Folding Study of Venus Reveals a Strong Ion Dependence of its Yellow Fluorescence under Mildly Acidic Conditions

Shang-Te Danny Hsu, Georg Blaser, Caroline Behrens, Lisa D. Cabrita, Christopher M. Dobson, and Sophie E. Jackson

Department of Chemistry, University of Cambridge
Lensfield Road, Cambridge, CB2 1EW, United Kingdom

1STDH and GB are joint first authors.
Address correspondence to: Shang-Te Danny Hsu (stdh2@cam.ac.uk) and Sophie E. Jackson (sej13@cam.ac.uk), Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, United Kingdom.

Venus is a yellow fluorescent protein (YFP) that has been developed for its fast chromophore maturation rate and bright yellow fluorescence that is relatively insensitive to changes in pH and ion concentrations. In this paper, we present a detailed study of the stability and folding of Venus in the pH range from 6.0 to 8.0 using chemical denaturants and a variety of spectroscopic probes. By following hydrogen-deuterium exchange of 15N-labelled Venus using NMR spectroscopy over 13 months, residue-specific free energies of unfolding of some highly protected amide groups have been determined. Exchange rates of less than one per year are observed for some amide groups. A super-stable core is identified for Venus and compared to that previously reported for GFP. These results are discussed in terms of the stability and folding of fluorescent proteins.

Under mildly acidic conditions, we show that Venus undergoes a drastic decrease in yellow fluorescence at relatively low concentrations of guanidinium chloride. A detailed study of this effect establishes that it is due to pH dependent, non-specific interactions of ions with the protein. In contrast to previous studies on E2GFP, which showed a specific halide ion binding site, NMR chemical shift mapping shows no evidence for specific ion binding. Instead, chemical shift perturbations are observed for many residues primarily located in both lids of the β-barrel structure which suggests that small scale structural rearrangements occur on increasing ionic strength under mildly acidic conditions and that these are propagated to the chromophore resulting in fluorescence quenching.

Since the identification of the green fluorescent protein (GFP) from the jellyfish Aequorea victoria in the early 1990s, a large number of fluorescent proteins (FPs) have been isolated from natural sources, primarily from marine animals and corals (1,2). These proteins have been developed further using protein engineering techniques to obtain a variety of FP with broad fluorescence emission spectra, ranging from blue to far-red (3). The FP family of proteins is now arguably the most versatile and commonly used biomarker employed in a myriad of applications in the Life Sciences (1,4-6). The impact that GFP and FP have had is clearly illustrated by the award of the 2008 Nobel Prize in Chemistry to Chalfie, Tsien and Shimomura.

The maturation of GFP and its variants involves the initial folding of the polypeptide chain into a native-like conformation that then undergoes an auto-catalytic cyclisation and dehydration of the tripeptide encompassing residues 65-67. This is then followed by a rate-limiting oxidation step to form the chromophore whose colour is determined by the side-chain composition of the tripeptide as well as its local environment (2,7,8). In the case of yellow fluorescent protein (YFP), a yellow variant of GFP, a number of mutations give rise to the red-shifted excitation and emission spectra (1). These include i) S65G, which prevents the formation of a hydrogen bond between Ser65 and Glu222, and ii) T203Y, which produces additional polarisability around the chromophore resulting in fluorescence quenching.

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state energy. Together with CFP (a cyan variant of GFP which contains a critical Y66W mutation that results in a chromophore with an indole rather than a phenol ring), YFP is often used as a fluorescence acceptor-donor pair in Förster Resonance Energy Transfer (FRET) measurements (1,4,5,9). This technique is commonly used both in vitro and in vivo to monitor changes in the intermolecular distances between CFP- and YFP-tagged interacting partners by measuring the relative intensities of both cyan and yellow fluorescence (9-11).

The spectroscopic properties of many GFP variants are known to be sensitive to pH (12) and halide ions (13). This property has been exploited in a number of applications where FPs have been developed to report on changes in the cellular environment (14,15). However, for FRET-based studies where changes in fluorescence serve as the basis for monitoring changes in distance, the sensitivity of GFP and its variants to the chemical environment is highly undesirable. For this reason, a number of GFP variants have been engineered with reduced pH and halide ion sensitivity. Citrine is one such example - it contains a Q69M mutation in addition to the four mutations that were originally introduced to derive a yellow variant eYFP (S65G/V68L/S72A/T203Y). The Q69M mutation reduces the pH and halide ion sensitivity. Citrine is one such example - it contains a Q69M mutation in addition to the four mutations that were originally introduced to derive a yellow variant eYFP (S65G/V68L/S72A/T203Y). The Q69M mutation reduces the pH and halide ion sensitivity and has an increased yield in terms of protein expression (1). Recently, a new variant of YFP called Venus has been reported to have further reduced sensitivity with respect to proton and halide ion concentrations; additionally, it has a significantly accelerated rate of chromophore maturation (within minutes rather than hours) and the highest quantum yield amongst all FP variants at the time that it was first reported (16). Instead of the Q69M mutation used in Citrine, Venus contains the mutation, F46L, which was found to accelerate the oxidation step during chromophore maturation, in addition to four others, F64L/M153T/V163A/S175G that were found to facilitate folding at 37 °C.

Despite the apparent robustness and high stability of many FPs under native conditions, emerging data suggest that the folding of GFP and its variants is highly complex, with intermediate states populated under equilibrium and non-equilibrium conditions, and parallel pathways existing on the unfolding and refolding energy landscape (17-25). In addition, hysteresis observed in the unfolding/refolding curves of one particular variant - superfolder GFP (sfGFP) – has been associated with the post-translational modification of chromophore formation (26,27). However, there remains much that is not yet known on how this important class of proteins fold.

Here, we report a study on the stability and unfolding of Venus in which solution-state nuclear magnetic resonance (NMR) spectroscopy was employed to extract the thermodynamic parameters of unfolding under native conditions by using hydrogen-deuterium exchange (HDX) experiments. In addition, fluorescence and far-UV circular dichroism (far-UV CD) spectroscopies were used to monitor the chemical denaturant-induced unfolding process under different conditions.

As a result of using the chemical denaturant guanidinium chloride (GdmCl) to study the stability and unfolding of Venus as a function of pH, we have also investigated the chloride ion sensitivity of the protein. In contrast to earlier reports (16), our results show that the fluorescence of Venus is sensitive to chloride ions at lower (pH 6.0) but not higher (pH 8.0) pHs. We report here a series of experiments using fluorescence and NMR spectroscopy to probe the nature of the chloride ion sensitivity, and use both chloride and nitrate salts to establish that the effects are not due to the specific binding of halide ions to Venus, as has been reported for GFP (13), but can be attributed instead to many small scale structural rearrangements in Venus which occur on increasing the ionic strength of the solution.

Our results contribute to the general knowledge and understanding of the stability and folding of FPs, in addition to providing important information on how these large β-barrel structures react to changes in environmental conditions. We prove that Venus is not as insensitive to chloride ions as originally thought (16), and show that small scale changes in structure can result in significant changes in the fluorescence properties of this FP.

EXPERIMENTAL PROCEDURES
Plasmid and Reagents. A plasmid containing the gene encoding Venus was a kind gift from Prof. Atsushi Miyawaki at the Brain Science Institute, RIKEN, Japan. The gene was subcloned into a pET21 vector with a hexahistidine tag at the N-terminus to aid purification. Ultra-pure guanidinium chloride (GdmCl) was purchased from MP Biomedicals, Cambridge. All other chemicals were of analytical grade and purchased from Sigma, BDH, or Melford Laboratories. Millipore-filtered, double-deionised water was used throughout. YFP (Venus) also known as SEYFP-F46L variant contains the mutations F46L, F64L, S65G, V68L, S72A, M153T, V163A, S175G, T203Y when compared to wild-type GFP.

Protein expression and purification. A single colony of transformed E. coli cells (BL21(DE3)) harbouring the Venus expression vector was picked from 2×TY ampicillin plates and used to inoculate 10 mL of 2×TY media containing 0.1 mg mL\(^{-1}\) of ampicillin. After growth at 37 °C overnight, this pre-culture was used to inoculate 1 L growth culture of 2×TY media containing 0.1 mg mL\(^{-1}\) of ampicillin. The medium was incubated at 37 °C on a shaker until the cell density at A\(_{600}\)nm reached 0.5–0.6. The cells were induced with 1 mL of 1 M IPTG and left shaking for four hours. The yellow fluorescent cells were harvested by centrifugation (SLC 4000 Sorvall rotor, at 5000 rpm, 8 °C for 10 min) and the cell pellet resuspended in 50 mM Tris, 0.3 M NaCl (pH 8.0) and lysed by sonication (Misonix Inc) on ice for 5 min (15 s pulse on and 45 s pulse off) power cycles at power level 8 with a 9 mm probe. The lysate was centrifuged at 18000 rpm (SS34 Sorvall rotor) at 8 °C for 45 min. The supernatant was pooled and loaded onto a HisTrap (GE Healthcare) column at a flow rate of 2 mL min\(^{-1}\). After washing with 25 mL of 50 mM Tris, 0.3 M NaCl (pH 8.0), Venus was eluted using a linear gradient to 50 mM Tris (pH 8.0), 0.3 M NaCl, 0.3 M imidazole run over five column volumes. The yellow fractions were pooled and concentrated (Vivaspin 20; Vivascience), which was pre-equilibrated in 50 mM Tris (pH 7.4) using a flow rate of 2.5 mL min\(^{-1}\). The purity of the YFP eluted was confirmed by SDS–PAGE and its concentration determined spectrophotometrically using an extinction coefficient of 23380 M\(^{-1}\) cm\(^{-1}\) at 280 nm.

Equilibrium unfolding experiments
The exact concentration of GdmCl solution was determined from its refractive index using an Atago 1T refractometer (Bellingham + Stanley Ltd., Kent, UK). GdmCl solution was then mixed with the appropriate buffer solution to give various concentrations of GdmCl (0 – 7 M) in 800 µL aliquots using a Hamilton Microlab apparatus (Taylor Scientific, MO, USA). 100 µL of protein solution in the appropriate buffer was added to yield a final concentration of the protein of 10 µM. 50 mM MES buffer was used for pH 6.0 and pH 6.6, and 25 mM Tris buffer for pH 7.6 and pH 8.0. The protein/denaturant mixtures were incubated at 25 °C or 37 °C for at least 7 days before measurements were taken.

Far-UV Circular Dichroism Measurements.
Far-UV CD spectra were recorded using a 0.1 cm path length cell on a Chirascan circular dichroism spectrometer (Applied Photophysics, Leatherhead, UK) with an emission band pass of 2 nm. Scans were taken between 210 nm and 250 nm at a scan rate of 1 nm s\(^{-1}\). The largest difference in signal between the native and denatured states of YFP was observed at 220 nm. The integral of the CD signal between 210-250 nm was used to monitor unfolding. The concentration of protein was 10 µM with various concentrations of GdmCl (0 to 7 M).

Fluorescence Measurements. Fluorescence measurements were taken with a Cary Eclipse 200 spectrometer (Varian, Palo Alto, CA, USA) using a 1 cm path length cuvette. For the yellow chromophore, the excitation wavelength was 488 nm with a band pass of 5 nm for both excitation and emission. The largest difference in fluorescence between the native and denatured states was observed at 527 nm, and the emission at this wavelength was used in the subsequent analysis. For tyrosine fluorescence, the excitation wavelength was 275 nm. The largest difference in fluorescence between the native and denatured states was observed at 308 nm.
Determination of the pK_a of the yellow chromophore

The pK_a of the p-hydroxy benzylidene imidazolidinone chromophore of Venus was determined by monitoring the fluorescence (527 nm) or absorbance (516 nm) of the protein as a function of pH in a low ionic strength 10 mM MOPS-MES buffer. The pK_a was measured in the absence of added salt in addition to the presence of 0.4 M NaCl or NaNO_3. The data were fitted to the equation 1:

\[ F = A + B \left(1 + 10^{(pK_a - pH)_{\text{fit}}}ight)^{-1} \]  

where \( F \) is the fluorescence, \( A \) and \( B \) are variables defining the baselines, and \( n_H \) is the Hill coefficient. UV-Vis measurements were taken with a Cary 400 Scan (Varian, Palo Alto, CA, USA).

Two-State and Multi-State Model Analysis

Equations for the fitting of unfolding data to a two-state and three-state model have been derived and described in detail elsewhere (22). Equation 2 is for a simple two-state model:

\[
S = \frac{(\alpha_N + \beta_D[D]) + (\alpha_D + \beta_N[D]) \exp \left( \frac{m_{D,N}}{RT} [D] - [D]_{50N} \right)}{1 + \exp \left( \frac{m_{D,N}}{RT} [D] - [D]_{50N} \right)}
\]  

where \( S \) is the observed intensity of the optical property, \( \beta_N \) and \( \beta_D \) are the slopes of the native and denatured baselines, respectively, and \( \alpha_N \) and \( \alpha_D \) are the intensity of optical properties of the native state and denatured state, respectively in the absence of denaturant, \( R \) is the gas constant, \( T \) is the absolute temperature, \( m_{D,N} \) is a constant related to the average fractional change in the degree of exposure of residues on unfolding, and \([D]\) is the concentration of denaturant. \([D]_{50N}\) is the midpoint of the unfolding transition and concentration of denaturant at which 50% of the sample is unfolded and 50% folded. \( \Delta G_{D-N}^H \) is the difference in Gibbs' free energy of native and denatured states, can be calculated using Equation 3.

\[ \Delta G_{D-N}^H = m_{D-N}[D]_{50N} \]  

The data from yellow fluorescence, tyrosine/tryptophan fluorescence and far-UV circular dichroism experiments were fitted globally to equation 1 using a shared \( m_{D,N} \) value and the software package from Graphpad Prism (Version 5).

NMR hydrogen-deuterium exchange (HDX) experiments. 600 µL of ^15N-labelled Venus, at a concentration of 140 µM and buffered in 20 mM Tris-HCl (pH 8.0), was flash-frozen and lyophilized overnight prior to the HDX experiment. 600 µL of 99.9% D_2O pre-warmed to 37 °C was added to the lyophilized NMR sample and the resulting solution was immediately transferred to a 5 mm NMR tube. A series of 2D [^15N-^1H] HSQC spectra were recorded over 48 hours at 37 °C using a 700 MHz Bruker AVANCE NMR spectrometer equipped with a cryogenic probehead (Bruker BioSpin, Karlsruhe, Germany). 1D proton spectra were recorded before and after the HDX series and the peak intensity of the most upfield-shifted methyl resonance (δ^1H = -1.0 ppm) was used as a reference for normalisation. Over the next 13 months, the NMR sample was sealed with parafilm and incubated at 37 °C in between the NMR measurements, with intervals from days to months. For each time point, a 1D proton spectrum was recorded for intensity normalisation with respect to the initial time point and a 2D [^15N-^1H] HSQC spectrum was recorded to monitor the intensity changes of individual amide ^15N-^1H correlations, using the previously reported assignments (BMRB entry 15826) (28). The individual peak intensities were normalised and fit to a single-exponential decay, \( I(t) = I_0 e^{-k_{ex} t} \), using the rate analysis module within Sparky (29). The observed HDX rate constants of amide protons, \( k_{ex} \), were used to calculate the free energy of unfolding, \( \Delta G_{HX} \), defined as

\[ \Delta G_{HX} = -RT \ln \left( \frac{k_{in}}{k_{ex}} \right) \]

under EX2 conditions. The sequence-dependent intrinsic HDX rate constants, \( k_{in} \), and the corresponding free energy of unfolding were calculated using an Excel spreadsheet obtained from the Englander laboratory (http://hx2.med.upenn.edu/download.html).
**RESULTS**

Residue-specific free energy analysis of folding equilibrium under native conditions by NMR HDX experiments. In order to make a direct comparison between the stability and unfolding of Venus with that previously reported for GFPuv (22,23), a detailed amide hydrogen/deuterium exchange (HDX) study on Venus was undertaken. These experiments have been widely used to probe (un)folding intermediates and to extract the free energy of unfolding of proteins at a residue-specific level (see ref. (30) and references therein). The latter requires that the system under investigation is in the so-called EX2 regime – the exchange rate is proportional to the catalyst concentration, i.e., pH for a base-catalysed exchange reaction – which has already been established for GFPuv under similar experimental conditions (22). Our previous studies on GFPuv revealed a super-stable core of amide groups that are highly resistant to exchange even in the presence of GdmCl over a period of three months (23). In order to obtain a similar quantitative description of the stability and super-stable core of Venus, we have extended the HDX experiments at 37 ºC to over 13 months in order to measure, with confidence, the rates of HDX for even the slowest exchanging amide groups. These groups have exchange rates, which are in the range of $10^{-6}$ min$^{-1}$, or once per year, e.g., M218 and Y106 (Figure 1). An initial qualitative comparison reveals that Venus exhibits a more extended degree of protection under native conditions (pH 8.0 and 37 ºC) than GFPuv (where the amides of 13 residues I14, L15, L60, Y92, V93, Y106, V112, K113, N120, N121, L201, M218 and L220 are protected after one month of HDX in the presence of 0.5 M GdmCl; underlined residues remained protected after 12 weeks of HDX) (23). The additional highly protected residues in Venus are clustered between β-strands 10 and 11 (A206, Y219 and L221) and at the ends of the β-barrel structure. Structural mapping of these highly protected amide groups reveal two well-defined hydrogen bond networks, centred in β-strands 5 and 11 with Y106 and M218 having the lowest exchange rates within the cluster. Structurally, these two hydrogen bond networks form an extended β-sheet (Figure 1C) while topologically they cluster at the N- and C-termini of the primary sequence, particularly the latter where four out of the six highly protected hydrogen bonds involve four consecutive residues, M218-L221, in the last strand, β11 (Figure 1D).

The rates of exchange of individual amide protons, spanning more than four orders of magnitudes, were used to derive the free energy of unfolding, $\Delta G_{\text{HDX}}$, at the resolution of individual amide groups under native conditions. Plotting the results as a function of residue number reveals that the free energies of unfolding are higher for residues in β-strands with an average value of 12.5 ± 0.9 kcal mol$^{-1}$ (n = 44), and lower for those in loop regions or α-helices with an average of 8.3 ± 0.8 kcal mol$^{-1}$ (n = 13). The overall pattern of the observed free energies of unfolding are in line with the observed rates of exchange, with amides in β4-β6, β10, and β11 exhibiting markedly higher stabilities compared to those in other β-strands (Figure 1).

**Chemical denaturation studies and the stability of Venus as a function of pH.** A number of studies have used the fluorescence of the post-translationally formed chromophore as a sensitive probe of the state of the protein to monitor both the un/folding and stability of a number of FP variants, including Citrine, GFPuv, sfGFP and DsRed.
(19-23,26,27,31-33). Previously, we have studied the chemical denaturation of both GFPuv and Citrine using the intrinsic green and yellow fluorescence, respectively (22,23,32). For GFPuv, large kinetic barriers to unfolding were observed, which result in the system taking a significant amount of time to reach equilibrium (approximately 3 months) (22). Here, we have measured the stability of Venus with the chemical denaturant GdmCl and, similar to the results described above, find that the chemical denaturation curves also shift as a function of incubation time (data not shown). Because of the very long timescales on which it takes the system to fully reach equilibrium, we have chosen to work under non-equilibrium conditions and samples were incubated at 25 °C for seven days prior to measurements (unless stated otherwise). Although the thermodynamic parameters that are obtained (Table 1) are not true equilibrium values they are a good measure of the stability of the protein under different conditions and a comparative analysis of the data is reliable.

The chemical denaturation of Venus was measured over the pH range 6.0 to 8.0. At higher pH values (7.6 and 8.0) the protein was found to be very stable, with a single unfolding transition which has a midpoint at approximately 5.8 M GdmCl (Figure 2A). Intriguingly, at pH 6.6 and below, an additional transition was observed at a much lower denaturant concentration (approximately 1.0 M GdmCl). This is very pronounced at pH 6.0 leading to a biphasic transition profile (Figure 2A). One explanation for these results is that a partially structured intermediate state with decreased fluorescence is populated at low denaturant concentrations under these conditions.

In order to investigate this further, tyrosine and tryptophan fluorescence were used as independent structural probes of the GdmCl-induced unfolding (Figure 2B). In good agreement with the results obtained using the yellow fluorescence, a progressive reduction in the stability of Venus was seen on decreasing the pH from 8.0 to 6.0. However, the initial decrease in signal observed at low concentrations of denaturant at pH 6.0 and 6.6 with yellow fluorescence was not detected. In addition, far-UV CD spectroscopy was also employed. In this case, a single unfolding transition was observed at all pH values (Figure 2C) consistent with the tyrosine and tryptophan data. Together, these results suggest that the initial loss in yellow fluorescence observed at pH 6.0 and 6.6 at low concentrations of GdmCl is not due to an unfolding event but instead that quenching of the fluorescence occurs possibly as a direct result of using GdmCl and the interaction of chloride ions with Venus.

**Urea and NaCl titration curves.** One possible explanation for the results described above is that, contrary to what has previously been reported (16), the fluorescence of Venus is sensitive to chloride ions at pH values of 6.0 and below. In order to investigate the origin of the loss in fluorescence at pH 6.0 in the presence of low concentrations of GdmCl, experiments in urea were performed.

In contrast to GdmCl, urea is a non-ionic chemical denaturant which has roughly half the denaturing strength of GdmCl. A titration of Venus with urea shows that the yellow fluorescence remains constant up to a denaturant concentration of 4 M (Figure 2D), strengthening the hypothesis that the initial loss of yellow fluorescence observed in the GdmCl titration is the result of interactions between the protein and chloride ions, rather than an unfolding-induced solvent exposure of the chromophore. In order to confirm this, an additional titration of native Venus with sodium chloride was undertaken and the results obtained are in good agreement with the GdmCl titration up to about 1 M in each case (Figure 2D). Together, the results strongly suggest that under mildly acidic conditions chloride ions (from GdmCl or NaCl) have a profound effect on the yellow fluorescence of Venus.

**Measurements of the pK_a of the p-hydroxy benzylidene imidazolidinone chromophore of Venus.** One mechanism by which chloride ions may lead to a quenching of the yellow fluorescence of Venus is by inducing a shift in the pK_a of the p-hydroxy benzylidene imidazolidinone chromophore, as it is well established that the anionic form of the phenolic chromophore has higher fluorescence at 527 nm compared with the neutral form (13). In order to investigate this possible mechanism, the absorbance and fluorescence of Venus was measured as a function of pH over the pH range 5.5 to 8.0 in a low ionic strength MOPS-MES buffer in the absence
and presence of 0.4 M chloride ions, Figures 3 and S3. As a control, 0.4 M NaNO₃ was also used to determine whether the effects were specific and due to halide ion binding, or non-specific and due to changes in ionic strength. The data in Figure 3A clearly show that both chloride and nitrate ions result in a significant loss in fluorescence which is most pronounced at lower pH. Analysis of the data, however, shows that there is relatively little change in the pKᵢ of the yellow chromophore on addition of either salt, Figure 3B, Table 2. Similar results were obtained when absorbance was used (Figure 3C,D, Table 2). The Hill coefficients varied from 0.7 to 1.2 but generally were close to one suggesting a single titratable group. Table 2. However, there was not a clear isobestic point in the absorbance measurements, Figure 3C, suggesting that the system shows complex behaviour on protonation/deprotonation. In addition, a blue shift in the peak corresponding to the neutral form of the chromophore was observed on addition of chloride or nitrate ions, Figure 3C. The results indicate that neither chloride nor nitrate ions shift the pKᵢ of the chromophore in Venus establishing that this cannot be the cause of the observed quenching. The results also suggest that the effects are non-specific and are most likely attributable to changes in ionic strength. The addition of 0.4 M NaCl or NaNO₃ to a solution of Venus in a high ionic strength citrate-phosphate buffer at pH 6.0 had no effect on the fluorescence (data not shown) confirming our hypothesis that the effects are largely due to changes in ionic strength.

Structural effects of chloride and nitrate ions on Venus. In order to investigate the effects of both chloride and nitrate ions further, NMR experiments were undertaken in which ¹⁵N-labelled samples of Venus at pH 6.0 were titrated with sodium chloride or sodium nitrate. Perturbations to the chemical shifts were monitored as independent structural probes. In both cases, the results of the titrations show marginal changes in the overall appearance of the [¹⁵N-¹H] correlations of Venus, indicating little significant conformational change upon the addition of either chloride or nitrate ions up to a maximum concentration of 0.4 M (Supplementary Material, Figure S1). Additionally, the combined (¹⁵N and ¹H) chemical shift perturbations (see Materials and Methods for definition) of individual residues showed only a few significant changes for residues scattered in a number of β-stranded regions, particularly in β-strands 2 and 8-10 (Figure 4A). Collectively, the [¹⁵N-¹H] correlations of Venus exhibit progressive down-field shifts changes on increasing chloride or nitrate ion concentrations (Figure 4A and Supplementary Material, Figure S1). Structural mapping of the residues that show significantly large chemical shift perturbations shows that most of these residues are located at the ends of the β-barrel structure; that is, in the loops or helices in both lids or at the ends of individual β-strands (Figure 4B and 4C). For example, while the backbone amide group of Trp57 (which is buried) shows limited chemical shift changes, those of the side-chain indole group (which is exposed) are significantly larger, suggesting that the effect may be associated with ion accessibility rather than specific coordination of an ion in the interior of the protein. The latter has been proposed based on previous crystallographic reports which have shown a well-defined halide ion binding site located in the proximity of the hydroxyl group of the tyrosyl ring of the chromophore, and close to His148, the residue that is associated with the pH sensitivity of GFP fluorescence (13). The maximum chemical shift perturbation of His148 in this study is very small (0.076 ppm). In conclusion, our NMR data show little evidence to support a specific chloride or nitrate ion binding site in Venus.

DISCUSSION

GFP and its family members are central to modern biological and medical sciences because of their versatility and robustness in a multitude of imaging and spectroscopic applications (1,4-6). Critical to the usefulness of FP variants is the ability to achieve efficient folding, rapid maturation of the chromophore, high quantum yields, i.e. brightness, and reduced sensitivity to environmental changes, such as pH and ion concentrations. For this reason, Venus has become one of the most commonly used FP variants for imaging and FRET studies both in vivo and in vitro, and it has been used in both ensemble measurements and single molecule experiments (1,16,34).
Recent studies on the stability and folding of GFP and some of its variants have established that the protein has a complex energy landscape with a number of different intermediate states, high energy barriers and multiple pathways (18-27,31,32,35). Fully characterising this energy landscape, and establishing whether there is a general underlying folding mechanism for the complex β-barrel topology of FPs represents a significant challenge. To gain a better understanding of the folding of FPs in general, we have carried out extensive biophysical studies on Venus using a variety of spectroscopic tools.

At pH values of 7.0 and higher, the native state of Venus is extremely stable with a midpoint of unfolding of over 5.8 M GdmCl (after 7 days of equilibration in denaturant at 25 °C). This is similar to previous studies which have reported high stabilities for GFP and other FPs (36). In our previous studies on the unfolding/folding behaviour of a truncated version of GFPuv, we observe that the protein takes a long time to fully equilibrate in the presence of chemical denaturants due to the high energy barriers of unfolding (22). Venus shows similar behaviour (data not shown), however, a full time-dependent analysis of the unfolding was not undertaken here. Instead, stability measurements at different temperatures and pHs were made after the same equilibration period (1 week) to enable us to perform a comparative analysis. The data clearly show that there is a rapid decrease in stability on lowering the pH from 7.6 to 6.0 (Figure 2 and Table 1).

It is difficult to compare quantitatively the stability we have measured here for Venus with that previously measured for other FPs, as different experimental conditions and equilibration times have been used. However, measurements have made on GFPuv at pH 6.0 and 25 °C, after 5 and 13 days of incubation in GdmCl. In this case, the midpoints for unfolding are approximately 2.75 and 2.50 M, respectively, which can be compared with the midpoint for Venus measured under similar conditions after 7 days of 3.5-3.6 M GdmCl (Table 1). Thus, Venus is more stable towards chemical denaturation than GFPuv.

In order to compare in further detail the unfolding/folding and stability of Venus with GFPuv, a comprehensive series of H/D amide exchange measurements were made. For GFPuv, we have established that many amide protons are very resistant to exchange, effectively undergoing no exchange at all even at high pH (8.6), and temperature (37 °C) and in the presence of 1 M GdmCl (22,23). With the aim of obtaining accurate exchange rates for even the most slowly exchanging amide protons of Venus, HDX measurements were made at pH 8.0 and at 37 °C for over 13 months and HDX rates spanning over four orders of magnitude were obtained (Figure 1). Free energies of exchange were calculated and are shown in Figure 1. For many of the amide groups in the β-strands of the barrel structure, high values for ΔGd,HX are obtained (approximately 12 kcal mol⁻¹), indicating that exchange probably occurs only on global unfolding, which under the conditions used is a rare event. In comparison, amide groups in loop and helical regions have lower values which cluster around 8 kcal mol⁻¹ suggesting that there are also local unfolding events which lead to exchange. A comparison between the free energies of unfolding calculated using the yellow fluorescence from the chemical-denaturation experiments and those calculated from the HDX measurements reveals that for some of the most protected amide groups in β-strands 4-6 and 11, values of ΔGd,HX are higher than for ΔGd,H 0 (Figure 1E). Although some caution has to be used when comparing the different datasets which are measured under different (equilibrium versus non-equilibrium conditions), the data suggest that these regions exhibit a higher stability than that reported by the chromophore and form a super-stable core within the structure.

For both GFPuv and Venus there is evidence that β-strand 7 is the least stable β-strand based on the NMR HDX data (Figure 1D). Consistent with this are previous simulation and NMR studies which have suggested that the formation of β-strand 7 is the last step in the folding pathway, given the observed internal dynamics that are most abundant in this region in the native state (37,38). In addition, a recent in vitro biomolecular fluorescence complementation (BiFC) study has provided further evidence that the folding of β-strand 7 is involved in the last step of the β-barrel closure of a circularly permutated and truncated GFPuv variant (39).
A comparison of the results reported here for Venus with those previously published for GFPuv (22,23) shows that the super-stable core of highly protected amide groups in Venus is similar to GFPuv and includes residues in β-strands 1-2 and 4-6. These residues form a mini β-sheet, which for GFPuv we have postulated forms a critical folding nucleus (22). However, the results on Venus show that this region is more extended and also comprises further residues located in β-strands 2, 4, and 6, but also many in β-strands 10 and 11. This leads to two hydrogen-bonded networks which centre around residues in β-strands 5 and 11 and which together cluster and link N- and C-terminal regions of the protein (Figure 1D). The exact reasons why Venus displays a larger super-stable core compared to GFPuv are not known, however, it is noted that Venus has a higher global stability than GFPuv.

For GFPuv, the highly protected super-stable core of amide groups is proposed to form a potential folding nucleus as it constitutes the most stable region of structure within the protein. For Venus, the folding nucleus is more extensive, and may well be more stable (Figure 1F). Notably, it incorporates regions of the protein at both the N- and C-termini of the structure as described above. It is therefore interesting to speculate that the formation of such a stable nucleus leads to faster folding kinetics and an accelerated maturation of the chromophore, as has been observed (16). This is approaching the time scale that is required for translation of the complete primary sequence of the protein. Therefore, the chromophore formation of Venus may be coupled to protein synthesis in a co-translational manner which we have devised an experimental strategy to probe by solution state NMR spectroscopy (40,41).

Robust co-translational folding of FPs is crucial in the context of studying protein stabilities through the use of FPs as fluorescent fusion tags (42-44). It has been shown that fusion of GFPuv at the N- or C-termini of the target proteins can lead to an increased aggregation propensity (17). While a number of “superfolder” FPs have been developed to achieve very robust refolding efficiencies (45), there is still room for improvement through protein engineering, e.g., by supercharging to prevent aggregation (46).

Not only is it crucial to obtain robust folding efficiencies and stabilities for full-length FPs but also enhanced structural integrities of FP fragments. In addition, spectroscopic properties that are insensitive of the local environment are critical for many applications including BiFC. In this technique, binary complex formation is monitored through the emergence of fluorescence upon FP fragment complementation (47). A recent in vitro BiFC study that used the superfolder YFP as a model system showed that BiFC can be biased by the nature of the binding events, which are salt-dependent: the two examples presented therein exhibited opposite salt-dependency when the yellow fluorescence was used as a readout (48). Interestingly, high pH (7.6-8.8) facilitated BiFC while a complete loss of BiFC was observed at neutral pH (7.2) in the presence of 150 mM NaCl.

Through the use of GdmCl in our unfolding experiments, in addition to other experiments reported here, we have established that Venus is sensitive to chloride ions under very mildly acidic conditions, Figures 2 and 3. This important result is in contrast to previous studies which suggested that Venus is less sensitive to proton and halide ion concentrations than other FPs (16).

In order to understand the mechanism by which chloride ions result in a decrease in fluorescence at pH 6.0, the pKₐ of the p-hydroxy benzylidene imidazolidinone chromophore of Venus was measured under different conditions, Figure 3 and Table 2. Although a significant loss in fluorescence was observed at pH 6.0 in the presence of 0.4 M chloride or nitrate ions (Figure 3A) it could not be attributed to changes in pKₐ which varied little (Table 2). The Hill coefficients associated with the pKₐ fits were found to be near unity suggesting a single titratable site (Table 2). However, the absorbance measurements did not show a clear isobestic point, Figure 3C, suggesting more complex behaviour. In addition, a blue shift in the absorbance of the neutral form of the chromophore was observed on addition of chloride or nitrate ions, Figure 3C.

A number of groups have studied the pH and anion dependence of different fluorescent proteins, and have shown that the pKₐ of the chromophore can be modified both by mutation of residues close to the chromophore,
or by halide ion binding. For example, the Remington group showed that mutation of His148 to glutamine or glycine, increases the pK\text{a} of the chromophore from 7 to 8 in a YFP variant(49). However, these mutations also caused the loss of the isobestic point in pH titrations(50), similar to our observations on Venus, indicating that multiple processes occur simultaneously. In addition, they observed a blue shift in the absorption maximum of the neutral form of the chromophore upon the addition of chloride(50). Venus also demonstrates this behaviour and there is a blue shift in the neutral form from 421 to 400 nm upon addition of 0.4 M chloride or nitrate ions, Figure 3C, which suggests that destabilization of the transition dipole of the neutral form results from a decrease in the polarity of the environment near the chromophore, providing evidence that this is a ground-state effect. CD analysis in the visible region showed two negative peaks at 395 nm and 516 nm (data not shown) corresponding to the anionic and neutral forms of the chromophore, respectively. The addition of either chloride or nitrate ions to Venus did not alter the peak positions or intensities in contrast to YFP-H148Q suggesting that Venus does not have a specific anion binding site, and the sensitivity of the protein towards chloride ions to the substitution of F64L that is thought to prevent chloride ion binding through a series of conformational rearrangements (51), the crystal structure of E\textsuperscript{2}GFP showed that the F64L replacement is responsible for creating a specific halide-binding site, and the sensitivity is further increased by the S65T and T203Y substitutions (13).

YFP-H148Q was reported to contain a specific halide-binding site (near the chromophore) altering its pK\text{a} from 7.14 in the absence of anions to 7.86 in 150 mM chloride(50)(52). A crystal structure of this variant identified this binding site(53).

A definitive binding site for either chloride or indeed nitrate ions however, was not apparent from the NMR titrations performed on Venus, Figure 4. Changes in absorbance and fluorescence with increasing chloride or nitrate ion concentrations, Figure 3, occur concomitantly and show these anions affect molar absorbance rather than quantum yield. In agreement with our findings, fluorescence lifetimes of YFP-H148Q were independent of both pH (6.0-8.0) and chloride ion concentration (0-400 mM) suggesting a ground state effect(50).

Galietta and coworkers screened a mutant library of 1536 clones of the YFP-H148Q and selected variants with increased halide ion sensitivity(52). They measured halide ion binding affinities and associated shifts in pK\text{a} for a subset of these. Similar to the results reported here, the fluorescence of the mutants was found to be both pH-sensitive and also influenced by the presence of anions(52).

In order to further understand the effects of chloride ions on Venus fluorescence, and to establish whether there is a halide ion binding site in the protein, NMR was used to obtain residue-specific information on the structural changes induced in Venus on the addition of high concentrations (up to 0.4 M) of sodium chloride. In particular, we wanted to see if chloride ions induced changes in the chemical shifts of the residues shown in E\textsuperscript{2}GFP and YFP-H148Q to be located near the halide ion binding site. In our NMR backbone assignments, we could not identify the resonances of L64 (adjacent to the chromophore in the central \(\alpha\)-helix), V224 (located in \(\beta\)-strand 11) and Y203 (adjacent to a segment of \(\beta\)-strand 10 that is at the centre of a patch of unassigned residues, the surface of which overlaps with the dimer interface of Venus in the crystal structure (28)). These residues are likely to undergo breathing motions as a result of the ill-defined hydrogen bonding network centred around \(\beta\)-strand 7. However, we were able to observe the chemical shift perturbations of the amide groups of Q69 and V24 upon titration with 0.4 M of chloride ions at pH 6.0 and found that they are moderate with corresponding \(\Delta\delta\) values of 0.16 ppm and 0.20 ppm,
respectively, compared to other loop residues that exhibit significantly larger changes: the mean chemical shift change \(<\Delta\delta>\) is 0.25 ppm with a standard deviation of 0.21 ppm (Figure 4). Many of these have charged groups and hence we attribute the dominant effect of chloride ions to non-specific electrostatic interactions or electrostatic screening (Figure 4). This is consistent with our results using sodium nitrate instead of sodium chloride.

The chemical shift perturbation profiles of the two titration series are essentially the same (Figure 4 and Supplementary Figure S1) with a mean pair-wise chemical shift difference, \(\Delta\Delta\delta\), of only 0.04 ppm and a standard deviation of 0.10 ppm (Figure 4). In fact, nitrate ions induced slightly larger chemical shift perturbations compared to chloride ions, \(<\Delta\delta>=0.29\) ppm. We have found a good spatial correlation between the observed chemical shift changes in response to changes in ionic strength with those observed in response to changes in pH values from 6.0 to 9.6 (28) that are localised in the top and bottom lids of the barrel structure (Supplementary Figure S2). In addition, we identified a number of cavities within the barrel structure of Venus that are located in close proximities to the residues that underwent significant chemical shift changes upon pH changes. Water molecules can in principle fit in these cavities and modulate the yellow fluorescence of the chromophore. It is therefore plausible that the addition of ions as well as changes in pH can induce a series of local conformational rearrangements leading to water-mediated fluorescence quenching.

Recent studies using intrinsic fluorescence (19-22,27), exogenous fluorophores (32) and NMR (22,23,28,31,38,54) in addition to the work we present here, have begun to map out the complex energy landscape for both the in vitro and in vivo folding of this class of protein. Although many unusual features such as parallel pathways and kinetic traps due to chromophore formation have been clearly established, much more work is needed to fully characterise and understand how large \(\beta\)-barrel FPs fold. In particular, it is becoming clear that FPs engineered to have different spectral and biophysical properties can show quite marked differences in their behaviour towards environmental factors and intrinsic differences in stability and folding.
FOOTNOTES

STDH is a recipient of a Human Frontier Science Program Long-term Fellowship (LT0798/2005) and supported by the Postdoctoral Research Abroad Program of National Science Council of the Republic of China, Taiwan (NSC97-2917-1-564-102). CB is holding a Studienstiftung des Deutschen Volkes scholarship and is supported by the Erasmus/Sokrates program. LDC is a NH&MRC C.J. Martin Fellow. CMD acknowledges funding from the Wellcome and Leverhulme Trusts. SEJ acknowledges funding from the BBSRC (BBF00219X1). We thank Prof. Atsushi Miyawaki for providing the DNA plasmid of Venus, Dr. John Christodoulou for helpful discussion, and the staff and the use of the Biomolecular NMR Facility, Department of Chemistry, University of Cambridge. Financial support by the Access to Research Infrastructures activity in the 6th Framework Program of the EC (Contract # RII3-026145, EU-NMR) for conducting the research at CERM is gratefully acknowledged.
Figure 1. NMR HDX experiments of Venus. (A) 700 MHz $[^{15}\text{N}-^{1}\text{H}]$ HSQC spectra of Venus recorded at 37 °C before (grey), after three months (blue) and after 13 months (red) of HDX. (B) Representative HDX profiles over a broad range of timescales. The region between the initial point and three months of HDX is highlighted in cyan. (C) Structural mapping of the highly protected amide groups in Venus. The backbone amide nitrogen atoms of the residues that retain significant crosspeak intensities after three months of HDX are shown in blue spheres and labelled with corresponding identities. The carbonyl carbon atoms which are involved in the stable hydrogen bonding with those highly protected amide groups are shown in yellow spheres; the corresponding hydrogen bonds are shown in sticks, bringing the hydrogen bond donors and acceptors. Additionally, those hydrogen bonds that correspond to
the amide protons whose NMR intensities remained intense over 13 months of HDX are coloured red. (D) Topological mapping of the highly stable hydrogen bond network of Venus. The labelling follows the same scheme as in (C), except that the hydrogen bonds that are stable between 3-13 months are coloured blue, instead of yellow in (C). The hydrogen bonds that are involved in the super-stable core in GFP, i.e., the amide proton that remained largely protected over three months of HDX under denaturing conditions, are highlighted in dashed green lines. The 11 β-strands are labelled according and colour ramped from blue to red. The three α-helices (α3-α5) connecting strands β3 and β4 are shown in light blue rectangular boxes. (E) Residue-specific free energy of unfolding, ΔGHX, derived from NMR HDX experiments under native conditions is shown as a function of residue numbers. The free energies of unfolding, ΔGHX, derived from GdmCl induced unfolding experiments using the yellow fluorescence chromophore (Chr) and tyrosine fluorescence (Tyr) are indicated as horizontal bars with widths corresponding the uncertainties of the fittings. Residues located in β-strands and α-helices are indicated in blue and gold, respectively. The position of the chromophore encompassing G65, Y66 and G67 is indicated in red. (F) Structural mapping of the residue-specific free energy. Stereo-representation of the backbone of Venus with the Cα atoms shown in spheres and ramp-coloured according to the corresponding free energies of unfolding (left panel). The chromophore is shown in yellow sticks and tyrosine and tryptophan residues are shown in semi-transparent white and green surfaces, respectively.
Figure 2. Chemical denaturation of Venus monitored by the (A) yellow fluorescence, (B) tyrosine fluorescence and (C) far-UV CD and (D) Yellow fluorescence of Venus as a function of GdmCl, urea and NaCl concentration. (A-C) 10 µM protein in 50 mM MES buffer at pH 6.0 (filled circles) and pH 6.6 (squares) or 25 mM Tris buffer for pH 7.6 (triangles) and pH 8.0 (diamonds). Measurements were taken after 7 days of incubation at 25 °C and 37 °C (open circles). The data are normalized relative to their individual maximum fluorescence intensity. (D) The normalised chromophore fluorescence intensity at 527 nm is plotted as a function of denaturant (closed symbols) or chloride ion concentration (open symbols). Aliquots of 10 µM Venus were incubated with various GdmCl or urea concentrations or NaCl in the absence of denaturants for seven days prior to the measurements. The samples were in 50 mM MES buffer for pH 6.0 and pH 6.6 or 25 mM Tris buffer for pH 7.6.
Figure 3. pKₐ measurements of the yellow chromophore in the presence of chloride and nitrate ions.

The left panels show the fluorescence (A) and UV absorbance (C) spectra of Venus (black lines) in the presence of 400 mM chloride ions (dark grey lines) or nitrate ions (light grey lines) at the lowest and highest analyzed pH values (5.5 and 8.0); the right panels show the fluorescence (B) and UV absorbance (D) profiles of Venus in the presence of 400 mM chloride ions (dark grey circles) or nitrate ions (light grey triangles) at various pH values ranging from 5.5 to 8.3. Data were fitted to equation 1, and the pKₐ values of the chromophore calculated are given in Table 2. Conditions: 10 mM MES/MOPS buffer mixture was used at 25 °C.
Figure 4. Structural mapping of chemical shift perturbations in Venus upon addition of chloride and nitrate ions. (A) Chemical shift perturbations ($\Delta\delta$) of Venus in the presence of 400 mM chloride (red) and nitrate ions (black) as a function of residue number. The weighted $\Delta\delta$ is defined as $[\Delta\delta(\text{H})^2+(0.65\times\Delta\delta(\text{N}))^2]^{1/2}$. The differences between chloride and nitrate ions ($\Delta\Delta\delta$) are shown in green with a shifted baseline to illustrate the similar effects induced by the two different ions. The dashed horizontal line indicates the values of two standard deviations (2$\sigma$) of all $\Delta\delta$. The residues that exhibit $\Delta\delta$ values larger than 2$\sigma$ are indicated with corresponding residue identities. Residues that are located in the $\alpha$-helical and $\beta$-stranded regions are shaded in pink an light blue, respectively, with the secondary structures shown on top of the panel. Structural mapping of the chemical shift perturbations induced by the addition of 400 mM chloride ions (B) and that induced by the addition of 400 mM nitrate ions (C). The backbone nitrogen atoms are shown in spheres with various sizes proportional to the observed chemical shift perturbations (as indicated in the middle). The chromophore within the beta-barrel is shown in yellow sticks.
Table 1: Apparent thermodynamic data from the chemical denaturation of Venus.

| conditions | $m_{D-N}$ (kcal mol$^{-1}$ M$^{-1}$) | $[D]_{50\%}$ (M) | $\Delta G_{D-N}^{H,\Theta}$ (kcal mol$^{-1}$) | $^a[D]_{50\%}$ (M) | $^a\Delta G_{D-N}^{H,\Theta}$ (kcal mol$^{-1}$) |
|------------|-----------------------------------|-----------------|-----------------------------------------------|-------------------|-----------------------------------------------|
| pH 8.0, 37$^\circ$C | YF | 2.22 ± 0.06 | 5.10 ± 0.01 | 11.3 ± 0.3 | 5.11 ± 0.02 | 8.74 ± 0.04 |
| | Tyr | 1.51 ± 0.07 | 5.24 ± 0.03 | 7.9 ± 0.3 | 5.21 ± 0.02 | 8.93 ± 0.04 |
| | CD | 2.08 ± 0.26 | 5.04 ± 0.05 | 10.5 ± 1.3 | 5.07 ± 0.06 | 8.7 ± 0.1 |
| pH 8.0 | YF | 1.83 ± 0.09 | 5.92 ± 0.03 | 10.8 ± 0.5 | 5.81 ± 0.02 | 9.9 ± 0.2 |
| | Tyr | 1.75 ± 0.11 | 5.80 ± 0.04 | 10.1 ± 0.6 | 5.89 ± 0.02 | 10.1 ± 0.2 |
| | CD | 1.83 ± 0.23 | 6.19 ± 0.15 | 11.2 ± 1.3 | 6.12 ± 0.03 | 10.5 ± 0.2 |
| pH 7.6 | YF | 1.91 ± 0.17 | 5.85 ± 0.06 | 11.2 ± 0.9 | 5.72 ± 0.03 | 9.8 ± 0.2 |
| | Tyr | 1.52 ± 0.08 | 5.93 ± 0.06 | 9.0 ± 0.4 | 5.77 ± 0.02 | 9.9 ± 0.2 |
| | CD | 1.65 ± 0.19 | 5.08 ± 0.15 | 8.4 ± 1.0 | 5.81 ± 0.03 | 9.9 ± 0.2 |
| pH 6.6 | YF | 1.99 ± 0.07 | 5.22 ± 0.02 | 10.4 ± 0.3 | 5.16 ± 0.04 | 8.8 ± 0.2 |
| | Tyr | 1.25 ± 0.05 | 5.23 ± 0.02 | 6.6 ± 0.3 | 5.23 ± 0.02 | 8.9 ± 0.2 |
| | CD | 2.09 ± 0.55 | 4.78 ± 0.08 | 10.3 ± 2.5 | 5.19 ± 0.04 | 8.9 ± 0.2 |
| pH 6.0 | YF | 2.20 ± 0.08 | 3.61 ± 0.02 | 8.0 ± 0.3 | 3.52 ± 0.17 | 6.0 ± 0.3 |
| | Tyr | 2.10 ± 0.12 | 3.23 ± 0.02 | 6.8 ± 0.4 | 3.19 ± 0.03 | 5.5 ± 0.1 |
| | CD | 2.48 ± 0.61 | 3.26 ± 0.10 | 8.8 ± 2.2 | 3.16 ± 0.03 | 6.2 ± 0.2 |

Values were obtained from the best fit of the yellow fluorescence (YF), tyrosine fluorescence (Tyr) and far-UV CD (CD) data to a two-state model, equation 2. For the YF data acquired at lower pHs, the intial drop in fluorescence which we have shown is not due to an unfolding event was not included in the fit. $^a$Calculated using a global fit using a shared $m_{D-N}$ value of 1.71 kcal mol$^{-1}$ M$^{-1}$.

Table 2. p$K_a$ and Hill coefficient $n_H$ values of the p-hydroxy benzylidene imidazolidinone chromophore of Venus in the presence and absence of additional salts.

| Conditions        | p$K_a$ Fluorescence | p$K_a$ Absorbance | $n_H$ Fluorescence | $n_H$ Absorbance |
|-------------------|---------------------|------------------|-------------------|------------------|
| MES-MOPS buffer   | 6.84 ± 0.04         | 7.01 ± 0.05      | 0.77 ± 0.07       | 0.68 ± 0.08      |
| + 0.4 M NaCl      | 6.89 ± 0.05         | 7.08 ± 0.07      | 0.95 ± 0.12       | 0.97 ± 0.16      |
| + 0.4 M NaNO$_3$  | 7.06 ± 0.04         | 7.15 ± 0.05      | 1.18 ± 0.11       | 1.19 ± 0.13      |

References.

1. Shaner, N. C., Patterson, G. H., and Davidson, M. W. (2007) Journal of Cell Science 120(24), 4247-4260
2. Tsien, R. Y. (1998) Ann. Rev. Biochem. 67, 509-544.
3. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) Trends in Biochemical Sciences 20(11), 448-455
4. Giepmans, B. N. G., Adams, S. R., Ellisman, M. H., and Tsien, R. Y. (2006) Science 312(5771), 217-224
5. VanEngelenburg, S. B., and Palmer, A. E. (2008) *Current Opinion in Chemical Biology* **12**(1), 60-65
6. Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002) *Science* **296**(5569), 913-916
7. Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) *Science* **273**
8. Reid, B. G., and Flynn, G. C. (1997) *Biochemistry* **36**, 6786-6791.
9. Shyu, Y. J., Suarez, C. D., and Hu, C. D. (2008) *Proceedings of the National Academy of Sciences of the United States of America* **105**(1), 151-156
10. Karasawa, A., Tsuboi, Y., Inoue, H., Kinoshita, R., Nakamura, N., and Kanazawa, H. (2005) *Journal of Biological Chemistry* **280**(51), 41900-41911
11. Millington, M., Grindlay, G. J., Altenbach, K., Neely, R. K., Kolch, W., Bencina, M., Read, N. D., Jones, A. C., Dryden, D. T. F., and Magennis, S. W. (2007) *Biophysical Chemistry* **127**(3), 155-164
12. Kneen, M., Farinas, J., Li, Y. X., and Verkman, A. S. (1998) *Biophysical Journal* **74**(3), 1591-1599
13. Arosio, D., Garau, G., Ricci, F., Marchetti, L., Bizzarri, R., Nifosi, R., and Beltram, F. (2007) *Biophysical Journal* **93**(1), 232-244
14. Li, C. J., Heim, R., Lu, P., Pu, Y. M., Tsien, R. Y., and Chang, D. C. (1999) *Journal of Cell Science* **112**(10), 1567-1577
15. Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M., and Miyawaki, A. (2004) *Proceedings of the National Academy of Sciences of the United States of America* **101**(29), 10554-10559
16. Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002) *Nature Biotechnology* **20**(1), 87-90
17. Chang, H. C., Kaiser, C. M., Hartl, F. U., and Barral, J. M. (2005) *Journal of Molecular Biology* **353**(2), 397-409
18. Chirico, G., Cannone, F., and Diaspro, A. (2006) *European Biophysics Journal with Biophysics Letters* **35**(8), 663-674
19. Enoki, S., Maki, K., Inobe, T., Takahashi, K., Kamagata, K., Oroguchi, T., Nakatani, H., Tomoyori, K., and Kuwajima, K. (2006) *J. Mol. Biol.* **361**, 969-982.
20. Enoki, S., Saeki, K., Maki, K., and Kuwajima, K. (2004) *Biochemistry* **43**
21. Fukuda, H., Arai, M., and Kuwajima, K. (2000) *Biochemistry* **39**, 12025-12032.
22. Huang, J. R., Craggs, T. D., Christodoulou, J., and Jackson, S. E. (2007) *Journal of Molecular Biology* **370**(2), 356-371
23. Huang, J. R., Hsu, S. T. D., Christodoulou, J., and Jackson, S. E. (2008) *Hfsp Journal* **2**(6), 378-387
24. Jackson, S. E., Craggs, T. D., and Huang, J. R. (2006) *Expert Review of Proteomics* **3**(5), 545-559
25. Mickler, M., Dima, R. I., Dietz, H., Hyeon, C., Thirumalai, D., and Rief, M. (2007) *Proceedings of the National Academy of Sciences of the United States of America* **104**(51), 20268-20273
26. Andrews, B. T., Gosavi, S., Finke, J. M., Onuchic, J. N., and Jennings, P. A. (2008) *Proceedings of the National Academy of Sciences of the United States of America* **105**(34), 12283-12288
27. Andrews, B. T., Schoenfish, A. R., Roy, M., Waldo, G., and Jennings, P. A. (2007) *Journal of Molecular Biology* **373**(2), 476-490
28. Hsu, S. T. D., Behrens, C., Cabrita, L. D., and Dobson, C. M. (2009) *Biomol. NMR Assign.* 3(1), 67-72
29. Goddard, T. D., and Kneller, D. G. *University of California, San Francisco*
30. Englander, S. W., Mayne, L., and Krishna, M. M. G. (2007) *Quarterly Reviews of Biophysics* 40(4), 287-326
31. Khan, F., Kuprov, I., Craggs, T. D., Hore, P. J., and Jackson, S. E. (2006) *Journal of the American Chemical Society* 128(33), 10729-10737
32. Orte, A., Craggs, T. D., White, S. S., Jackson, S. E., and Klenmer, D. (2008) *Journal of the American Chemical Society* 130(25), 7898-7907
33. Zapata-Hommer, O., and Griesbeck, O. (2003) *Bmc Biotechnology* 3
34. Cai, L., Friedman, N., and Xie, X. S. (2006) *Nature* 440(7082), 358-362
35. Xie, J. B., and Zhou, J. M. (2008) *Biochemistry* 47(1), 348-357
36. Stepanenko, O. V., Verkhusha, V. V., Kazakov, V. I., Shavlovsky, M. M., Kuznetsova, I. M., Uversky, V. N., and Turoverov, K. K. (2004) *Biochemistry* 43(47), 14913-14923
37. Helms, V., Straatsma, T. P., and McCammon, J. A. (1999) *Journal of Physical Chemistry B* 103(16), 3263-3269
38. Seifert, M. H. J., Georgescu, J., Ksiazek, D., Smialowski, P., Rehm, T., Steipe, B., and Holak, T. A. (2003) *Biochemistry* 42(9), 2500-2512
39. Huang, Y. M., and Bystroff, C. (2009) *Biochemistry* 48(5), 929-940
40. Hsu, S. T. D., Cabrita, L. D., Fucini, P., Christodoulou, J., and Dobson, C. M. (2009) *Journal of the American Chemical Society* 131(24), 8366+
41. Hsu, S. T. D., Fucini, P., Cabrita, L. D., Launay, H., Dobson, C. M., and Christodoulou, J. (2007) *Proceedings of the National Academy of Sciences of the United States of America* 104, 16516-16521
42. Rucker, E., Schneider, G., Steinhauser, K., Lower, R., Hauber, J., and Stauber, R. H. (2001) *Protein Expr. Purif.* 21(1), 220-223
43. Wang, H. Y., and Chong, S. R. (2003) *Proceedings of the National Academy of Sciences of the United States of America* 100(2), 478-483
44. Yen, H. C. S., Xu, Q. K., Chou, D. M., Zhao, Z. M., and Elledge, S. J. (2008) *Science* 322(5903), 918-923
45. Pedelacq, J. D., Cabantous, S., Tran, T., Terwilliger, T. C., and Waldo, G. S. (2006) *Nature Biotechnology* 24(1), 79-88
46. Lawrence, M. S., Phillips, K. J., and Liu, D. R. (2007) *Journal of the American Chemical Society* 129(33), 10110+
47. Kerppola, T. K. (2006) *Nature Reviews Molecular Cell Biology* 7(6), 449-456
48. Ottmann, C., Weyand, M., Wolf, A., Kuhlmann, J., and Ottmann, C. (2009) *Biological Chemistry* 390(1), 81-90
49. Elsliger, M. A., Wachter, R. M., Hanson, G. T., Kallio, K., and Remington, S. J. (1999) *Biochemistry* 38, 5296-5301.
50. Jayaraman, S., Haggie, P., Wachter, R. M., Remington, S. J., and Verkman, A. S. (2000) *J. Biol. Chem.* 275, 6047-6050.
51. Rekas, A., Alattia, J. R., Nagai, T., Miyawaki, A., and Ikura, M. (2002) *Journal of Biological Chemistry* 277(52), 50573-50578
52. Galietta, L. J. V., Haggie, P. M., and Verkman, A. S. (2001) *Fecs Letters* 499(3), 220-224
53. Wachter, R. M., Yarbrrough, D., Kallio, K., and Remington, S. J. (2000) *Journal of Molecular Biology* 301(1), 157-171
54. Seifert, M. H., Ksiazek, D., Azim, M. K., Smialowski, P., Budisa, N., and Holak, T. A. (2002) *Journal of the American Chemical Society* **124**(27), 7932-7942
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J. Biol. Chem. published online November 9, 2009

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