed into a 4x SDS-gel loading buffer to quench the reaction. For dark measurements, all steps were performed under dim red light. For light measurements, the samples were illuminated with a blue LED panel just prior to and throughout the course of the experiment. Samples were subjected to SDS-PAGE analysis; dried gels were exposed overnight and bands were visualized by phosphorimaging. Band intensities were measured using Fiji (Rueden et al., 2017; Schindelin et al., 2012); initial velocity values were calculated in Microsoft Excel from the linear region of the curve and were plotted in RStudio and edited in Adobe Illustrator CS6.

Results

Bioinformatics analysis reveals numerous alphaproteobacterial EL346 homologs

To identify potential additional monomeric histidine kinases, we searched for homologs to the full-length EL346 sequence as a well-validated, functional monomer. Taking the top 100 BLASTp hits (in addition to EL346), all belonging to the *Alphaproteobacteria*, we generated a multiple sequence alignment of proteins which all contained the same domain architecture of a single N-terminal PAS or LOV domain followed by a C-terminal HWE/HisKA2 histidine kinase, and without any predicted transmembrane segments. A distance tree analysis revealed three clades (**Fig. 1a**) which split on sensor type: PAS-HKS (non-LOV), LOV-HKS, and EL346-like LOV-HKS. Interestingly, a subgroup of the EL346-like LOV-HKS is missing the conserved phosphoacceptor histidine (**Fig. S1**). We also included the related EL368 (dimer) and EL362 (blind monomer) histidine kinases from *E. litoralis* HTCC2594 that we had previously identified

(Correa et al., 2013), as well as the *Caulobacter crescentus* LovK (dimer) (Purcell et al., 2010) in the alignment; all three of these proteins clustered in the LOV-HKs clade. Additionally, our search identified a third PAS-HK clade with proteins containing non-LOV PAS domains with 17-24% sequence identity to the EL346 LOV domain. Notably, many of these domains contained a conserved tryptophan in place of the cysteine utilized in LOV-type photochemistry (Crosson & Moffat, 2001) (**Fig. S1**). These data show that the PAS/LOV-HK architecture is widespread throughout *Alphaproteobacteria* and contains members which could well respond to stimuli other than blue light.

	Organism	Protein Name	Uniprot ID	NCBI ref.	Identity to EL346	Oligomeric State
EL346-like LOV-HKs	<i>Erythrobacter litoralis</i> HTCC2594	EL346	Q2NB77	YP_457861.1	N/A	Monomer
	<i>Rubellimicrobium thermophilum</i> DSM 16684	RT349	S9SB69	WP_021096554.1	42.3%	Dimer
	<i>Roseivivax halodurans</i> JCM 10272	RH376	X7EIB8	ETX15640.1	35.8%	Dimer
LOV-HKs	<i>Methylobacterium extorquens</i> DM4	ME354	C7CCX6	YP_003069551.1	29.6%	Dimer
	<i>Erythrobacter litoralis</i> HTCC2594	EL368	Q4TL45	ABC62688.1	25.5%	Dimer
	<i>Erythrobacter litoralis</i> HTCC2594	EL362	Q2N9L9	WP_011414456.1	25.8%	Monomer
PAS-HKs	<i>Rhizobium etli</i> CFM 42	RE356	Q2KD32	YP_4679981.1	30.1%	Monomer/ Dimer
	<i>Methylocella silvestris</i> BL2	MS367	B8ELH4	YP_002360925.1	28.7%	Monomer/ Dimer
Other	<i>Bacillus subtilis</i> / <i>Bradyrhizobium japonicum</i>	YF1 (engineered YtvA/FixL fusion)	N/A	N/A	25.5%	Dimer

Table 1: Oligomeric states of known LOV-HKs and PAS-HKs include mix of monomers and dimers. Soluble HKs shown here are separated by clade with the same coloring as Fig. 1 (EL346-like LOV HKs – pink, LOV-HKs – blue, PAS-HKs – purple, Other – grey) and include those investigated in this study (EL346, RT349, RH376, ME354, RE356, and MS367) as well as those identified in literature (EL368, EL362, and YF1) (Correa et al., 2013; Diensthuber et al., 2013). Oligomeric states were determined in this study by SEC-MALS.

LOV-HK homologs are light-sensing and appear mostly dimeric

We posited that the EL346-like LOV-HK clade might contain other monomeric HKs similar to EL346, in addition to other members which might adopt the more commonly-observed dimeric assembly seen in the engineered LOV-HK YF1 (Diensthuber et al., 2013), as illustrated in **Fig. 1b**. To test this hypothesis, we selected several HKs from each clade and characterized their oligomeric state and function with a variety of biochemical assays. We started by determining the ability of the LOV-containing proteins to bind flavin chromophores and undergo typical LOV photochemistry via UV-visible absorbance spectroscopy. The dark and light spectra of four representative proteins, EL346, ME354, RT349, and RH376 (see **Table 1** for protein details) all show the characteristic flavin-LOV triplet (~450 nm) in the dark state which disappears upon illumination (**Fig. 2a**). Upon incubation in the dark, the 450 nm absorbance returns with exponential kinetics and a time constant that varies by protein: the other LOV-HKs measured here, such as ME354 (236 min), RT349 (240 min), and RH376 (62 min), had longer reversion constants τ than EL346 (33 min) in the presence of 1 mM ATP (**Fig. 2b**). The notably long reversion time constants of ME354 and RT349 can be explained by the presence of several “slowing” mutations in their LOV domains relative to that of EL346, specifically at positions 19, 21, 32, and 101 (EL346 numbering), as collated in a review by Pudasaini and coworkers (Pudasaini et al., 2015).

We determined the oligomeric state of these LOV-HKs in the presence of ATP and either dark or light state, using size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS), starting by confirming that EL346 is monomeric under both conditions with minimal change in shape upon illumination as revealed by SEC elution profiles (**Fig. 2c**). In contrast, the three other LOV-HK proteins are stable dimers (via MALS data) but undergo a range of light-dependent shape changes which impact SEC elution times. These range from minimal, with ME354 eluting as a single peak that does not noticeably change between dark and light, to substantial shifts to later elution times for both RT349 and RH376 consistent with a light-induced compaction. Overall, our results suggest that other LOV-HKs are generally dimeric and exhibit varying global conformational changes in response to light.

Non-LOV PAS-HKs show mix of monomers and dimers, both of which are active

The two PAS-HKs we characterized, from *Rhizobium etli* CFN 42 (RE356) and from *Methylocella silvestris* BL2 (MS367), appear as a mixture of dimer and monomer, as well as an oligomeric or aggregated fraction, as confirmed by SEC-MALS (**Fig. 3a**). These two proteins exhibit 45% sequence identity to one another (32% over the PAS domain) and behave very

Figure 2: UV-visible absorption spectra and SEC-MALS reveal diversity of LOV-HK dark state reversion kinetics and large scale responses to illumination. a) UV-visible absorption spectra of EL346, ME354, RT349, and RH376, superimposing dark state scans (black) with scans post-illumination (red). All spectra display the disappearance of the 450 nm triplet upon exposure to light, consistent with LOV photochemistry. **b)** Dark state reversion kinetics, measured by the return of absorbance at 446 nm post illumination (black), fit to single exponentials (red) reveal an 8-fold range of time constants. **c)** SEC-MALS elution peaks are represented by dRI in arbitrary units and MALS-derived masses are solid lines under the peaks. MWs and elution volumes of six standard proteins are shown in grey in the top panel. MALS traces show that EL346 is monomeric in solution, while ME354, RT349 and RH376 are all predominantly dimeric. A light-dependent shift in elution peak shape and position is seen in both RT349 and RH376.

similarly in our biophysical tests and activity assays. While these proteins lack a known trigger analogous to light for the LOV-HKs, we still wanted to determine whether both proteins exhibited any autophosphorylation in their default state as purified from *E. coli*. Both MS367 and RE356 displayed autophosphorylation activity approximately one order of magnitude higher than that of EL346 as determined previously (Dikiy et al., 2019; Rivera-Cancel et al., 2014) and confirmed in this study (see below), with the characteristic plateau indicating an equilibrium between kinase and phosphatase activity (**Fig. 3b**). The activity of RE356 was slightly higher than that of MS367, with approximately 1.5-fold greater initial velocity. It is unclear whether this level of

activity indicates a high basal activity or whether binding to the putative trigger turns off the kinase.

We wondered whether oligomeric state (monomer vs dimer) affects autophosphorylation activity. We found that the monomeric and dimeric states of MS367 and RE356 are in slow equilibrium, and could be separately injected onto SEC-MALS, as shown for MS367 in **Fig. 4a**. We also determined autophosphorylation activity separately for the monomer and dimer fractions of MS367 and found that the dimer is approximately 4-fold more active than the monomer fraction (**Fig. 4b**). However, the dimeric fraction also exhibited a large proportion of oligomer/aggregate, suggesting that the dimeric state is less stable or prone to further oligomerization (**Fig. 4a**). Using size-exclusion chromatography, we determined that the monomeric state of RE356 is stable on the order of days at 4 °C, while the dimer slowly oligomerizes or aggregates on this timescale (**Fig. 4c**). These data illustrate that active monomeric HKs are to be found in this family, but dimerization may serve as a regulatory mechanism.

Figure 4: PAS-HK monomer/dimer states can be separated, dimer is more active than monomer, and monomer is more stable over time. **a)** MS367 monomers and dimers were separated and injected onto a Superdex 200 GL 10/300 SEC column. The resulting MALS-derived masses (solid lines under elution peaks) show that both remain stable upon separation. **b)** MS367 autophosphorylation assays (phosphor images, top and plots, bottom) of the monomer (red traces) and dimer (blue traces) of MS367 show that the dimer has more autophosphorylation activity. **c)** SEC traces of RE356 dimer and monomer after 0, 2, and 4 days at 4 °C are shown, revealing that the monomer is stable over time, while the dimer tends to aggregate.

Figure S3: Pairwise alignment of EL346 and RH376 reveals “gaps” in EL346 sequence (Supplemental to Fig. 5). The pairwise alignment of EL346 and RH376 sequences was performed by MUSCLE (Edgar, 2004). “Insertion” regions of RH376 termed RH1-4 are highlighted with dashed boxes. Secondary structure prediction by JPred (Drozdetskiy et al., 2015) (red = helices, green = sheets) for RH376 and from the 4R3A PDB entry for EL346 (Rivera-Cancel et al., 2014) as well as domain locations (LOV, DHp, and CA) are shown next to the amino acid sequences.

Figure 5: RH376 mutant deletion regions are designed in predicted dimer interfaces. RH376 deletion mutants RH1, RH2, and RH3 are highlighted in 3 schematics: **a)** Domain architecture with the predicted secondary structure at the location of each deletion. RH1 is located in the A'α helix of the LOV domain, RH2 in the linker between the LOV domain and α1 of the DHp domain, and RH3 is in the ATP lid. **b)** Monomeric homology model of RH376 based on the structure of EL346 (Rivera-Cancel et al., 2014). **c)** Dimeric homology model of RH376 based on the engineered YF1 sensor kinase (Diensthuber et al., 2013). All LOV domains are colored blue, DHp domains green, and CA domains red. Mutations are highlighted and circled.

Engineering a “monomerized” LOV-HK

With the possibility of monomer-dimer transition as a regulatory mechanism, we sought to isolate the determinants of dimerization in this family of sensor HKs. During evolution, many factors (e.g. changes to protein sequence) can influence the multimeric arrangement of proteins belonging to the same family (Perica et al., 2012; Pillai et al., 2020). To investigate such determinants of the oligomeric state, we compared monomeric EL346 with selected dimeric HKs from all three clades using sequence alignment. These pairwise alignments highlighted four regions of difference in the primary sequence between EL346 and the other HKs; these regions were distributed differently throughout the three clades. However, the dimeric RH376 from the EL346-like clade contained all four regions of insertions of amino acids between the two proteins (**Fig. S3, 5a**). We named the regions RH1-4 and set out to test the hypothesis that one or more of these might be determinants of oligomeric state. Region RH1 is an insertion of 5 amino acids in RH376 compared to EL346, likely in the A'α helix of the LOV domain. Region RH2 is an insertion of 7 amino acids in RH376 compared to EL346, located in the linker between the LOV domain and α1 of the DHp domain. These residues may be in the unstructured linker or form part of an elongated α1 helix; secondary structure prediction by JPred (Drozdetskiy et al., 2015) suggests that these residues are helical (**Fig. S3**). Region RH3 consists of two insertions, the largest by far, of 19 and 11 amino acids in RH376 compared to EL346, in the ATP lid region. This insert region is predicted to have some helical structure (**Fig. S3**), unlike the ATP lid of EL346. Region RH4, located at the very C-terminus, is the only unique insertion in EL346, of 10 residues. Coincidentally, RH4 corresponds almost entirely to the highly dynamic C-terminal residues we deleted in an NMR construct of EL346, removal of which had no effect on the monomeric state of that protein (Dikiy et al., 2019). Thus, we did not pursue RH4 any further as a driver of monomer/dimer state.

Observing that the insert regions are either in previously reported dimerization interfaces for LOV domains (RH1, A'α helix) (Banerjee et al., 2016; Diensthuber et al., 2013) and HKs (RH2, DHp domain) (Qin et al., 2000; Tomomori et al., 1999), or large insertions with predicted secondary structure elements (RH3), we hypothesized that they could contribute to the dimerization of RH376 and perhaps be important for dimerization in other HKs. A homology model of the RH376 sequence based on the engineered dimeric LOV-HK YF1 (Diensthuber et al., 2013) illustrates the potential involvement of these residues in dimerization interfaces (**Fig. 5c**). We thus deleted each of the RH1, RH2, and RH3 regions from the RH376 sequence, expressed the proteins, and compared them to RH376 WT in terms of LOV photocycle, oligomeric state, and autophosphorylation activity.

Figure S4: RH376 mutant UV/visible absorbance and dark state reversion kinetics show that mutants are light sensitive with recovery times comparable to the WT (Supplemental to Fig. 6).
a) Absorbance of dark and light states along with **b)** dark state reversion kinetics show that RH376 WT and mutants are light sensitive. Based on tau values extracted from a single exponential decay fit, the photocycle of these mutants is comparable to the WT.

RH1, RH2, and RH3 proteins display the characteristic LOV absorbance triplet centered at 450 nm and thermal reversion to the dark state with time constants comparable to that of the WT protein (**Fig. S4**), suggesting that the deletions do not perturb the folding and function of the LOV domain. In RH2, we detected a small change in the relative heights of the triplet peaks, indicating that flavin binding is likely adversely affected (**Fig. S4**) despite the deletion and flavin binding occurring in separate domains (DHp, LOV). We determined the oligomeric state of each deletion mutant by SEC-MALS and found that none of the single deletion constructs elutes as a monomer regardless of dark or light conditions (**Fig. 6**). However, in each case, the apparent light-induced conformational shift seen in the WT protein is lost. In addition, the measured mass for RH2 is lower than that expected for a full dimer, suggesting, along with the changed elution volume, that the protein samples both dimeric and monomeric conformations.

Protein	Deletion	Oligomeric State	Tau (min)	Initial Velocity $\frac{nmol PO_4}{mg protein} min^{-1}$		
				Dark	Light	Light:Dark
RHWT	N/A	Dimer	61.8	8.53E-03 ± 59.9%	1.31E-02 ± 19.8%	1.54
RH1	A'α	Dimer	64.3	2.54E-03 ± 37.8%	5.41E-03 ± 98.4%	2.13
RH2	LOV/DHp linker	Monomer/ Dimer	75.4	n.d.	n.d.	N/A
RH3	ATP lid	Dimer	60.9	8.57E-04 ± 12.1%	5.71E-04 ± 11.6%	0.67
RH1+3	A'α and ATP lid	Monomer	70.2	n.d.	n.d.	N/A

Table S1: RH376 Deletion Mutant Measurements (Supplemental to Fig. 6). Oligomeric states were determined by SEC-MALS. Tau represents the time constant of the thermal reversion of the light state as fit to a first-order exponential decay (Fig. S4). Initial velocities were calculated from the linear region of autophosphorylation activity curves. Error is represented by the percent bias between trials of the same conditions. The ratio of light to dark initial velocities represents the degree of light activation that each protein undergoes.

We suspected that these deletions would not have a major impact on autophosphorylation activity of RH376, given their modest effects on photocycle and oligomerization state. As a baseline, we found that the RH376 WT protein displays robust autophosphorylation activity in the dark, comparable to that of RE356, as well as a ~1.5x increase in initial velocity upon illumination (**Fig. 6**). The RH1 mutant also shows autophosphorylation activity, although lower than that of the WT protein, with an initial velocity in the dark of about 0.3x of the WT. However, there is still an increase in the initial velocity of

autophosphorylation in the light. By contrast, RH2 shows no detectable autophosphorylation in the dark or light (**Table S1, Fig. S5**), while RH3 shows detectable but severely attenuated activity in the dark (~0.08x of the WT initial velocity) with no light-dependent increase in activity (**Fig. 6**). By comparison, EL346 shows autophosphorylation activity dramatically lower than that of RH376 WT, on the same order as that of RH3, but with a detectable light-mediated increase of ~2x (**Fig. 6**). In the case of RH376, both the RH2 and RH3 variants dramatically decrease autophosphorylation activity despite having different effects on dimerization.

Given that neither RH1 nor RH3 perturbed dimeric state yet had widely divergent effects on activity and light-activation, we sought to combine these deletions to see whether there

would be a synergistic effect. We characterized the LOV absorbance spectrum and photocycle of the so-called RH1+3 (**Fig. S4**) and saw no large perturbations. By contrast, SEC-MALS showed a dramatic change in oligomeric state, with RH1+3 existing mainly as a monomer (**Fig. 6**), though neither deletion alone showed monomeric character. Interestingly, this monomeric protein also retains the apparent compaction of the protein upon illumination, which was not seen for either RH1 or RH3 separately. Additionally, we detected no autophosphorylation activity in RH1+3 at conditions similar to the other proteins tested (**Table S1, Fig. S5**). Thus, loss of activity seems to be correlated with RH2 and RH3 deletions, with loss of RH3 region driving a loss of activity in combined mutants as well.

Our data suggest that neither of the segments targeted by RH1 and RH3 are sufficient for dimerization, as detected by SEC-MALS. However, they are apparently both necessary, as loss of both regions results in a primarily monomeric protein. For autokinase activity, all regions contribute to activity, with the order of RH1<RH3<RH2 in terms of increasing necessity for activity. Thus, activity and dimeric state are not entirely directly correlated, with the dimeric RH3 deletion lacking activity and light-activation as well as the monomeric RH1+3.

Discussion

Prior to this study, the vast majority of characterized sensor HKs were known to be dimeric; we are aware of only the monomeric LOV-containing EL346 and EL362 from *E. litoralis* HTCC2594 (Correa et al., 2013; Rivera-Cancel et al., 2014) as exceptions. Our work further expands the behavior of these enzymes to include two proteins in a slow monomer:dimer equilibrium along with three obligate dimers which exhibit a range of large-scale conformational changes upon activation. Our approach to identify these novel sensor HKs relied on sequence homology searches to find homologs of EL346, revealing a large family of related LOV- and PAS-HKs in the *Alphaproteobacteria*. Most of these proteins were Pfam (El-Gebali et al., 2019) HisKA_2 or HWE-HK-type histidine kinases, which have not been thoroughly characterized structurally (Herrou et al., 2017; Rinaldi et al., 2016; Rivera-Cancel et al., 2014), and we hypothesized that a subset of these sequences with the highest homology to monomeric EL346 might contain other monomers.

Contrary to this simple expectation, we found a diverse range of assemblies and conformational changes among five new sensor HKs characterized here. For the LOV-HK proteins, we found several dimers, including two members of the EL346-like clade of LOV-HKs (RT349 and RH376) that were dimeric in SEC-MALS analyses in both light and dark conditions. A newly-characterized member of the other LOV-HK clade (ME354) is also clearly dimeric, as

are the established EL368 and *C. crescentus* LovK (Correa et al., 2013; Purcell et al., 2010), which also cluster within that second clade.

While these new LOV-HKs are all dimeric, we observed an unexpected diversity of large-scale structural changes among them in response to blue light activation as assessed by SEC-MALS. While ME354 shows minimal change upon illumination, both RT349 and RH376 exhibit light-dependent shifts towards later elution volumes, suggesting that the proteins adopt more compact structures in the light while not changing the apparent MW of either protein. Notably, RT349 in the light appears to consist of two overlapped peaks, indicating multiple conformations, one of which corresponds to a globular dimer and the other to an expanded monomer state (**Fig. S6**); in contrast, RH376 appears to shift to a single, compacted structure. These shifts appear to be more substantial than the light-dependent motions reported in the activation of either the engineered dimeric YF1 or natural monomeric EL346 LOV-HKs (Berntsson et al., 2017; Diensthuber et al., 2013; Dikiy et al., 2019; Rivera-Cancel et al., 2014). Further studies are needed to more fully characterize these motions, particularly to determine

how they relate to the large conformational changes postulated to accompany activation from crystal structures of inactive HKs (Casino et al., 2014; Mechaly et al., 2014).

Turning to the two PAS-HKs, both RE356 and MS367 interconvert slowly enough between dimeric and monomeric states that we could separate them by size exclusion chromatography. The stable PAS-HK MS367 monomer is also active in autophosphorylation, albeit at a lower rate than the dimer, suggesting that shifts in the monomer/dimer equilibrium could regulate sensor HK enzymatic activity, as has been demonstrated or postulated for a range of signaling proteins including certain sensor HKs (Lee et al., 2008), receptor tyrosine kinases (Yarden & Schlessinger, 1987), and photoreceptors (Liao et al., 2020; Sang et al., 2005; Zoltowski & Crane, 2008; Zoltowski et al., 2013). Dimerization may be modulated by potential ligand binding in the yet-uncharacterized PAS domains of these HKs, as in other PAS-domain containing signaling proteins (Scheuermann et al., 2009), or via other mechanisms. We also suggest that the relative instability of the dimer state, which forms higher-molecular-weight aggregates over time, may serve as an intrinsic timer on the more active signaling species to temporally limit a biological response post-activation.

Given the integral role of dimerization in this possible regulation, along with its obvious necessity in *trans*-phosphorylation (Ashenberg et al., 2013) within HKs, we sought to understand the sequence determinants of dimerization with an engineering approach. Using RH376 as a model dimer, we systematically removed combinations of the three insertions it contains compared to the monomeric EL346 (**Figs 5 and S3**), hypothesizing that one or more of these changes could create a monomeric LOV-HK. While each deletion mutant was well-folded as evidenced by binding flavin chromophores and undergoing canonical LOV photochemistry, we found that all three single deletion mutants (RH1, RH2, RH3) remained largely dimeric despite the removal of 5-30 amino acid residues apiece. Each, however, lost the light-induced compaction seen in SEC-MALS analysis of the wildtype protein. Remarkably, the combination of two of these deletions to generate the RH1+3 construct both monomerized the protein and restored the light-induced conformational shift seen in the WT parent sequence. From these studies, we arrive at two general conclusions: 1) multiple regions determine oligomeric states in this family of sensor HKs, and 2) light-induced conformational changes have a complex relationship with autophosphorylation activity and do not appear to be restricted to dimeric or monomeric states.

The first of these conclusions, the use of distributed interfaces for dimerization, is consistent with structural studies on the dimeric YF1 engineered sensor kinase (Diensthuber et al., 2013) showing intermonomer contacts via residues in both the LOV domain A'α helix and

DHp domain, which are typical dimerization interfaces for LOV photosensors (Diensthuber et al., 2013; Key et al., 2007) and canonical HisKA histidine kinases (Ashenberg et al., 2013; Dutta et al., 1999) respectively. These correspond to our RH1 and RH2 deletions; neither of these changes, nor the RH3 deletion in a large loop near the kinase ATP binding site, were sufficient on its own to generate monomers, but each had markedly different effects on autophosphorylation. These data suggest different types of dimers may be generated by these deletions, some of which retain function better than others. Of surprise to us, the RH3 deletion – which removes a large loop near the ATP binding site – affected dimerization when paired with RH1. While this site has not been previously described as being involved in dimerization to the best of our knowledge, we note that ATP itself bridges interactions between multiple domains in both known HWE/HisKA2 HK crystal structures (Rinaldi et al., 2016; Rivera-Cancel et al., 2014), laying some precedent for interactions at or near the nucleotide to be involved in dimerization. Clearly, further structural work is needed to more fully assess how dimerization is encoded among multiple HKs.

In addition, our work underscores the diversity of structural changes which occur as part of the functional activation process among HKs, another long-discussed topic within the community. Considering the low-resolution conformational changes we observe by SEC-MALS upon photoactivation of the LOV-HKs tested here, we see a wide span of responses. It is clear from the detailed structures available among HKs, chiefly on inactive forms, that substantial rearrangements must be required to let catalytic domains transit long distances to phosphorylate substrate histidines within DHp domains (Diensthuber et al., 2013; Marina et al., 2005; Rivera-Cancel et al., 2014). Solution integrated structural biology approaches are beginning to reveal such changes with fewer experimental caveats than crystallographic approaches (Berntsson et al., 2017; Dikiy et al., 2019; Ghosh et al., 2019) but this essential process still remains undercharacterized at present.

The diversity of oligomeric states in a family of sensor HKs that we uncover here opens the door to novel regulatory modes within this family, such as control of dimerization, whether by ligand or inherent thermodynamic stability, or cooperativity. The closely related bacterial chemoreceptor proteins, for instance, form large arrays in order to increase signaling output through cooperativity (Muok et al., 2020). Similar use of monomer/dimer transitions and evolution of variable quaternary structure is at the heart of acquiring cooperativity and novel regulatory modes in the classic hemoglobin family of oxygen-binding proteins, which started as ancestral monomers prior to dimerizing by gene duplication events and the acquisition of a relatively small number of mutations (Pillai et al., 2020). Our findings suggest that different

oligomeric states within the sensor HK family could allow for different potential for cooperativity, perhaps depending on the input signal or output pathway, or integrating multiple points of control or regulation to be added to the canonical signal transduction pathway of two-component systems.

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Competing interests

The authors declare no competing interests.

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