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Supporting information for article:

An unusual disulfide-linked dimerization in the fluorescent protein rsCherryRev1.4

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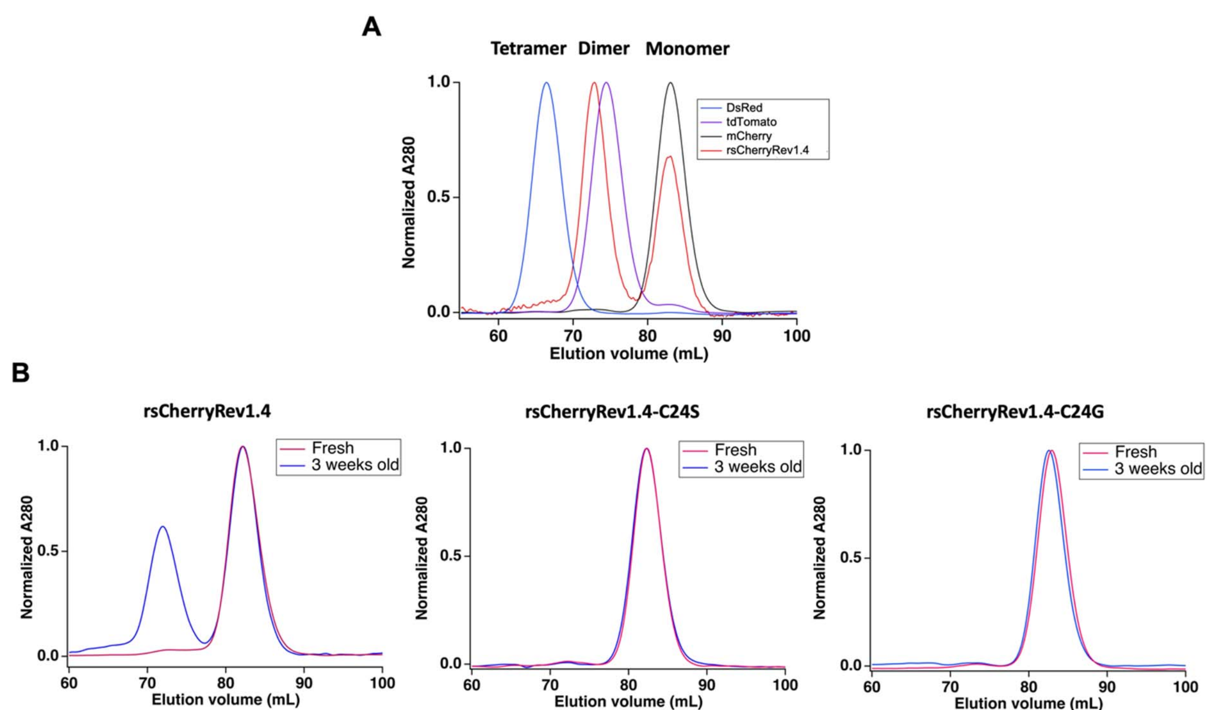

Figure S1 Size exclusion chromatography profiles of rsCherryRev1.4 and its mutants. (A) Oligomer states of rsCherryRev1.4 compared to DsRed (tetramer), tdTomato (dimer) and mCherry (monomer). (B) Oligomer states of rsCherryRev1.4 and its mutants C24S and C24G after three weeks of storage at 4°C.

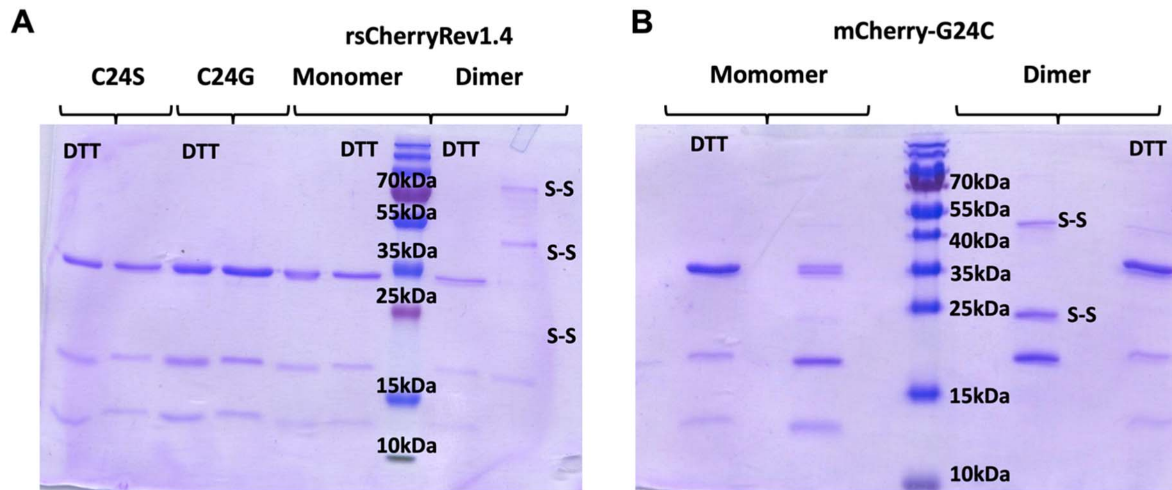

Figure S2 SDS-PAGE analysis of rsCherryRev1.4, its mutants and mCherry-G24C under reducing or non-reducing conditions. The samples were collected from the eluted peaks after size exclusion chromatography. Multiple bands in SDS-PAGE analysis of monomeric and dimeric rsCherryRev1.4 can be explained as a result of an acylimine cleavage which is frequently observed for SDS-PAGE analysis of DsRed derivatives (Gross *et al.*, 2002).

Note:

N-terminal after an acylimine cleavage: Mass ~ 11.6 kDa

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPMVSKGEEDNMAIIKEFMRFKVHMEG
SVNCHEFEIEGEGEGHPYEGTQTAKLKVTKGGPLPFAWDILSPQF

C-terminal after an acylimine cleavage: Mass ~ 18.8 kDa

MYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLCG
TNFPSDGPVMQKKTMGWFACSEQMYPEDGALKGLSKMRLKLKDGGHYDAEFKTTYKAKK
PVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK

The molecular mass of the precursor peptide is 30.4 kDa. The backbone cleavage occurs at the acylimine bond linking the chromophore and Phe65. Therefore, both reduced/non-reduced monomeric rsCherryRev1.4 and non-reduced dimeric rsCherryRev1.4 result in 30.4, 18.8 and 11.6 kDa bands in SDS-PAGE analysis. Dimeric rsCherryRev1.4 under non-reducing possibly results in the following fragments bands in SDS-PAGE analysis:

+ 60.8 kDa (dimer with a S-S bond)

+ 30.4 + 11.6 = 42 kDa (1 monomer + N-terminal due to a S-S bond at Cys24-Cys24)

+ 11.6 + 11.6 = 23.2 kDa (2 N-terminals due to a S-S bond at Cys24-Cys24)

+ 18.8 kDa (C-terminal)

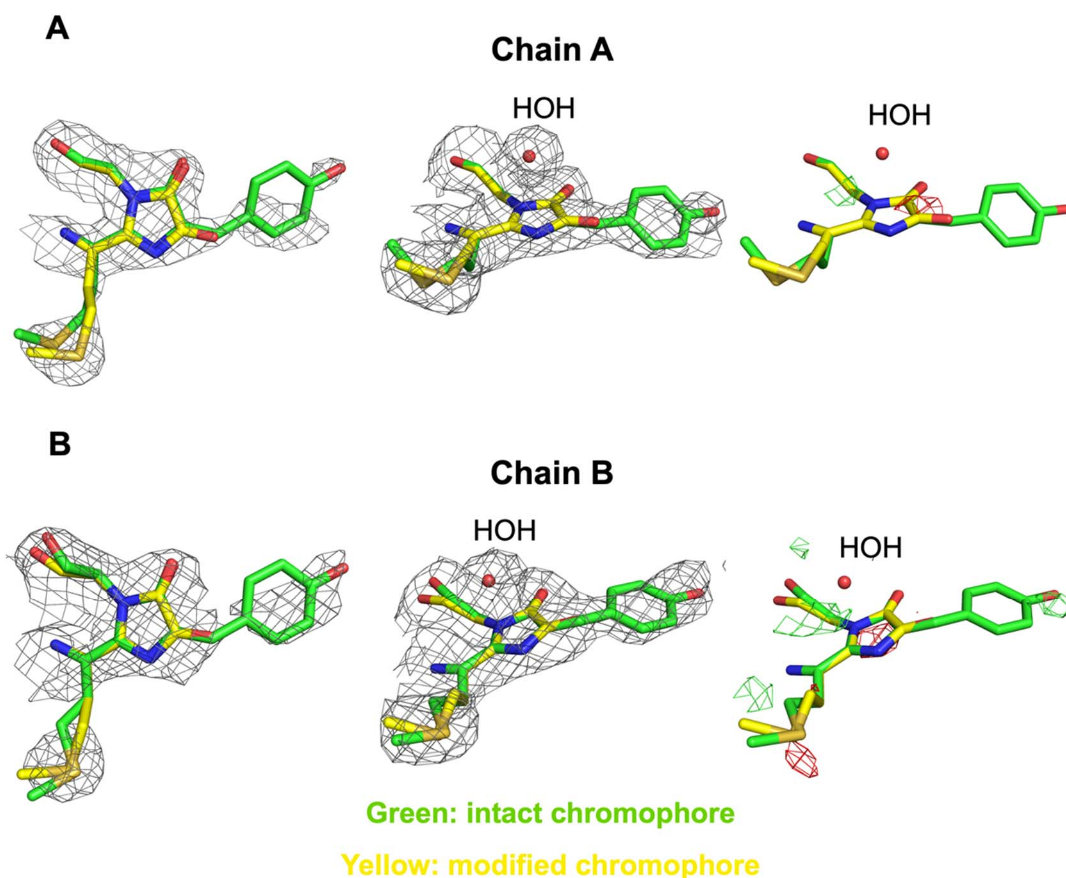

Figure S3 Electron density maps and chromophore structures of two protomers (chain A and B) in the rsCherryRev1.4 structure. The chromophores are depicted as sticks. The intact chromophore and modified chromophore are colored green and yellow, respectively. (Left) $2F_o$-F_c maps contoured at a level of 1 r.m.s.d., showing the incomplete electron density at the position of the intact chromophores. (Middle) $2F_o$-F_c maps contoured at a level of 0.5 r.m.s.d., indicating the presence of the intact chromophore with some residual electron density attached to the imidazolinone ring. (Right) F_o-F_c maps contoured at a level of 3 r.m.s.d., showing some positive and negative electron density surrounding the chromophores. Images were generated using PyMOL (The PyMOL Molecular Graphics System, Version 2.5.2, Schrödinger, LLC).

Note: The $2F_o$-F_c map contoured at a level of 1 r.m.s.d. of the rsCherryRev1.4 displays reduced electron density at the positions where the intact chromophores should occur (Figs. S3A, B-left). This suggests that rsCherryRev1.4 underwent chromophore degradations with cleavage of the *p*-hydroxyphenyl ring from the chromophore skeleton (Bui *et al.*, 2023). A trace of the intact chromophore was still detected in the rsCherryRev1.4 solution with an absorbance peak at approximately 410 nm (Fig. S4). It could be explained by the absorbance of the intact chromophore in the protonated form or the chromophore of the pre-activated form which is similar to PAmCherry (Subach, Malashkevich *et al.*, 2009) (Fig. S4). Therefore, the structure of rsCherryRev1.4 was refined with both the intact and modified chromophore, resulting in occupancies of 0.25 and 0.75,

respectively. The model fits rather well to the electron density maps despite some residual electron density in the $2F_o$-F_c maps contoured at a level of 0.5 r.m.s.d. (Figs. S3A, B-middle) and a few positive/negative electron density peaks in the F_o-F_c maps contoured at a level of 3 r.m.s.d. (Figs. S3A, B-right). This residual electron density suggests that additional structures are possibly present at the chromophore position. However, the limited resolution of the x-ray structure makes it difficult to identify other modified structures formed during the chromophore degradation, which requires more investigations to address these modifications.

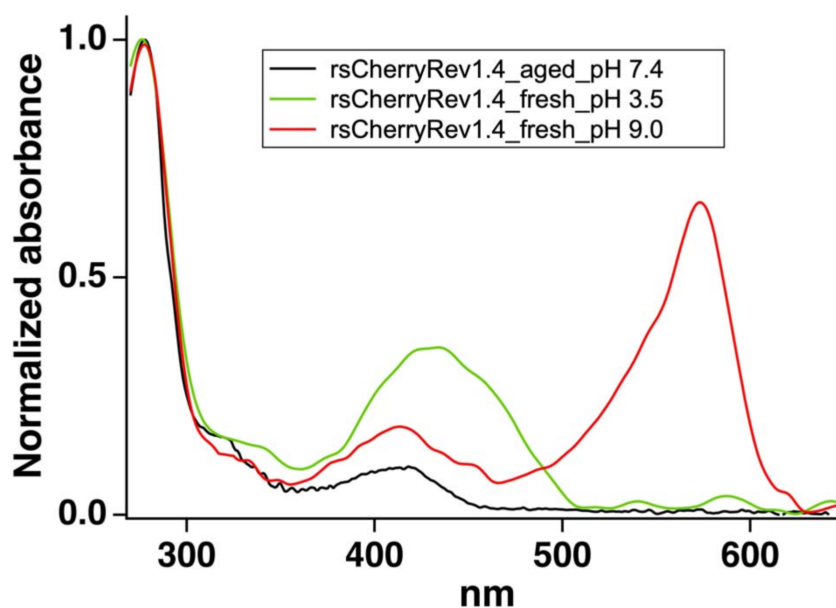

Figure S4 The absorbance spectra of rsCherryRev1.4 in solutions collected with the freshly purified samples (at pH 3.5 and 9.0) and the sample used to crystallize (at pH 7.4).

Table S1 List of used primers

Name of primer	Primer sequence
rsCherryRev1.4-C24G_forward	5’-GGGCTCCGTGAACGGCCACGAGTTCGA-3’
rsCherryRev1.4-C24S_forward	5’-GGGCTCCGTGAACAGCCACGAGTTCGA-3’
mCherry-G24C_ forward	5’-GGGCTCCGTGAACTGCCACGAGTTCGA -3’

Table S2 Interface residues and atoms in the dimeric rsCherryRev1.4 structure, using the web-based sever PDBePISA

Chain A			Chain B		
Residue name	Residue number	Interface atoms	Residue name	Residue number	Interface atoms
A:SER	21	CB, OG	B:SER	21	CB, OG
A:CYS	24	N, CA, O, CB, SG	B:CYS	24	N, CA, O, CB, SG
A:GLU	26	CG, OE2	B:GLU	26	CG, OE2
A:GLU	94	OE1	B:GLU	94	OE1
A:VAL	96	CB, CG1, CG2	B:VAL	96	CB, CG1, CG2
A:ASN	98	OD1, ND2	B:ASN	98	OD1, ND2
A:GLY	102	CA, O	B:GLY	102	CA, O
A:VAL	104	O, CB, CG1, CG2	B:VAL	104	O, CB, CG1, CG2
A:THR	106	CB, OG1, CG2	B:THR	106	C, CB, OG1, CG2
A:VAL	107	C	B:VAL	107	C
A:THR	108	OG1	B:THR	108	OG1
A:LYS	123	CG, CD, CE	B:LYS	123	CE, NZ
A:CYS	125	C, O, CB, SG	B:CYS	125	C, O, CB, SG
A:GLY	126	N, C, O	B:GLY	126	N, C, O
A:THR	127	CB, OG1, CG2	B:THR	127	CB, OG1, CG2
A:ASN	128	N, CA, C, O, CB, CG, OD1, ND2	B:ASN	128	N, CA, CB, OD1, ND2
A:PHE	129	C, O	B:GLU	176	OE1
A:PRO	130	CA	B:LYS	178	NZ
A:LYS	178	NZ			