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RESEARCH ARTICLE

aeBlue Chromoprotein Color is Temperature Dependent

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Abstract: ***Background*:** Marine sessile organisms display a color palette that is the result of the expression of fluorescent and non-fluorescent proteins. Fluorescent proteins have uncovered transcriptional regulation, subcellular localization of proteins, and the fate of cells during development. Chromoproteins have received less attention until recent years as bioreporters. Here, we studied the properties of aeBlue, a a 25.91 kDa protein from the anemone *Actinia equina*.

***Objective*:** To assess the properties of aeBlue chromoprotein under different physicochemical conditions.

***Methods*:** In this article, during the purification of aeBlue we uncovered that it suffered a color shift when frozen. We studied the color shift by different temperature incubation and physicochemical conditions and light spectroscopy. To assess the possible structural changes in the protein, circular dichroism analysis, size exclusion chromatography and native PAGE was performed.

***Results*:** We uncover that aeBlue chromoprotein, when expressed from a synthetic construct in *Escherichia coli*, showed a temperature dependent color shift. Protein purified at 4 °C by metal affinity chromatography exhibited a pinkish color and shifts back at higher temperatures to its intense blue color. Circular dichroism analysis revealed that the structure in the pink form of the protein has reduced secondary structure at 4 °C, but at 35 °C and higher, the structure shifts to a native conformation and Far UV- vis CD spectra revealed the shift in an aromatic residue of the chromophore. Also, the chromophore retains its properties in a wide range of conditions (pH, denaturants, reducing and oxidants agents). Quaternary structure is also maintained as a tetrameric conformation as shown by native gel and size exclusion chromatography.

***Conclusion*:** Our results suggest that the chromophore position in aeBlue is shifted from its native position rendering the pink color and the process to return it to its native blue conformation is temperature dependent.

Keywords: Aeblue chromoprotein, color shift, protein secondary structure analysis, chromophore, cold chain reporter, bioreporters.

1. INTRODUCTION

Marine sessile organisms display a wide variety of colors due to the expression of eukaryotic chromoproteins [1]. These proteins show strong structural similarity to the best-known green fluorescent protein or GFP.

Non-fluorescent chromoproteins (also called pocilloporins [2]) are characterized by intense light absorption with a molar extinction coefficient, in some cases, exceeding 100,000 and presenting virtually no fluorescence.

The chromophore conformation in chromoproteins is an isomerized non-coplanar version of the DsRed-like chromophore [3]. These proteins have a distinctive difference with most fluorescent proteins, they absorb mainly visible light, which is detectable with the naked eye [1]. Alieva and colleagues [1] have uncovered a wide variety of chromoproteins that can be used for different reporter applications.

Chromoproteins have become relevant for molecular biology applications as reporters, but only few have been studied in detail for their biochemical and spectroscopic properties.

Non-fluorescent proteins, acting as photoprotectants in the marine organisms [4], can be detected by naked eye

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inspection [5]; due to the chromophore spatial configuration [6]. The pocilloporin chromophore is composed of the typical triad Gln-Tyr-Gly which is identical to DsRed (also detected easily by naked eye inspection) but the tyrosine ring is in a non-coplanar *trans* configuration, rendering a stable chromophore resistant to different physicochemical conditions [6]. The simple detection of these proteins renders them as ideal for many molecular biology applications [7, 8]. The most important disadvantage of these proteins is that they usually form oligomers, making them not useful for protein tagging.

While designing a dual biosensor, we characterized the aeBlue chromoprotein since there are few examples of characterized blue chromoproteins reported to date [9]. We began to work with aeBlue chromoprotein, isolated by Shkrob and colleagues form *Aquinia equina* [10]. We used a synthetic construct for its expression in *Escherichia coli*, noticing that when expressed and kept inside the cellular milieu, the protein remained blue, but after lysis using freeze-thaw cycles and then kept at 4 °C, the protein turned pink. The color returned to blue when incubated at higher temperatures.

In the present work, we show that purified aeBlue shows a temperature dependent color shift from pink to blue, that is irreversible. Circular dichroism analysis revealed that the pink form shows reduced secondary structure and by size exclusion chromatography, showed that it retains its tetrameric conformation.

2. MATERIALS AND METHODS

2.1. Cell Culture and Media

Cells were grown in LB media supplemented with kanamycin (50 µg/ml) for cells transformed with ATUM pJ201 plasmid. Cells transformed with pQE30 plasmid were grown with 200 µg/ml ampicillin. Protein induction was achieved with 0.5 mM IPTG after cells reached 0.5 OD_{600}. Cells grown in the dark were incubated in a 125 ml flask covered with aluminum foil.

2.2. Strains Used in this Study

XL1-Blue strain (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F′ proAB lacIqZΔM15 Tn10 Tet*^{r}) was used to propagate plasmids and express the aeBlue protein. We also tested protein expression in M15/pREP4 (Qiagen) and BL21 Rosetta (Novagen) without observing differences (data not shown).

2.3. Plasmid Construction

aeBlue protein sequence entry in iGEM database is under the accession number BBa_K864401. This is a codon-optimized sequence. We used instead the sequence DQ159069.1 from GenBank [10]. Codon analysis rendered a CAI of 0.69 (https://www.genscript.com/tools/rare-codon-analysis) in *E. coli*.

Gene synthesis was done by ATUM in plasmid pJ201. Upon reception, the synthetic gene was amplified by PCR using primers listed in Supplementary Table **S1**, adding *Bam*HI and *Hind*III restriction sites. PCR was done with Herculase II fusion DNA polymerase (Agilent Technologies using the following amplification protocol: 94° C for 2 min, 30 cycles of 94 °C for 20 secs, 50 °C for secs and 72 °C for 30 secs, and a final 5 min 72 °C extension. PCR product and digested pQE30 vector (rendering an N-terminal 6xHis tag protein) were purified by gel electrophoresis and using PureLink Gel Extraction kit (Invitrogen) according to the manufacturer's instructions.

Ligation was done with T4 DNA ligase (Promega) according to the manufacturer's instructions. Ligation was transformed into XL1-Blue chemical competent cells and plated on LB agar plates supplemented with 100 µg/ml of ampicillin. Pale blue colonies were selected and further analyzed by growing them and induce protein expression by adding 0.5 mM IPTG. The plasmid was confirmed by automated sequencing. The same strategy was used for the cloning of JuniperGFP (accession number BBa_J97001), DsRFP (accession number BBa_E1010) and AmilCP (accession number BBa_K592009).

2.4. Protein Purification

Overnight pre-culture was grown in LB supplemented with 200 µg/mL of ampicillin. Then, 200 mL of LB supplemented with 200 µg/mL of ampicillin with constant vigorous shaking at 37 °C until an OD_{600} of 0.5 was reached. 1 mM of IPTG was added to the culture and kept in vigorous shaking at 37 °C for 18 hrs. Protein expression and maturation was verified by withdrawing 1 ml of cell culture and spinning down cells by centrifugation (2500 x g for 5 min at 4 °C) to confirm color.

Cells were collected by centrifugation (2500 x g for 5 min at 4 °C) and lysed on ice using 10 µg/ml lysozyme in PBS plus 5% glycerol for 30 min., and then two freeze-thaw (between -80 °C and 4 °C) cycles were used to complete the lysis. After lysis is completed, cell debris were removed by centrifugation at 10,000 RPMs for 10 min. The protein obtained is pink (aeBlue-pink).

For the blue form of the protein, an equally prepared lysate was incubated at 25 °C (aeBlue-blue) for 60 min, reaching a total change to the blue form.

Both supernatants were subjected to metal affinity chromatography (Qiagen) following the manufacturer's protocol for soluble protein purification, but aeBlue-pink and aeBlue-blue purification was carried at 4 °C to prevent protein degradation.

Protein purity was verified by SDS-PAGE. Protein was quantified using the DC Protein Assay (Bio-Rad).

2.5. Physicochemical Tests

Protein temperature color shift was conducted by using 10 µg of protein in PBS buffer and incubated for 10 min at the indicated temperature.

pH tests were conducted by preparing 1 M stock buffer solutions and then adjusting 10 µg of protein with the desired buffer at the indicated pH value at a final concentration of 100 mM. pH 7 controls were generated to show that the buffer did not render an effect on the protein color. For pH 1.5, HCl-KCl solution was used. The citrate-phosphate

solution was used at pH 3 and 7 to rule out the effect of the buffer itself. Citrate solution was used for pH 5. Tris-HCl buffer was used at pH 9 and NaOH-KCl solution was used at pH 12.

Tests were conducted in 0.5 ml centrifuge tubes incubated at 4 °C or at 35 °C. To record images, solutions were quickly transferred to a 96-well plate and incubated at the indicated temperature. Final pH value was confirmed using pH strips.

Urea denaturation analysis was conducted using a 4 M urea solution mixed with 10 µg protein and incubated in a 96-well plate at the indicated temperature for 10 min. Protein-urea solution was gently mixed by pipetting.

Hydrogen peroxide effect was performed by diluting H_2O_2 solution with 10 µg of protein in a 96-well plate to achieve the desired final H_2O_2 concentration. Reactions were incubated at the indicated temperature for 10 min unless stated in the figure.

β-mercaptoethanol effect was performed by diluting β-mercaptoethanol solution containing 10 µg of protein in a 96-well plate to achieve the desired final concentration. Protein was incubated at the indicated temperature for 10 min unless stated in the figure.

Light effect on the formation of chromophore was conducted as described for protein purification protocol, except that the incubation flask was covered with aluminum foil and kept in the dark while centrifuging the cells.

After the incubation time of each experiment, plates were photographed with an in-house made device using a 41-megapixel PureView Zeiss digital camera.

All experiments were conducted in independent triplicates showing the same results consistently. In all figures, we show one representative experiment.

2.6. Size Exclusion Chromatography and Native PAGE Analysis

100 µg of purified protein in PBS buffer was loaded into a previously calibrated with known standards (200, 150, 66, 29, and 12.4 kDa) Superdex S200 FPLC column at a flow rate of 0.25 ml/min and analyzed the retention time. Detection was carried at 280 nm and confirmed by SDS-PAGE (12%).

Native gel PAGE analysis was conducted in a 6% gel using Tris/glycine buffer. Samples were loaded using 20% sucrose without tracking dye. The oligomeric state was predicted in osFP web server [11].

2.7. Far-UV and Near UV-visible CD-spectra and Thermal Denaturation an Analysis

19 µM protein solution in PBS was subjected to Far-UV and Near UV-Visible CD-spectra scan and Far-UV CD-thermal denaturation analysis in an AVIV Biomedical Circular Dichroism spectrophotometer (Lakewood, NJ, USA), model 202-01.

2.8. Spectrophotometric Analysis

19 µM protein solution in PBS was analyzed by light scan spectrophotometry using a Shimadzu UV160U spectrophotometer equipped with a temperature control chamber.

2.9. Protein Model and Analysis

The protein sequence was modeled using the Phyre2 and Swiss model web servers [12, 13]. The structural model was validated with Verify 3D web server, achieving a 98.24% of the residues show an average in 3D-1D score of >=0.2 [14, 15]. Visualization of models was done with PyMol [16]. The structural alignment was generated with the TM-align online tool [17].

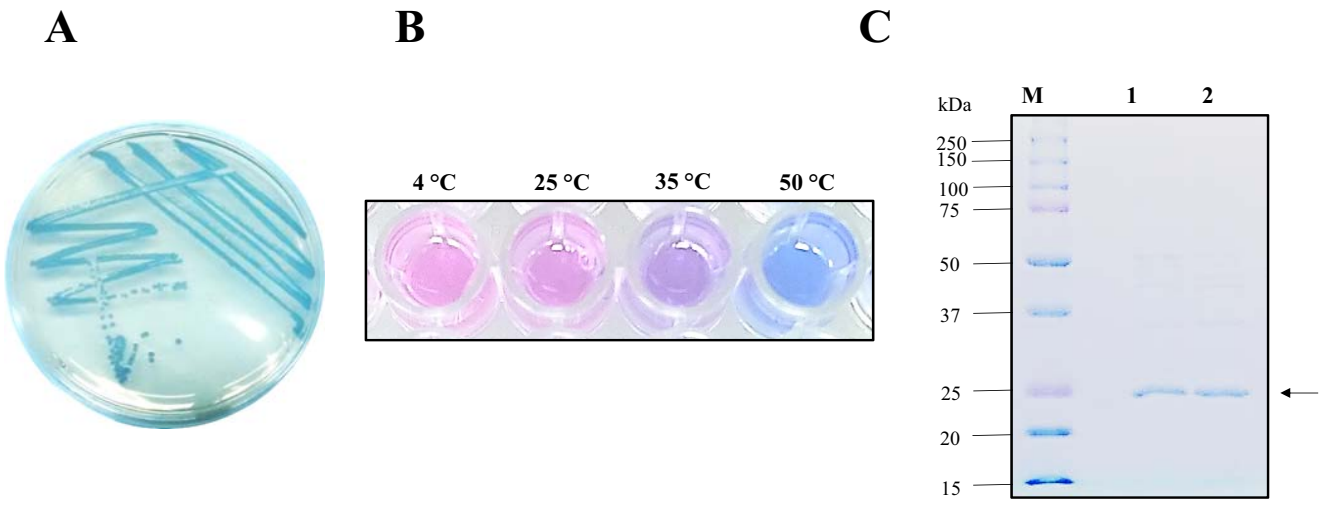

Figure 1. aeBlue expressed in *Escherichia coli* shows a color shift that depends on freezing and the temperature that is kept. Panel **A**, an LB plate of *E. coli* cells expressing aeBlue protein. Panel **B**, Purified protein at 4 °C was incubated at the indicated temperatures for 10 min. to show the color shift. Panel **C**, IMAC purified aeBlue-blue (lane 1) and aeBlue-Pink (lane 2) SDS-PAGE analysis. M, molecular size markers (Precision Plus Protein Dual color standards, Bio-rad). The black arrow indicates the expected protein size band obtained.

3. RESULTS

3.1. The Color-Shift Finding

The aeBlue protein coding sequence was synthesized and expressed in *E. coli*. As reported [7], transformants showed blue and pale blue colonies after transformation with the expression plasmid. The strong blue colonies (Figure **1** panel A) were further analyzed.

During plate storage at 4 °C, we did not notice the phenotype reported previously [7], showing that in three days colonies shift from blue towards pink. On our hands and using a different strain (XL1-Blue instead of MG1655 as previously reported [7]).

The purified protein after lysis and two freeze-thaw cycles to achieve complete cell lysis (see Materials and Methods), the resulting supernatant kept at 4 °C showed a pink color instead of the blue color observed in the cell pellet, suggesting that the freezing process may have modified the protein. Faster thawing at 25 °C also rendered the same result (data not shown).

After incubating the cell lysates or the purified protein, the color shifted from pink to blue depending on the incubation temperature, reaching the strong blue color at 35 °C after 60 min or 50 °C for 5 min. (Figure **1**, panel B and Supplementary Movie **S1**), suggesting that the transition from pink to blue at certain temperatures both forms co-exists.

Also, no difference in the color shift was observed when a cryoprotectant was present (25% glycerol, data not shown).

Shifting the purified protein at higher temperatures to achieve the blue form and then placing it back to ice or freezing, the color remained blue, showing that this process is irreversible (data not shown). We refer to aeBlue-pink to the pink form of the protein and aeBlue-blue to the blue form of the protein that was purified to near homogeneity at 4 °C or 25 °C respectively (see Materials and Methods).

In Figure 1 panel C shows the SDS-PAGE profile of both aeBlue-Pink and aeBlue-Blue of the protein (estimated molecular weight of 25.9 kDa) that were used throughout the experiments. In contrast with previous observations [10], no protein fragmentation was detected under the conditions tested here.

3.2. Absorption Spectra of the Two-color Forms of the aeBlue Chromoprotein

Spectroscopic analysis at different incubation temperatures was conducted, showing that regardless of the temperature, the aeBlue-blue form color remained unchanged (Figure **2**, panel A).

The aeBlue-pink form of the protein showed a transition from 539 nm to 593.5 nm, which corresponds to the maximum absorption peak of the aeBlue-blue form. This is confirmed in the absorption spectra at 35 °C where an increase in the blue form absorption peak is observed. The spectra also show that both forms co-exist with the transition in color as temperature rises. The pink form exhibits a lower light absorption than the blue form, even though that in all cases, the same protein amount was used during measurement. In the aeBlue-blue spectrum, a fraction of the pink form is detected at 4 °C or 25 °C as a shoulder of the spectra, suggesting that the pink form is part of the chromophore pathway formation that leads to the blue form.

3.3. Reduced Secondary Structure Content is Related to the Color Shift of aeBlue Chromoprotein.

With the above observations, Circular Dichroism (CD spectra) analysis was conducted to assess secondary structure differences between the pink and blue forms of aeBlue.

In Figure **3**, panel A the relative molar ellipticity of aeBlue-blue form is shown, indicating native secondary structure conformation.

As shown in Figure **2**, the pink color is shifted back to blue at higher temperatures, and in the CD spectra this is confirmed by an increase in secondary structure content, supporting this finding. In Figure **3**, panel B, the CD spectra of the pink form shows reduced secondary structure content at 4 °C. At higher temperatures, the protein remains less structured than the blue form throughout the temperature scan. The spectra at 35 °C shows a smother spectrum and the secondary structure content resembles the native conformation, which correlates with the blue color of the protein. This result suggests that the pink form of the protein is in an intermediate state of the folding process of the native protein or a partially denatured state by the freezing process, This is not extensive on the overall protein structure or oligomeric state. For further explore the aromatic residue chiral environment, we performed a near UV-visible CD

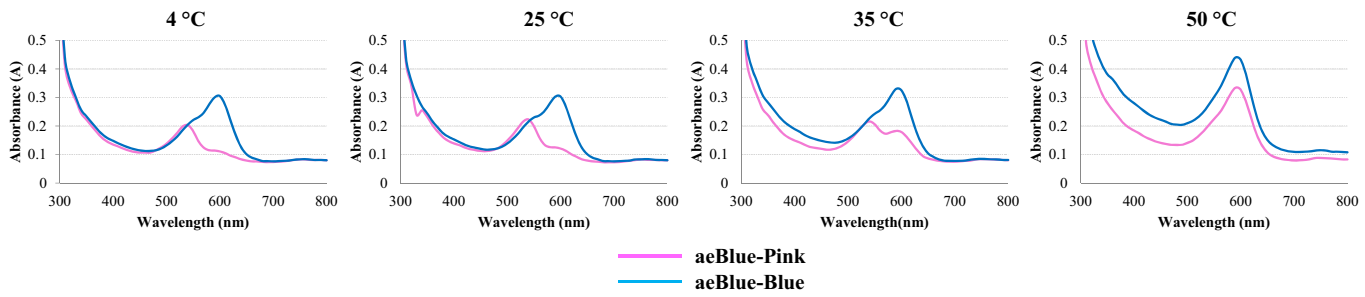

Figure 2. aeBlue protein exhibits a color transition that is temperature dependent. Absorption spectra scan using the purified aeBlue-blue and aeBlue-pink proteins at different temperatures showing the transition from pink to blue. Protein concentration was set to 19 µM concentration of both color forms.

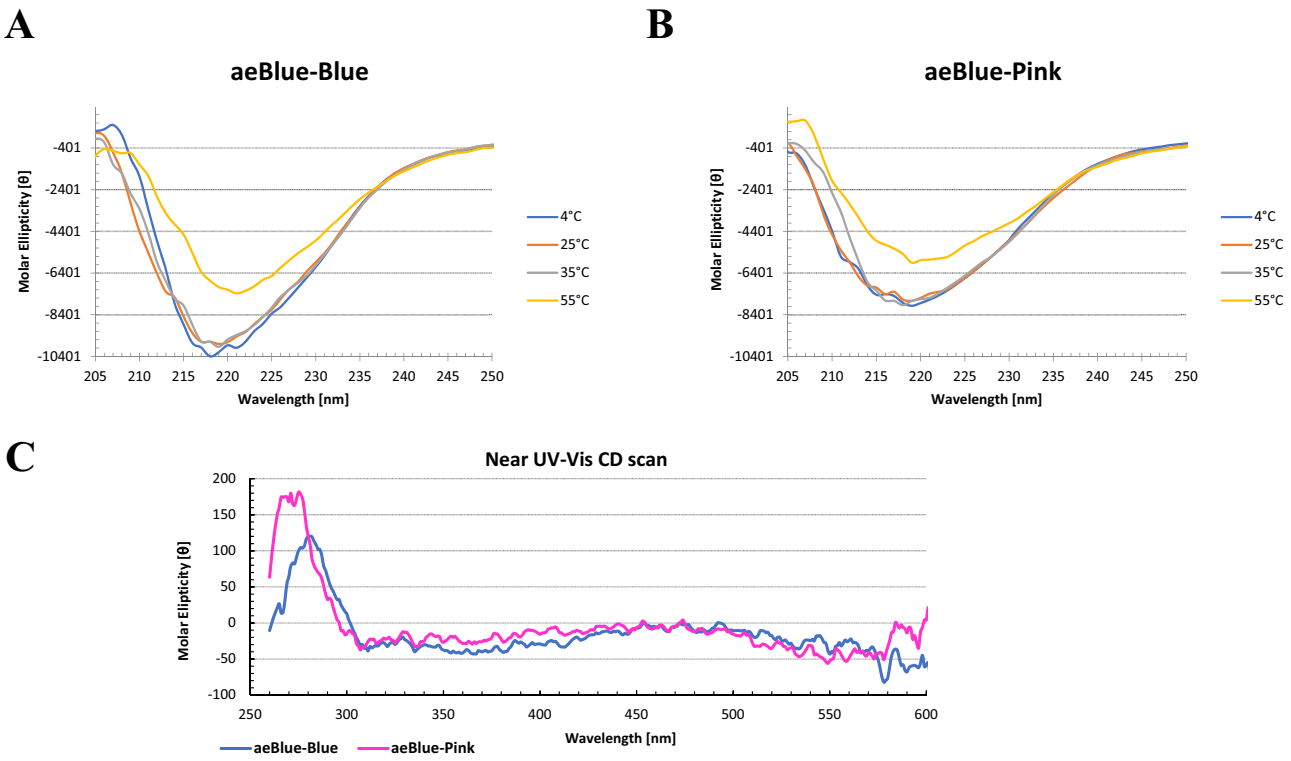

Figure 3. Circular Dichroism analysis of 19 µM samples of aeBlue-blue and aeBlue-pink proteins. Each measurement was done by triplicate and smoothing of the plot was done. The data is shown as molar ellipticity at each wavelength measured. Panel A shows the CD spectra of the Blue form of aeBlue. Panel B shows the CD spectra of the Pink form of aeBlue. Panels C the CD scan in the near UV and visible spectra of the Blue and Pink form respectively, color indicates the aeBlue form analyzed.

analysis. As shown in Figure **3**, panel C using CD spectral analysis for both forms of the protein in the near UV-visible region, suggests that either the quaternary structure is affected or the aromatic residue is shifted. With these results, the color change may be dependent on the local arrangement of the chromophore (Figure **2**) or quaternary structural modification. This observation is also supported by the slow maturation rate observed in other proteins such as the fluorescent timers [18].

Melting curve analysis coupled with CD spectra shown in Figure **4**, panel A, the denaturation rate of the pink form in comparison with the blue form at the same protein concentration (19 µm) is higher in the aeBlue-pink protein than in aeBlue-blue. At 50 °C, the pink form is turned blue and the secondary structure content is similar to the aeBlue-blue protein; suggesting that the overall structure remains mostly unchanged, and the color change is related to the chromophore positioning and not to the whole protein secondary structure.

To assess the accessibility of aeBlue-pink chromophore to denaturing agents due to relaxed secondary structure, we conducted an assay using urea at varying concentrations and incubated the protein mixture at 4 °C and 35 °C. As shown in Figure **4**, panel B, urea at different concentrations is not severely affecting the aeBlue-blue form of the protein under the conditions tested. As controls, fluorescent proteins and a blue purple chromoprotein were included in this analysis, which showed color retention or fluorescence at 1 M urea. In contrast, the pink form of the protein only retained color at 0.1 M urea, whereas at higher concentration lost its color.

At 35 °C, in a time course shown in Figure **4**, panel B, the aeBlue-Pink protein loses its color in 1M urea after 120 min, the 0.1 M exposed protein shows blue color recovery and faint blue color is shown in the higher urea concentrations. The selected proteins as controls do not change color at the same temperatures tested.

Overall, we concluded that the protein secondary structure is modified in the chromophore vicinity and the proper positioning is temperature dependent.

3.4. Chromophore Maturation by Light and Oxidants

Chromophore maturation at two different conditions was evaluated. One, growing the cells without light or exposing the purified protein to a reducing agent and two, by the exposure of the purified protein to hydrogen peroxide at different concentrations.

In Figure **5**, panel A cells grown with or without light showed the strong blue color, ruling out that the chromophore acylimine moiety requires a photoactivation step [19]. Also, the purified protein showed only a reduced intensity of color when incubated with the reducing agent β-mercaptoethanol, ruling out the need for disulfide or thiol reduction for color development (Figure **5**, panel A).

The exposure to the oxidant hydrogen peroxide did not perturb the color appearance of the pink form, by turning it

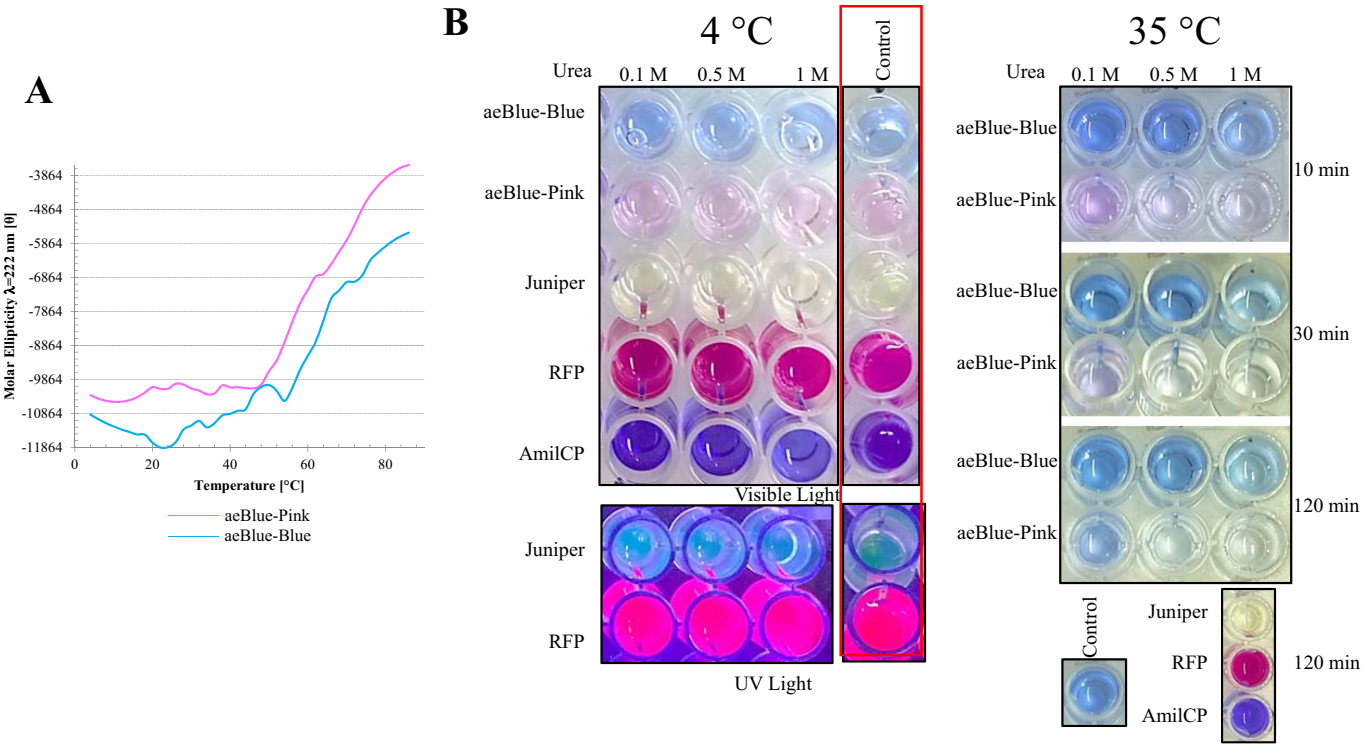

Figure 4. Denaturing profile of the aeBlue-blue and aeBlue-pink proteins. Panel **A**, melting scan CD spectra of the two forms of aeBlue. Molar ellipticity of the two proteins at different temperatures. Panel **B**, the effect of different urea concentrations. 30-minute incubation at 4 °C or at 35 °C of the different proteins is indicated. 35 °C measurements included a control condition indicated below the 35 °C figure (120 min. incubation in PBS buffer). JuniperGFP, DsRFP and AmilCP proteins were also tested as controls. DsRFP and JuniperGFP were also recorded using UV light.

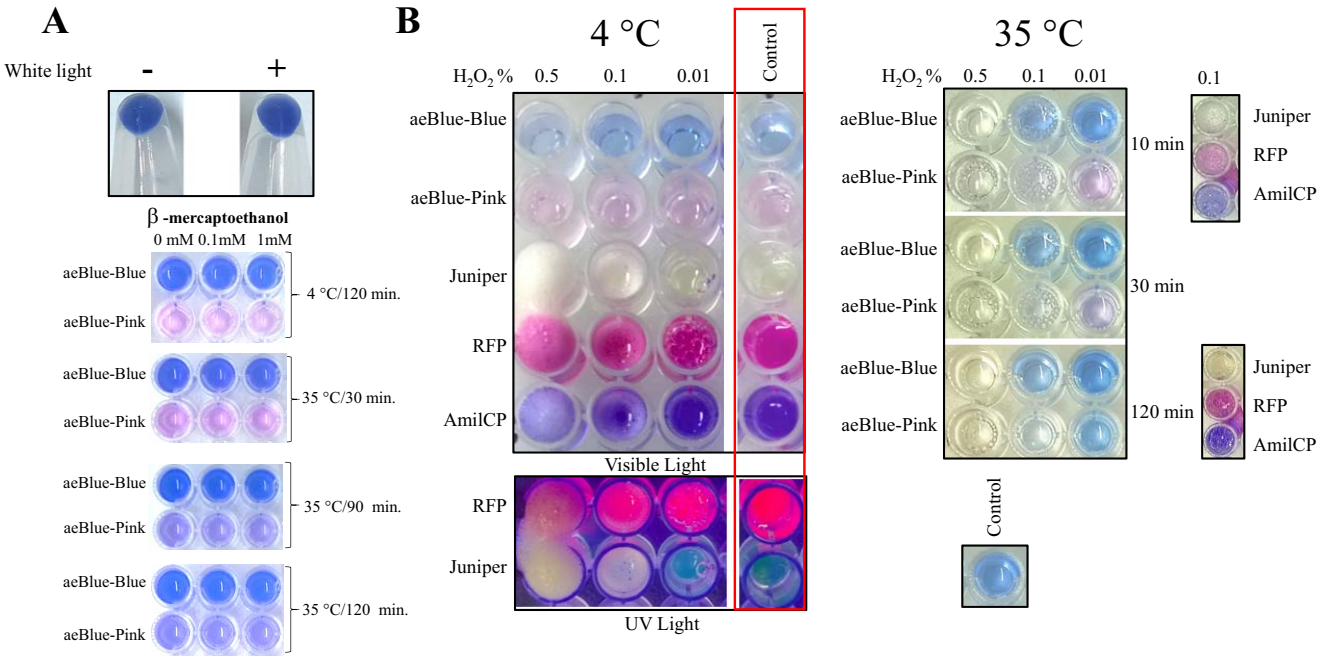

Figure 5. Chromophore perturbing conditions tests of the aeBlue-blue and aeBlue-pink proteins. Panel **A**, aeBlue *E. coli* expressing cells were grown in the absence of white light. Cell cultures were spun down by centrifugation and the cell pellet was photographed. The lower panel shows the effect of incubating the purified proteins exposed to β-mercaptoethanol at 4 °C and 35 °C at the indicated times. Panel **B**, the effect of three different concentrations of H_2O_2 on the protein color. 30-minute incubation at 4 °C or at 35 °C of the different proteins is indicated. 35 °C measurements included a control condition indicated below the 35 °C figure (120 min. incubation in PBS buffer). JuniperGFP, DsRFP and AmilCP proteins were also tested as controls. DsRFP and JuniperGFP were also recorded using UV light.

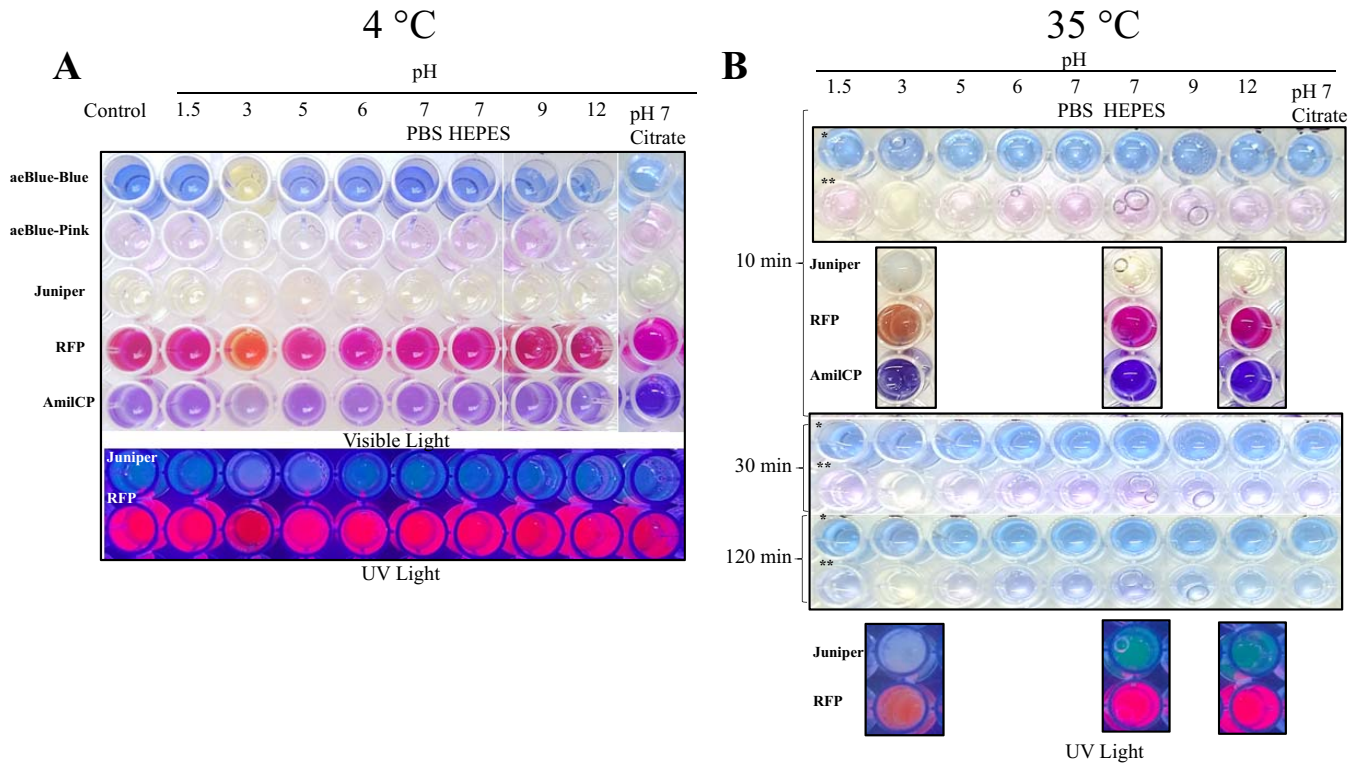

Figure 6. Chromophore stability at different pH. Panel **A**, the purified protein was incubated at different pH values for 30 minutes at 4 °C. pH is indicated at the top of the figure. pH 3 showed a reduction of color in aeBlue, DsRFP and JuniperGFP, therefore the citrate buffer was adjusted to pH 7 to rule out the effect of the buffer itself (citrate pH7 lane). PBS, HEPES and citrate buffers were used to perform this experiment. Panel **B**, same conditions tested as in Panel **A**, but incubating the proteins at 35 °C for 10, 30, and 120 min, also, we show only the controls for juniperGFP, DsRFP and AmilCP for pH 3, 7 (HEPES) and 12 since no differences were observed besides at pH 3. Proteins are indicated at each row. DsRFP and JuniperGFP were also recorded using UV light. pH 7 was tested using PBS, citrate, and HEPES to show that no effect is observed due to the buffer used. * Indicates the row for aeBlue-blue and ** indicates the row for aeBlue-pink.

blue at 4 °C (Figure **5**, panel B), only showing a reduction of color at higher concentrations. This is also observed in fluorescent proteins JuniperGFP and DsRFP.

The color shift at 35 °C is still observable in the pink form in the presence of hydrogen peroxide. The fluorescent proteins JuniperGFP and DsRFP showed an intense reaction with hydrogen peroxide (bubble formation, due to the rapid conversion of H_2O_2 into H_2O and O_2) and color reduction at high concentrations of the oxidant. JuniperGFP protein lost its fluorescence at 0.1% H_2O_2, indicating rapid oxidation of the chromophore.

3.5. pH Effect on Chromophore Formation in aeBlue

The chromophore in most fluorescent proteins (GFP-like) are pH sensitive [19, 20]. The effect of pH at 4 °C and 35 °C may be relevant to the color shift. To rule out the possible contribution of buffer alone or the counter ion on temperature color shift, controls at pH 7 were included.

Figure **6** shows that specifically pH 3 at 4 °C reduces the color of the blue and pink forms of aeBlue, while AmilCP also exhibited color loss at this specific pH value. At 35 °C, the aeBlue-blue form is resistant to this pH. Additionally, PBS adjusted to pH 3, showed the same result (data not shown). In comparison, GFP at pH 1 retains its spectroscopic properties [21], suggesting that another mechanism is responsible for color lose or chromophore positioning.

The theoretical pI of aeBlue is 6.79, while Juniper is 5.61, DsRFP is 5.65 and AmilCP is 7.6 which may partially suggest that aeBlue chromophore is sensitive at a pH value closer to its pI. DsRFP and Juniper fluorescent proteins maintained most of their color and fluorescence at the pH values tested.

Previously, a DsRFP optimized for *E. histolytica* is fluorescent in the vesicles of this parasite which are acidic [22]. JuniperGFP protein is also resistant to a wide range of pH, rendering it suitable for a broader application in non-model living organisms.

The analysis carried at 35 °C showed that the protein retained the capacity to shift color regardless of the pH in the solution after incubating at 35 °C (Figure **6**, panel B). The color shift was slower at acidic pH values, but even in longer incubation times, the color shift was completed to the blue form.

3.6. Molecular Weight Estimation of aeBlue in both Color Forms

The color shift may be related to the dissociation of the monomers of aeBlue, affecting tertiary and quaternary

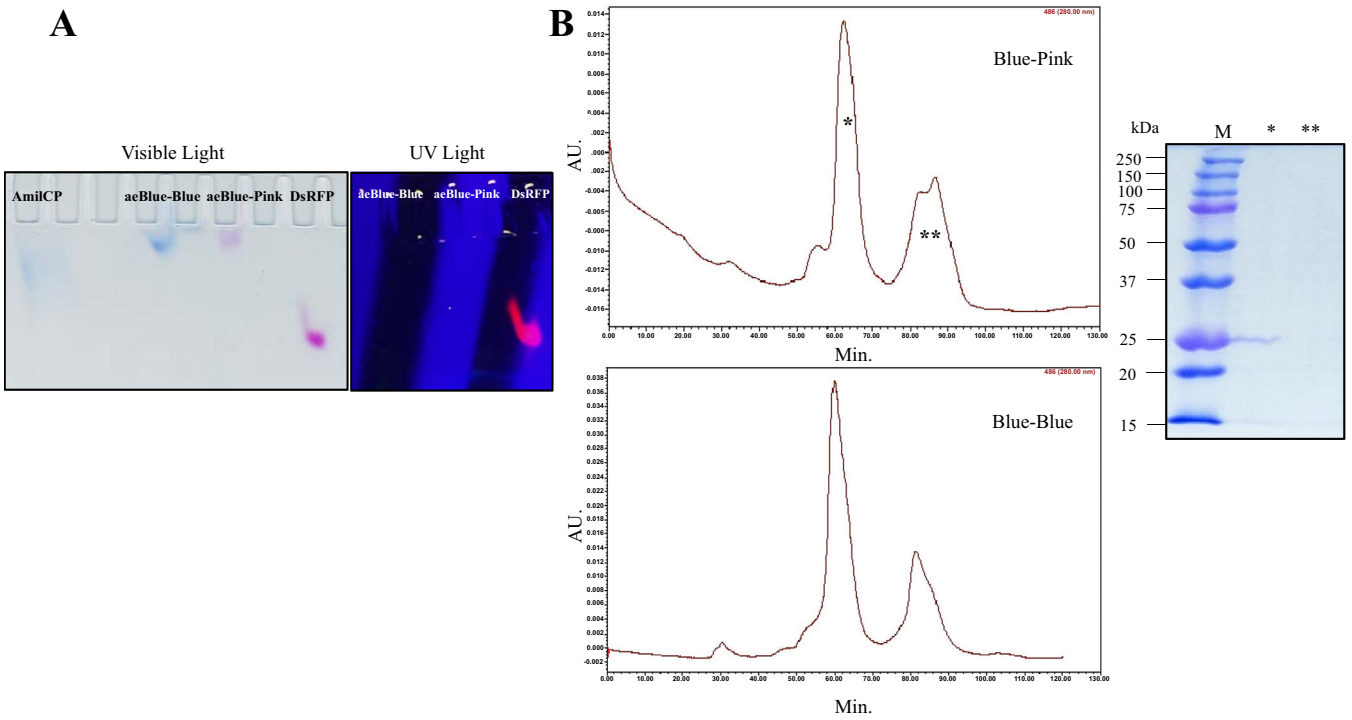

Figure 7. Molecular size estimation of the aeBlue protein forms by native PAGE analysis and size exclusion chromatography. Panel **A**, native PAGE of purified AmilCP, aeBlue-Blue, aeBlue-Pink and DsRFP proteins was conducted at 4 °C to preserve the aeBlue-pink color. UV light imaging of the same gel is shown. Panel **B**, FPLC size exclusion chromatography analysis in a Superdex 200 column of aeBlue-blue and aeBlue-pink forms. Fractions were analyzed by SDS-PAGE (right of chromatogram). M, molecular size markers (Precision Plus Protein Dual color standards, Bio-rad). Lane 1, a sample of the highest protein content in peak indicated with * in the aeBlue-pink analysis. Lane 2, secondary peak indicated by **, no protein is detected in this fraction.

structure. The native conformation predicted for DsRFP is monomeric while for aeBlue and AmilCP is predicted to be tetrameric [11]. Oligomeric conformation analysis of the two forms of aeBlue and purified AmilCP and DsRFP proteins were used for estimating molecular weight in size exclusion chromatography and net charge and migration rate in native gel electrophoresis.

Figure **7**, panel A shows that AmilCP, aeBlue-Blue and aeBlue-Pink exhibited a slow migration in a native gel, while DsRFP had much faster mobility, in agreement with the maintenance of protein net charge and perhaps oligomeric state. Strongly indicating that the tertiary and quaternary structure is maintained.

To determine the molecular weight of both forms, we used size exclusion chromatography (Figure **7**, panel B). We estimated the molecular weight for the pink form of 99.75 kDa, corresponding to a 3.84 subunit (near tetrameric) and the blue form of 115.5 kDa, which corresponds to a 4.45 subunit composition, slightly above the tetramer. The secondary shoulder shown cannot be detected by SDS-PAGE, but the absence of color suggests that is not related to aeBlue. This result indicates that the protein is in the same oligomeric conformation (tertiary and quaternary structure), but the chromophore is positioned differently between aeBlue-pink and aeBlue-blue forms.

4. DISCUSSION

In this report, aeBlue chromoprotein has been shown to change color in a temperature dependent manner, confirming a similar previous observation [7]. The data presented here suggest that the chromophore position is sensitive to freezing and relocates in a temperature dependent manner regardless of the physicochemical environment. The predicted structural model suggests that the chromophore in aeBlue is in a *cis* configuration for the aromatic residue (Supplementary Figure **S1**, panel A and B for the structural alignment of the indicated chromophores and Supplementary Figure **S2** for the orientation of the chromophore residues) and the required histidine of the chromophore is located farther in this protein family. In contrast, DsRFP-like chromophore is located in the half portion of the protein, suggesting a transition between these two positions in aeBlue.

Unlike a previous observation [7], the protein shows color change after freeze-thawing and kept at 4 °C and not *in vivo* at the same temperature. The color change can be attributed to a relocation of the chromophore due to freezing and the protein is locked in this conformation at low temperatures. CD spectra showed that the pink form of the protein exhibits a lower secondary structure but as shown by CD analysis in the near UV and visible region the aromatic

residue is most likeley to be shifted in its position, since size exclusion chromatography and native PAGE shows that aeBlue retains its tetrameric conformation.

Light, pH, denaturants, reducing and oxidant agents do not have an effect on the protein color, suggesting that this protein chromophore accesibility differs with known chromophores of fluorescent proteins [1].

4.1. Color Development

Color change may be related to the position of the chromophore residues and other relevant residues that may also participate in this process.

The model in Supplementary Figure **S1**, panel A shows the chromophore residues highlighting the relevant triad for chromophore formation and the accessory residues for chromophore color.

Residues corresponding to positions 148 and 165 in GFP numbering are needed in mutant proteins for the conversion to red and far-red emission.

The maximum fluorescence is observed in mutants in positions Ser143, Ala158 and Cys 143 pairs shifting the overall emission (Supplementary Figure **S1**, Panel A and B, [10]). This model was validated as previously reported (see Materials and Methods and ([14,15]). These residues are not present in aeBlue except for a Met residue at the equivalent Cys 143 (GFP numbering), and is part of the triad, suggesting that the pink color that is non-fluorescent in red fluorescent proteins can render an intermediate state shifting light emission into the 539 nm wavelength light absorption, as for aeBlue pink form.

Also, this protein is not affected by the reducing agent β-mercaptoethanol, the sample just showed a slight reduction in color intensity, ruling out the involvement of a thiol group in structure stability or chromophore positioning.

In Supplementary Figure **S2** shows the predicted structural position of the triad related to the chromophore of the closest structural homologous proteins crystalized to date, aeBlue shows differences regarding the position of the chromophore residues (aromatic residue in magenta), suggesting that the predicted position of the phenolic group and the histidine residue may be responsible for the color shift by positioning the phenolic group to a red-like position (lower panel), only when the structure is altered by freezing. The lower part of Supplementary Figure **S2** indicates the possible chromophore displacement, resembling the FRquadricolor phenolic group position. This protein shows a green to red color shift over time.

aeBlue mutant showing a redshift requires Lys6 and Lys7 (GFP numbering) additional mutations to maintain protein solubility (AQ14 mutant, [10]). The pink state is maintained only at 4 °C, suggesting that as described by Shkrob and colleagues [10], the Lys6 and Lys7 mutations stabilize the chromophore positioning towards red. Also, the obtained color shift to red in the mutant AQ143 is attributed to the hydration of the C-N bond of the chromophore acylimine moiety by an inner water molecule [10], supporting the shift towards pink in chromophore position in aeBlue. The size difference in size exclusion suggests that the protein is modified in its conformation slightly, showed by reduced mobility during chromatography, also a state prone to denaturing perhaps due to unspecific residue bonding.

4.2. Relationship with Fluorescent Protein Timers

The complexity of the conformation of the native chromophore will be under further scrutiny due to the native gel analysis and size exclusion chromatography that indicates that the pink and blue conformations of the protein are tetrameric, suggesting a local rearrangement of the chromophore or the surrounding residues to this region (Supplementary Figure **S2**). Finally, the conversion of color, for example, blue to red and *vice versa* occurs in other models, like in the fluorescent timers derived from mCherry [18], that change color over time.

The chromophore positioning in the pink form may be similar to the key residues involved in color change in the fluorescent timer described by Pletnev and colleagues [23] (Supplementary Figure **S1**, panel C, FRquadricolor), where residues 70, 83 and 146 (GFP numbering) are needed for the conversion from green to red over time, these residues are present in aeBlue in the same positions.

We attempted to generate random mutants to either obtain mutants unable to shift color or to retain the pink color regardless of the temperature, but no mutants were obtained with this behavior (data not shown), also targeted mutations to the chromophore triad showed only colorless colonies (data not shown).

The freezing step may partially denature the protein allowing to relocate the aromatic residue in the chromophore. This is supported with the findings in other model proteins, that after thawing deeper contact with the ice-liquid interface is generating partially denatured proteins; when slow thawing is used, the recovery of active enzyme is reduced [24], thus making aeBlue a candidate to further study the protein conformation changes due to freezing. The model presented in Supplementary Figure **S2**, panel **B**, suggest the displacement of the aromatic residue to a position similar to those proteins rendering a red color, such as the FRquadricolor protein timer. This model is supported by the results presented here in the near UV-visible CD scan and size exclusion chromatography and native PAGE.

Studies of these proteins in sea anemones and corals can provide insight if the color change is related to modifications in the water temperature along with the wellbeing of the symbionts living with them that may impact on protein folding. The organisms that attach to the sea floor display intense colors that their function is still largely unknown [25]. In some cases, the health of the organism is related to the color scheme presented and has been associated with the reduction of GFP and GFP-like protein expression [26]. Also, there is evidence regarding their role as oxygen radical quenchers were these proteins are expressed differentially in the animal during stressing conditions [27, 28]. For *A. equina*, the optimal growth rate is restricted to 18.7-19.9 °C as an optimum temperature [29, 30] suggesting that aeBlue color shift may be related to the biology of this organism.

The underlying mechanism rendering this color shift is not related to the protein fragmentation at the chromophore

as described previously [31, 32], but a conformational stable oligomeric state that the chromophore is sensitive to freezing and relocation of the chromophore residues is temperature dependent when the protein is in *in vitro* conditions.

Overall, this protein could be useful in biotechnological applications for the generation of cold storage and cold chain transportation reporter.

CONCLUSION

The present study shows that aeBlue protein exhibits a color change that is temperature dependent. Further research is needed to obtain accurate structural data from both protein states to fully comprehend the mechanism of color change.

LIST OF ABBREVIATIONS

CD spectra	=	Circular Dichroism spectra
GFP	=	Green Fluorescent Protein
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PBS	=	Phosphate Buffer Saline
PCR	=	Polymerase Chain Reaction
SDS-PAGE	=	Sodium Dodecyl Sulfate-Polyacrylamide Electrophoresis

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

In the manuscript the accession numbers used are cited. No database data (datasets) are used in this work.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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