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Structural Analysis of the Immature Form of the GFP Homologue DsRed

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Abstract—The crystal structures of DsRed have shown that it contains an unusual non-proline containing *cis* peptide linkage. We have shown that it is also present in the precyclized immature form of DsRed, thereby eliminating the possibility that *cis*/*trans* isomerization drives the formation of the acylimine, which is responsible for DsRed's red fluorescence. Two mechanisms have been proposed for chromophore formation in green fluorescent protein (GFP), a 'reduced' and an 'oxidized' mechanism. DsRed adopts a tight turn conformation, such as that found in GFP, in the immature intermediate proposed in the oxidized mechanism, but not in the one predicted by the reduced mechanism.

DsRed (drFP583 in the original Matz nomenclature[1] is a red-emitting fluorescent protein from *discosoma* that is commonly used as a tracer molecule. It is commercially available and has been studied in some detail.[2] It is an obligate tetramer,[3,4] although a monomeric mutant has been formed.[5] The structure of the chromophore in DsRed is known and a mechanism for its formation has been proposed[6] (see Fig. 1). The immature DsRed forms an intermediate green species by cyclization (dehydration) and oxidation (dehydrogenation) in the same way that the green fluorescent protein (GFP) chromophore is formed. A subsequent dehydrogenation of Gln66 forms the mature red chromophore (see Fig. 1). Quantum mechanical calculations[6] and small molecule studies[7] have confirmed that the extended conjugation due to the acylimine accounts for the observed red-shift. The formation of the mature red chromophore takes from hours to days and is incomplete.[6] Point mutations such as P37S, K83R, N42H and T217S stabilize the green immature form of DsRed, which has an absorption peaking at 480 nm.[8] Another intermediate has also been found with an absorption maximum at 408 nm; it has been suggested that the two intermediates correspond to the protonated and deprotonated forms of the chromophore, as observed in GFP.[8]

Resonance coherent anti-stokes Raman scattering experiments suggest that more than just π-bonding chromophore system extension might be required for DsRed chromophore formation.[9] Sacchetti et al. have presented a model according to which oligomerization of DsRed is required for stabilization of the chromophore and plays a crucial role in maturation of the fluorophore in wild type DsRed.[10]

Two groups have independently solved and reported the crystal structure of DsRed.[11,12] DsRed is found as a tetramer, the monomers consist of 11-stranded β barrels, and the chromophore has been extended by an acylimine, relative to that of GFP. An unusual feature of the structure is that the amide bond preceding the acylimine has a rare *cis* configuration. It has been suggested[11] that isomerization of the *cis* peptide bond between Phe65 and Gln66 is a key step in the formation of the acylimine.

The main reason that DsRed and GFP are such useful and popular tracer molecules is that their chromophore is formed in an autocatalyic post-translational step. This means that they require no other chemical to form the chromophore. We have shown that the chromophore-forming region of immature GFP is preorganized in a unique conformation required for chromophore formation. This 'tight turn' conformation has an *i* carbonyl carbon to $i+2$ amide nitrogen distance of less than 2.90 Å with $\phi = 60 \pm 30°$ and $\psi = 30 \pm 15°$.[13] A similar

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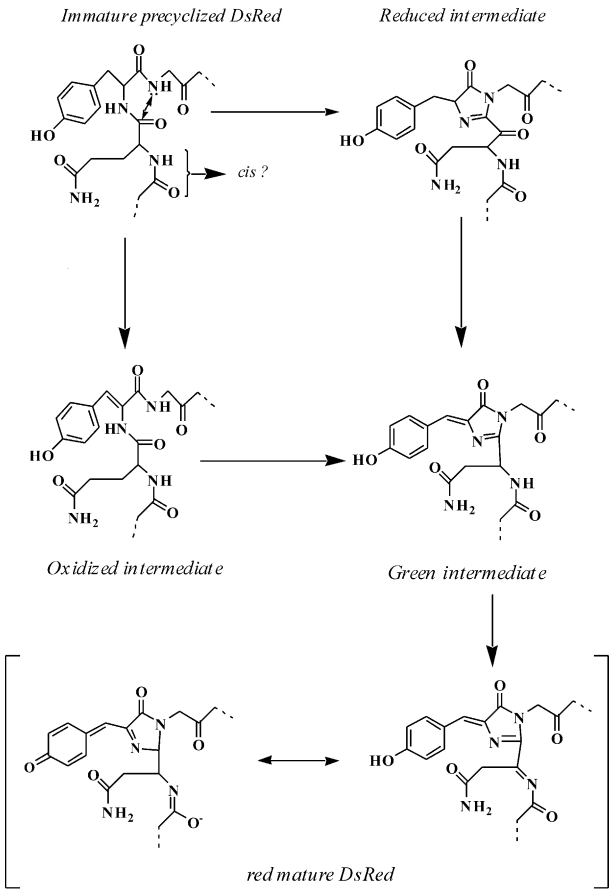

Figure 1. Proposed mechanism for the chromophore formation in DsRed. The pathway through the top-right reduced intermediate is the commonly accepted mechanism.[6] The oxidized mechanism, which passes through the dehydro-Tyr67 intermediate shown in middle left structure, is based on a mechanism we have proposed for GFP[16] and HAL.[14]

tight turn has been observed in histidine ammonium lyase (HAL).[14]

In this paper we describe calculations used to establish whether the same tight turn motif present in the immature precyclized form of GFP[13–16] is present in immature DsRed. We have also used computational methods to determine whether the *cis* amide dihedral angle of Gln66 found in the crystal structures of mature DsRed is also present in the low energy conformations of immature DsRed.

Modeling

The coordinates for the crystal structure of DsRed were obtained from the file 1GGX[11] in the Protein Data Bank. MacroModel v7.1[17] was used to graphically replace the posttranslationally formed chromophore with the precyclized amino acid sequence, henceforth referred to as the immature form. Hydrogen atoms were added to both protein atoms and solvent atoms as required.

All calculations were done with the AMBER* forcefield in MacroModel v7.1. An 8 Å 'hot' sphere from the chromophore (residues 66, 67 and 68) with secondary constrained spheres of 2 Å ($k = 100$ kJ/Å) and 2 Å ($k = 200$ kJ/mol) were used in all Monte Carlo dihedral and molecular positional conformational searches.[18,19]

During the Monte Carlo dihedral and molecular positional conformational searches all the backbone torsion angles of residues 62–70 were randomly varied between 0 and 180°. The backbone bond of residue 70, and the amide bond between residues 61 and 62 were designated as closure bonds with minimum and maximum closure distances of 0.5 and 2.5 Å, respectively. The chirality of all backbone carbons was maintained. The crystallographically-determined water molecules were incorporated into all calculations. All confirmations within 50 kJ/mol of the global minimum were saved. The Monte Carlo conformational searches consisted of 15,000 MC steps with 500 iterations per step. Structures were considered to be unique when a pair of related atoms was separated by more than 0.025 Å after a least squares superimposition of all non-hydrogen atoms.

Chromophore Formation

The chromophore in the solid state structure of wild-type DsRed was computationally converted from the red mature DsRed solid state structure to immature precyclized DsRed (see Fig. 1 for nomenclature). A thorough dihedral Monte Carlo multiple minimum search was undertaken to find the most stable conformations of DsRed prior to chromophore formation. Fifteen thousand Monte Carlo steps were taken and the resulting conformations were minimized and combined as described in the experimental section. *cis* Non-proline peptide bonds are extremely rare, and therefore dihedral angles around the amide bonds are rarely rotated, however since the DsRed crystal structure contained one *cis* bond we randomly rotated all the amide dihedral angles in the substructure. Four thousand and eighteen unique conformations were obtained. The lowest energy conformation had a distance of 3.72 Å between the carbonyl carbon of Gln66 and the amide nitrogen of Gly68, and the average distance for all the low energy conformations was 3.22 Å, which is significantly longer than that found for GFP[13] and HAL.[14] The Ramachandran plot for the GFP-chromophore forming sequence located within the β barrel of GFP, shows that the ϕ/ψ space is extremely restricted. The peptide can only adopt conformations with $\phi = 60 \pm 30°$ and $\psi = 30 \pm 15°$; these are the 'tight turn' conformations. None of the low energy conformations of DsRed has Tyr67, which corresponds to Tyr66 in GFP, with dihedral angles falling in the tight-turn range. We therefore conclude that immature precyclized DsRed does not adopt a tight turn conformation, which we believe is required for cyclization.

Density functional calculations of the cyclization in GFP[16] and HAL[14] have shown that the commonly accepted mechanism in which cyclization precedes dehydrogenation (reduced mechanism) is not energetically viable, while the alternative oxidized mechanism where a dehydration occurs prior to the formation of

the ring yields reasonable energetics for the system. A dihedral Monte Carlo multiple minimum search of the immature oxidized intermediate (see Fig. 1 for nomenclature), was therefore performed as described in the experimental section. Five hundred and eighty-nine structures were found. The lowest energy conformation had a distance of 2.91 Å between the carbonyl carbon of Gln66 and the amide nitrogen of Gly68, and the average distance for all the low energy conformations was 2.93 Å, which is significantly shorter than that found for the reduced immature conformations. The Tyr67 ϕ and ψ dihedral angles of all the low energy conformations were within the tight turn region found in GFP. Our GFP analysis[14] showed that immature GFP would adopt a tight turn in both the reduced and oxidized mechanism, with the oxidized dehydro-Tyr66 intermediate having the tighter turn. In DsRed the oxidized mechanism has an intermediate in the tight turn conformation, while the reduced mechanism does not have a pre-organized tight turn conformation.

Is the Phe65-Gln66 Peptide Bond *cis* in Immature DsRed?

Ranganathan and co-workers found that the peptide bond between Phe65 and Gln66 was in the *cis* configuration in their crystal structure of DsRed.[11] They suggested that the *cis*/*trans* isomerization of this peptide bond was critical to the last step of the chromophore formation, which is responsible for the change from green to red fluorescence. However, all the low energy conformations found in the conformational searches described above, had a *cis* peptide bond in the same position as that found in the crystal structure of DsRed. No other *cis* amide linkages were found. The lowest energy conformation with a *trans* amide linkage between Phe65 and Gln66 was found 33 kJ/mol higher in energy than the lowest energy *cis* conformation.

Figure 2 shows the distribution of the dihedral angles of the peptide bond between Phe65 and Gln66 of the five hundred lowest energy conformations found in the three conformational searches. Both the immature precyclized (blue) and oxidized (red) forms of GFP are in the *cis* conformation. The only *trans* conformations were found at higher energies or by restraining the dihedral angle to a *trans* conformation (green). Examination of the residues surrounding the unusual *cis* peptide bond, showed that the *cis* conformation induces a deviation from planarity in the amide between Gln64 and Phe65. The deviation is more pronounced for the oxidized form (pink) than the reduced immature precyclized form (turquoise), and is non-existent in the oxidized form that has been constrained into a *trans* conformation (yellow).

In conclusion if chromophore formation in DsRed occurs by the commonly accepted reduced mechanism then our calculations clearly show that the immature form of the chromophore does not adopt a tight turn conformation. The precyclized immature intermediate expected on the basis of the reduced mechanism is not preorganized for cyclization. The tight turn conformation,

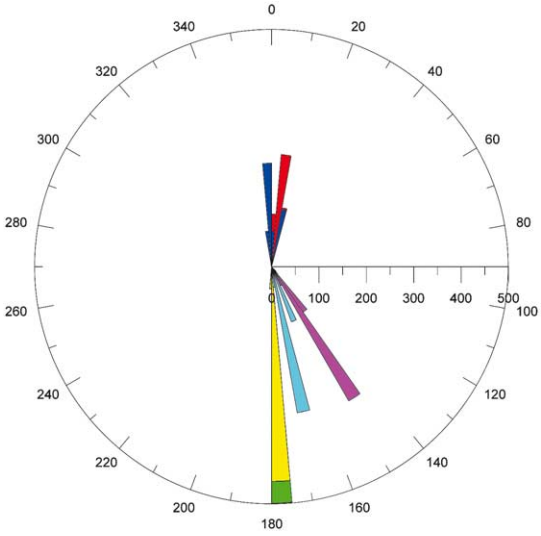

Figure 2. Distribution of the Phe65-Gln66 dihedral angles found in the 500 lowest energy conformations of the oxidized intermediate (red), reduced intermediate (blue) and oxidized intermediate (green) with imposed *trans* geometry. The Gln64-Phe65 linkage is non planar in the oxidized intermediate (pink) and the reduced intermediate (turquoise), but not in the oxidized conformations with imposed *trans* Phe65-Gln66 dihedral angles.

which brings the amide nitrogen and carbonyl carbon that form the five-membered ring in close proximity, is found in the oxidized mechanism.

The *cis* conformation found between Phe65 and Gln66 in the crystal structure of mature red fluorescent DsRed is also found in both the reduced and oxidized immature intermediates of DsRed. Since these intermediates form the green intermediate (see Fig. 2), there is no way that the *cis*/*trans* isomerization can be responsible for the acylimine formation.

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