Denaturation and partial renaturation of a tightly tetramerized DsRed protein under mildly acidic conditions

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Abstract The red fluorescent protein, DsRed, recently cloned from coral *Discosoma* sp. has one of the longest fluorescence waves and one of the most complex absorbance spectra among the family of fluorescent proteins. In this work we found that with time DsRed fluorescence decreases under mildly acidic conditions (pH 4.0–4.8) in a pH-dependent manner, and this fluorescence inactivation could be partially recovered by subsequent re-alkalization. The DsRed absorbance and circular dichroism spectra under these conditions revealed that the fluorescence changes were caused by denaturation followed by partial renaturation of the protein. Further, analytical ultracentrifugation determined that native DsRed formed a tight tetramer under various native conditions. Quantitative analysis of the data showed that several distinct states of protein exist during the fluorescence inactivation and recovery, and the inactivation of fluorescence can be caused by protonation of a single ionogenic group in each monomer of DsRed tetramer. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Red fluorescent protein; pH dependence; Denaturation; Renaturation; Tetramer; Kinetics

1. Introduction

The fluorescent proteins are a growing family of 25–30 kDa homologous chromoproteins found among the classes hydrozoa and anthozoa. The most extensively characterized member of this family, a green fluorescent protein (GFP) from jellyfish *Aequorea victoria* and its improved mutants have become widely used tools in biochemistry, biotechnology, cellular and developmental biology [1]. Recently, a novel red fluorescent protein, drFP583 or DsRed, has been cloned from reef coral *Discosoma* sp. [2]. The wild-type DsRed has a significantly red-shifted fluorescence spectrum providing good complementation to existing GFP-based in vivo approaches, including double- and triple-labeling cultured cells and whole organisms, studies of protein–protein interactions using fluorescence resonance energy transfer, and various fluorescent sensors for intracellular messengers [1,3]. Although the basis of the DsRed chromophore consists of the similar cyclized GFP-like tripeptide motif, -Gln-Tyr-Gly- [2,4], still little is known about the further backbone modifications of DsRed resulting in the complex red chromophore formation.

Because of a presumable external β-barrel structure [2,4], DsRed similarly to GFP presents a good model for studying the denaturation and renaturation of predominantly β-sheet proteins. The formation of native topology in the folding pathway, the protein intermediates and their structural nature, and specific interactions that stabilize the unique native state are the key questions regarding the folding of these proteins [5]. They bear particular significance for DsRed and GFP, because the inactivation and recovery of fluorescence are critically dependent on the fidelity of protein folding.

The kinetics of fluorescence development is also important for the use of DsRed as a reporter and pH-sensor in different intracellular environments, including its targeting to organelles under acidic conditions. It has been shown that wild-type *Aequorea* GFP and GFP-like protein from *Renilla reniformis* gradually lost their fluorescence at a pH below 6.0 because of protein denaturation [6]. Further, the fluorescence could be partially recovered by subsequent renaturation at neutral pH [7], but the kinetics of neither of these pH-dependent processes were analyzed in detail. To date, the only exception was the kinetics of chromophore formation studied for *Aequorea* GFP/Ser65Thr mutant renatured from 8 M urea and *Escherichia coli* inclusion bodies [8].

In the present work, we analyze pH-dependent kinetics for the inactivation and recovery of DsRed fluorescence under mildly acidic conditions, and elucidate the molecular mechanism underlying these fluorescence changes. We show that DsRed exists in solution as a tetramer, and present a kinetic mechanism for the acid-dependent DsRed behavior.

2. Materials and methods

The cDNA of wild-type DsRed was amplified by polymerase chain reaction (PCR) with an *Nde*I–*Bam*HI fragment using the pDsRed1-N1 vector (Clontech) as template followed by recloning into pET-11c vector (Invitrogen). A polyhistidine tag was added to the C-terminus during PCR amplification. The resulting pET-11c-DsRed-Hisx6 plasmid was transfected into BL21(DE3) *E. coli* strain (Invitrogen). Expression of the histidine-tagged DsRed was induced by 0.5 mM iso-

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Abbreviations: GFP, green fluorescent protein; CD, circular dichroism

propyl β-D-thiogalactopyranoside (Sigma) at 37°C during overnight culture, and recombinant DsRed was purified using Ni-NTA agarose (Qiagen). A stock solution of DsRed was made to a concentration of 12 mg/ml in 100 mM citric-phosphate buffer at pH 7.6 using Ultra-free-4 centrifugal filters (Millipore). Recombinant DsRed was at least 95% pure according to SDS–PAGE. Protein concentrations were determined by BioRad protein assay.

For spectroscopic studies the aliquots of DsRed stock solution were diluted 20–1200 times into 10–100 mM phosphate or citric-phosphate buffers with different pH values. For kinetic measurements the predetermined amounts of 5 M phosphoric acid were added to DsRed in 100 mM citric-phosphate buffer at pH 5.0 to achieve the necessary degree of acidification. If necessary, the pH of DsRed solution was further readjusted to pH 5.0 by addition of predetermined volumes of 10 M NaOH. Changes of pH were performed directly in spectrometric cuvettes under intensive mixing, therefore, the time-lag for establishing spatially equal pH did not exceed 10 s in any of the cases. As a rule, the sample for kinetic studies contained 0.01 mg/ml of DsRed, it was excited at 556 nm, and emission was detected at 583 or 590 nm. For absorbance and circular dichroism (CD) studies, base-renatured DsRed was further purified by gel filtration using superfine Sephadex G-75 (Pharmacia).

The Shimadzu UV-1601 spectrophotometer, Perkin Elmer LS50B spectrofluorimeter, and Jasco-720 spectropolarometer were used for absorbance, fluorescence and CD measurements, respectively. The Beckman XL-1 analytical ultracentrifuge was used for equilibrium centrifugation at 15 000 rpm for 48 h. All spectroscopic and centrifugation measurements were performed at 25 and 20°C, respectively. The data were further analyzed by Microcal Origin v. 5.0 (Microcal Software) and MatLab v. 5.3 (MathWorks).
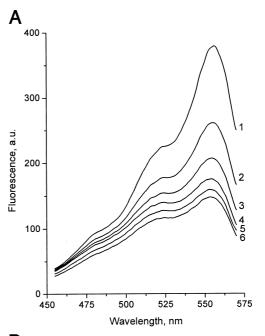
3. Results and discussion

DsRed fluorescence hardly changed from pH 5.0 to pH 12.0, but decreased outside this range (not shown). In weak acid (pH 4.0–4.8) DsRed fluorescence intensity exhibited a rather gradual and time-dependent decrease simultaneously at the excitation wavelengths 450–570 nm (Fig. 1A). Therefore, the kinetics of these fluorescence changes were further analyzed at acidic pH. Acidification below pH 5.0 accelerated the decrease of fluorescence intensity and also caused a decrease in the altitude of residual fluorescence intensity at different pH values (Fig. 1B). We have found that this time-dependent decrease of fluorescence intensity at each pH can be described by the two-exponential equation:
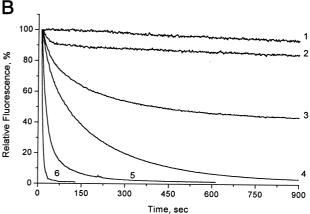
$$I_A = A_0 + A_1\exp(-t/t_{1A}) + A_2\exp(-t/t_{2A}) \quad (1)$$

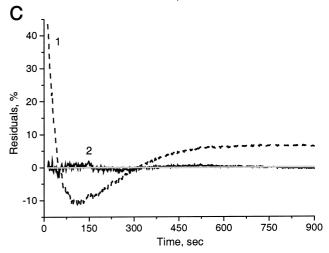where t is the time, I_A is the fluorescence intensity, A_0 is the residual fluorescence intensity at infinite time, and A_1, A_2, t_{1A} and t_{2A} are kinetic parameters depending on pH values. It is worth noting that the two exponents are minimum numbers of function that fit the kinetic data (Fig. 1C).

A decrease in pH value caused a two-order increase in A_1, and a similar decrease in A_0, t_{1A} and t_{2A}, while A_2 exhibited a bell-shaped dependence reaching a maximum at pH 4.4. At a pH value of 4.55 the residual intensity A_0 for DsRed was just half of the initial value, but at pH 4.4 DsRed had already lost all its fluorescence ($A_0 = 0$) (Fig. 2A). The rate of fluorescence
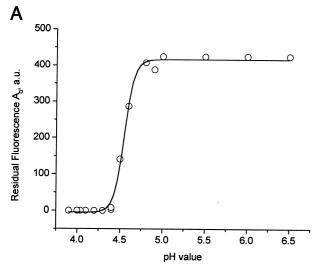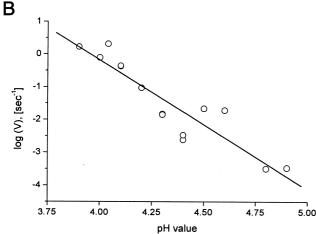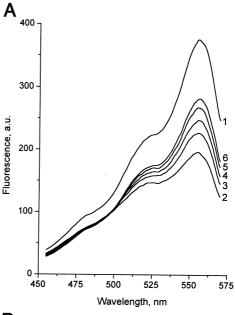

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Fig. 1. A: Excitation spectra of DsRed at pH 5.0 (1), and after incubation for 29 s (2), 62 s (3), 104 s (4), 244 s (5) and 495 s (6) at pH 4.3. The spectrum at pH 5.0 was not changed significantly during the same period (not shown). B: The degree of DsRed fluorescence inactivation under acidic conditions is presented as the time course for the relative fluorescence intensity in the buffers at pH 4.8 (1), 4.5 (2), 4.4 (3), 4.3 (4), 4.2 (5) and 3.9 (6). C: Example of residual errors from different theoretical approximations of DsRed fluorescence inactivation at pH 4.3 (curve 4 in B). Fits of this experimental curve by the one-exponential (1, black dashed line) and two-exponential (2, black solid line) equations are shown. Fit by three exponents resulted in approximation identical to the line (2). Grey straight line represents zero residuals.

A

B

Fig. 2. A: The dependence of DsRed residual fluorescence intensity at infinite time, A_0, from pH value of the incubation buffers. The experimental data (open circles) and fitting Boltzman curve (solid line) are presented. B: The dependence of the relative initial rate of fluorescence decrease, V, from pH value of DsRed incubation buffers in logarithmic coordinates. The values of V were calculated from tangents of the slope at initial time for the kinetic curves presented in Fig. 1B. The two-exponential approximation given by Eq. 1 was used for the calculations. The experimental data (open circles) and fitting straight line (solid line) are shown.
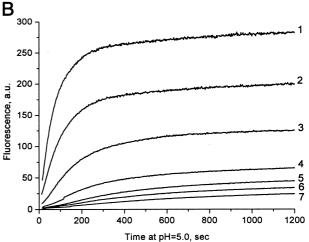
was in contrast to rather smooth spectroscopic changes in the cases of *Aequorea* GFP and *Renilla* GFP, where a complete loss of fluorescence was observed in the regions pH 4.2–6.5 [9] and pH 2.0–6.5 [10], respectively.

Further, we examined the possibility of recovering the fluo-

A

B

C

decrease was also significantly affected by pH value. The relative initial rate of fluorescence decrease, V, depended logarithmically on pH (Fig. 2B). This dependence is fitted in logarithmic coordinates by a straight line with the slope equal to -4.0 ($P < 0.001$). The sharp decrease of DsRed fluorescence
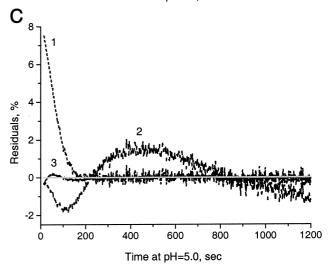
$\rightarrow$

Fig. 3. A: Excitation spectra of DsRed at pH 5.0 (1), and at pH 5.0 after 90 s incubation at pH 4.4 (2–6). The incubation time after re-alkalization from pH 4.4 to 5.0 was 68 s (2), 134 s (3), 200 s (4), 355 s (5) and 839 s (6), respectively. B: The DsRed fluorescence recovery at pH 5.0 after incubation at pH 4.1 during 30 s (1), 50 s (2), 99 s (3), 200 s (4), 372 s (5), 435 s (6), and 710 s (7). C: Example of residual errors from different theoretical approximations of DsRed fluorescence recovery at pH 5.0 after incubation at pH 4.1 during 435 s (curve 6 in B). Fits of this experimental curve by the one-exponential (1, black dashed line), two-exponential (2, black dotted line), and three-exponential (3, black solid line) equations are shown. Grey straight line represents zero residuals.

rescence of DsRed by re-adjusting the pH value to pH 5.0 after an incubation below pH 4.8. We have found that re-alkalization to pH 5.0 caused a significant recovery of the fluorescence, although still below the initial level. The fluorescence intensity gradually increased at excitation wavelengths of 510–570 nm (Fig. 3A). However, this recovery was almost absent in the range 480–500 nm, which probably corresponds to the excitation spectrum of the cyclized GFP-like tripeptide. The longer DsRed was incubated for at pH 5.0 after re-alkalization, the higher the level of fluorescence recovery observed. The fluorescence recovery reached saturation after prolonged incubation.

We analyzed the kinetics of this fluorescence recovery for DsRed samples incubated at different times at acidic pH 4.1, where the residual intensity was $A_0 = 0$ (Fig. 3B). We have determined that at excitation wavelength 556 nm a time-dependent increase of the fluorescence intensity after re-alkalization to pH 5.0 can be well described by a three-exponential equation (see Fig. 3C):

$$I_B = B_0 - B_1\exp(-t/t_{1B}) - B_2\exp(-t/t_{2B}) + B_3\exp(-t/t_{3B})$$

$$(2)$$

where I_B is the fluorescence intensity, B_0 is the maximal fluorescence recovery at infinite time, and B_1, B_2, B_3, t_{1B}, t_{2B} and t_{3B} are kinetic parameters which depend on the time of DsRed incubation at pH 4.1. The increase of DsRed incubation time at pH 4.1 caused a one-order decrease of B_0, B_1, B_2, B_3, and a similar increase of t_{1B}, t_{2B} and t_{3B} parameters, respectively. The longer the time DsRed was incubated in acid, the higher the amount of irreversibly inactivated DsRed and the lower the level of fluorescence recovery, B_0, observed (Fig. 4).

To elucidate the molecular mechanism underlying the inactivation and recovery of DsRed fluorescence, we performed CD and absorbance measurements. Fig. 5A shows the effect of acidic pH 4.1 treatment on the secondary structure of DsRed. The CD spectrum of DsRed at pH 5.0 had a strong negative deflection near 219 nm, indicating a significant spectral contribution from the β-structure. The secondary structure was disrupted at pH 4.1 and the resulting CD spectrum was indicative of a disordered coil. No further changes in the spectrum were observed following prolonged (48 h) incuba-
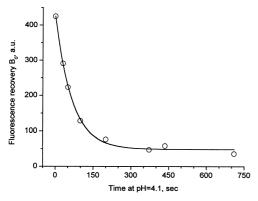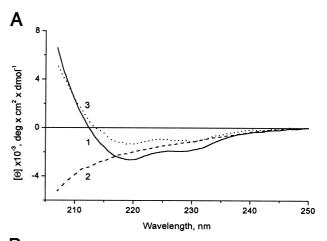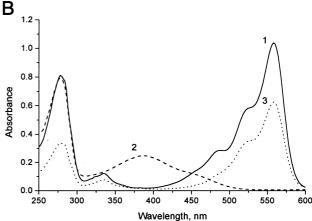

Fig. 5. A: CD spectra of DsRed at pH 5.0 (1), at pH 4.1 after incubation for 480 s (2), and at pH 5.0 after 60 s incubation at pH 4.1 (3). DsRed samples (1) and (2) were dissolved in 10 mM citric-phosphate buffers at protein concentration 0.24 mg/ml. Curve (3) represents the spectrum of combined fluorescent fractions after re-alkalization of protein sample from pH 4.1 to 5.0. The DsRed sample was incubated during 1 h at pH 5.0, and the fluorescent fractions were isolated by gel filtration (see Section 2). The final volume was adjusted to the volume of the initial sample. [Q] designates the mean residue ellipticity. The spectrum qualitatively similar to (2) was already observed after 60 s incubation at pH 4.1 (not shown). B: Absorbance spectra of DsRed at pH 5.0 (1), at pH 4.1 after incubation for 480 s (2), and at pH 5.0 after 60 s incubation at pH 4.1 (3). DsRed samples (1) and (2) were dissolved in 100 mM citric-phosphate buffers at protein concentration 0.6 mg/ml. Sample (3) was processed as in A.

tion at pH 4.1. These data are consistent with previous CD studies of GFP denatured by guanidine HCl [6]. The absorbance spectrum of DsRed at pH 4.1 completely lacked the absorbance pattern of native DsRed at pH 5.0 with three structured peaks (Fig. 5B). Instead it had a wide absorbance peak in the visible range with a maximum at 387 nm indicating significant changes of the microenvironment of the DsRed chromophore.

DsRed incubation at pH 4.1 during 60 s followed by re-alkalization to pH 5.0 resulted in the renaturation of DsRed detected by CD spectrum. The fraction of DsRed with recovered fluorescence separated by gel filtration had a similar deflection region to the original DsRed sample (Fig. 5A). Absorbance of this fraction had the structured pattern of a spectrum rather similar to native DsRed at pH 5.0 (Fig. 5B). All these data suggest that observed DsRed fluorescence

Fig. 4. The dependence of the maximal fluorescence recovery at infinite time, B_0, in the buffer with pH 5.0 from the time of DsRed incubation at pH 4.1. The experimental data (open circles) and fitting theoretical curve $B_0 = 377\exp(-1.51 \times 10^{-2}t) + 48.3$ (solid line) are shown.
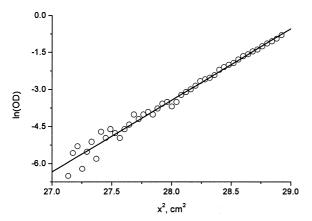
Fig. 6. Absorbance (OD) of DsRed sample at 280 nm along the length, x, of the centrifugation cell after equilibrium centrifugation. The DsRed sample was dissolved in 100 mM citric-phosphate buffer, pH 5.0 at 0.15 mg/ml. The experimental data (open circles) and fitting straight line (solid line) are shown.

inactivation and recovery were caused by DsRed denaturation at a pH below 5.0 and further protein renaturation at pH 5.0.

To further clarify the nature of factor 4 for the decrease of the initial rate of DsRed fluorescence (Fig. 2B) we performed a sedimentation equilibrium analysis of the DsRed sample at pH 5.0. A typical DsRed concentration profile in the centrifugation cell obtained after 48 h is shown in logarithmic coordinates in Fig. 6. A single straight line could fit the experimental data with a slope equal to 2.90 ($P < 0.001$). No further changes in the number of fitting curves and in the slope of the fitting line were observed after more prolonged centrifugation. This indicated that we have reached a thermodynamic equilibrium with a single type of DsRed particles. The molecular weight (MW) of the particles was further calculated on the basis of the slope value as described in [11].

The determined MW was 115.2 kDa indicating that DsRed formed tetrameric oligomers. This DsRed tetramerization did not depend on the protein concentration in the range 0.15–1.0 mg/ml tested (i.e. as low as 5.6 μM) and was observed at pHs from 5.0–8.0 (not shown). This suggests that DsRed exists as a tight non-dissociable tetramer in solution. Similarly, tetrameric organization of wild-type DsRed was detected by dynamic light scattering as a monomodal distribution (T. Mizukoshi, personal communication). It has also been reported that many GFP-like proteins from anthozoa species, other than *A. victoria*, form tight dimers, which can only be dissociated after irreversible denaturation [6]. Generally, oligomerization of proteins is a frequently observed phenomenon that occurs in the Golgi and endoplasmic reticulum [12]. It is thought that it serves to increase the stability of monomers and to perform protein functions. Oligomerization-assisted monomer folding was also reported [13–15].

Finally, we propose a consecutive mechanism for the simplest description of DsRed behavior under mildly acidic conditions:

$$\mathrm{DsRed_0} \underset{k_{1R}}{\overset{k_{1F}}{\rightleftarrows}} \mathrm{DsRed_1} \underset{k_{2R}}{\overset{k_{2F}}{\rightleftarrows}} \mathrm{DsRed_2} \underset{k_{3R}}{\overset{k_{3F}}{\rightleftarrows}} \mathrm{DsRed_3} \overset{k_{4F}}{\longrightarrow} \mathrm{DsRed_4} \qquad (3)$$

where DsRed_n designates the DsRed tetramer, n is a number of intermediate state, and k_{nF}, k_{nR} are forward and reverse rate constants, respectively. Because renaturation was shown with the three-exponential process (see Eq. 2), this mechanism

should include at least four states of DsRed tetramer. Moreover, at least one more state should not have the ability to renaturate ($\mathrm{DsRed_4}$). In the simplest case, the tetramer can acquire the same intermediate states during both denaturation and renaturation. Also, the two-exponential equation for denaturation (Eq. 1) suggests that at least two tetramer states are fluorescent (i.e. $\mathrm{DsRed_0}$ and $\mathrm{DsRed_1}$).

Draft estimations of the reverse rate constants in Eq. 3 can be made for pH 4.1 from kinetic parameters t_{1B}, t_{2B} and t_{3B} determined from Eq. 2: 2.6×10^{-3} s$^{-1} < k_{1R} \sim t_{1B}^{-1} < 1.5 \times 10^{-2}$ s^{-1}, 6.4×10^{-4} s$^{-1} < k_{2R} \sim t_{2B}^{-1} < 1.5 \times 10^{-3}$ s^{-1}, and 1.4×10^{-2} s$^{-1} < k_{3R} \sim t_{3B}^{-1} < 4.6 \times 10^{-2}$ s^{-1}. These values differ from those reported for renaturation of monomeric *Aequorea* GFP, $3–5 \times 10^{-3}$ s^{-1} [7] and 2.44×10^{-3} s^{-1} [8], probably because of the influence of the rather rigid tetramer organization of DsRed. Although determination of forward rate constants k_{1F}, k_{2F} and k_{3F} is complicated from present data, the rate constant for irreversible denaturation, k_{4F}, can be estimated from the fluorescence recovery experiments (Fig. 4), and at pH 4.1 $k_{4F} = 1.51 \times 10^{-2}$ s^{-1}.

The fact that DsRed fluorescence is inactivated in a rather narrow region of pH (Fig. 2A), and that the decrease of the initial rate of fluorescence depends on a pH factor of 4 (see Fig. 2B), the fluorescence inactivation results from the simultaneous protonation of several (at least four) ionogenic groups of DsRed. If all DsRed monomers in the tetramer denature completely independently, then each monomer should acquire four protons for the fluorescence loss. Alternatively, if the denaturation of DsRed monomers is mutually dependent or cooperative, the loss of fluorescence occurs after protonation of only a single ionogenic group in each monomer. Our kinetic mechanism favors the latter case of protonation. In both cases, an estimation for the upper limit of the pK_a value, p$K_a < 4.0$, can be done for ionogenic groups with equal dissociation constants [16].

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