An unfolded C_H1 domain controls the assembly and secretion of IgG antibodies

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- Supplemental Material -

Supplemental Methods

Protein production. The C_H1 domain (Thr123-Arg215 of the pdb file 1FH5; numbering starts with one in this work) and an Ala113Cys mutant of the C_L domain were amplified from the murine IgG1 MAK33 cDNA. Additionally, the murine IgG1 MAK33 cDNA was used as a template to amplify the gene segments encoding the C_L domain with the native C-terminal cysteine residue (amino acids Ala112-Cys215 of the pdb file 1FH5) and the C_H1 domain with the native C-terminal cysteine residue (Thr123-Cys217 of the pdb file 1FH5). All genes were cloned into the pET28a (Novagen, Gibbstown, NJ, USA) vector without a tag, except for the wild type C_L domain which was essentially purified as published (Feige et al., 2007). Isotope labeled C_H1 for NMR experiments was expressed in M9 minimal medium. For FRET measurements, the Ala113Cys mutant of the C_L domain was used. In the case of C_H1, the native C-terminal cysteine residue was used for labeling in FRET experiments. Proteins were expressed as inclusion bodies overnight at 37°C in BL21-DE3 cells in selective LB medium. Inclusion bodies were isolated as described (Feige et al., 2004). Briefly, inclusion bodies were solubilized in 50 mM Tris/HCl, pH 7.5, 10 mM β-mercaptoethanol, 10 mM EDTA, 8 M urea and subsequently applied to a Q-Sepharose column equilibrated in 50 mM Tris/HCl, pH 7.5, 10 mM EDTA, 5 M urea. All proteins of interest did not bind to the column under these conditions. Refolding was carried out by dialysis as published (Feige et al., 2007). After refolding, all proteins were applied to a Superdex 75pg (26/60) gel filtration column (GE Healthcare, München, Germany) equilibrated in PBS. Labeling of C_L Ala113Cys with lucifer yellow iodoacetamide (Invitrogen, Carlsbad, CA, USA) was performed according to the manufacturer's protocol. For FRET experiments between BiP, C_L and C_H1, C_L Ala113Cys was labeled with the ATTO532 maleimide dye (ATTO-TEC,

Weidenau, Germany), and murine BiP, which possesses two free cysteines, was labeled with ATTO594 maleimide. For FRET experiments between BiP and C_H1, C_H1 was labeled with ATTO532 at its native C-terminal cysteine residue. All proteins were separated from the free dye *via* a Superdex200 10/300GL HPLC column equilibrated in HKM buffer. All vectors in this study were sequenced and protein masses were verified by mass spectrometry.

Preparation of reduced C_H1. To reduce the internal disulfide bond of the C_H1 domain, the protein was unfolded in 2 M GdmCl in PBS at 25°C for 2 h in the presence of 10 mM TCEP. Subsequently, the protein was applied to a Superdex75 10/300GL HPLC column equilibrated in PBS supplemented with 0.1 mM TCEP.

Optical spectroscopy. A Jasco J-720 spectropolarimeter was used for all measurements (Jasco, Gross-Umstadt, Germany). A 2 mm quartz cuvette was used for near-UV CD spectra and kinetics (observed at 280 nm). 100 µM protein respectively 100 µM of each domain were used. Spectra of the C_H1 domain in the complex were calculated by substraction of the spectrum of the isolated C_L domain from the spectrum of the complex, measured after a 4 h equilibration step at 25°C. All spectra were averaged 16 times and buffer corrected.

Analytical HPLC experiments. For all experiments, a Shimadzu HPLC system (LC-20AT, SPD-20A, RF-10A_{XL}, SIL-20AC) (Shimadzu, München, Germany) was used. To assess complex formation between C_L and C_H1, both proteins were incubated at 25°C with a domain concentration of 25 µM each. After varying times, 100 µl of the sample were applied to a Superdex75 10/300GL HPLC column (GE Healthcare, München, Germany) in PBS at a flow rate of 0.75 ml/min. The peak height

corresponding to the dimer was analyzed over incubation time. To assess whether C_H1 can be oxidized if bound to BiP, C_H1 was reduced in HKM buffer with 2 mM TCEP for 1 hour. Complex formation between 30 µM reduced C_H1 and 50 µM Bip was performed at 37°C for 2 hours in HKM buffer supplemented with 1 mM ADP and 1 mM TCEP. The sample was applied at a flow rate of 0.25 ml/min to a Superdex 200 10/300GL column equilibrated in HKM buffer supplemented with 4 mM GSSG and 1 mM TCEP. Only the peak of BiP-bound C_H1 was collected and free sulfhydryls were reacted with 10 mM NEM for 15 minutes at room temperature. The samples were subsequently analyzed by MALDI-TOF/TOF mass spectrometry which showed peaks for oxidized as well as NEM-reacted C_H1. As a control, reduced, BiP-bound C_H1 was reacted under the same conditions with NEM and only showed peaks for NEM bound C_H1.

SDS-PAGE experiments. To assess the formation of the disulfide-bridged C_L/C_H1 dimer, both proteins were co-incubated at a concentration of 25 µM each in PBS at 25°C. If present, the concentration of CypB was 5 µM. At the indicated times, a 20 µl aliquot was withdrawn, supplemented with 10 µl 3x Laemmli sample buffer (without β-mercaptoethanol) and boiled for 1 min at 95°C. Subsequently, 20 µl of each sample was applied to a 17.5% SDS-PAGE gel and run for 75 min at 25 mA. The bands were quantified with ImageQuant TL (GE Healthcare, München, Germany).

Supplemental References

Feige,M.J., Hagn,F., Esser,J., Kessler,H., and Buchner,J. (2007). Influence of the internal disulfide bridge on the folding pathway of the C-L antibody domain. Journal of Molecular Biology *365*, 1232-1244.

Feige,M.J., Walter,S., and Buchner,J. (2004). Folding mechanism of the C(H)2 antibody domain. Journal of Molecular Biology *344*, 107-118.

Supplemental Figure Legends

Supplemental Figure 1 – Formation of tertiary and quaternary structure upon C_H1/C_L association. (A) The isolated C_L domain shows a well defined near-UV CD spectrum (cyan), whereas the isolated C_H1 domain shows the featureless spectrum of an unfolded protein (blue). The complex between C_L and C_H1 is shown in green and the spectrum of the C_H1 domain in the complex in red. The change in near-UV CD signal at 280 nm upon the co-incubation of C_L and C_H1 is shown in (B). The trace could be fitted with a single exponential function with a time constant of τ = 50 ±2 min (C) Formation of stable C_H1/C_L quaternary structure was assessed by analytical HPLC-experiments using 25 µM of each protein. The elution profiles after 1 min of co-incubation of C_L and C_H1 (dashed line) and after 5 h of co-incubation (straight line) are shown. In (D), the normalized intensity of the peak corresponding to the C_L/C_H1 dimer (15 min) is plotted over time. It could be fitted with a single exponential function (τ = 65 ± 5 min). All measurements were performed in PBS at 25°C.

Supplemental Figure 2 – Influence of the redox status of the C_H1 domain on its folding status and its association-coupled folding process. (A) The far-UV CD spectrum of the reduced C_H1 domain is characteristic of an unfolded protein. (B) 5 µM oxidized (straight line) or reduced C_H1 (dashed line) were added to 1 µM lucifer yellow labeled C_L. The change in anisotropy over time could be well described as a single exponential function for oxidized C_H1 (k_{obs} = 0.19 min^{-1}), for reduced C_H1 no change was observed. (C) For oxidized C_H1 (straight line), folding in the presence of C_L could be observed by far-UV CD-spectroscopy at 205 nm (τ = 44 ±4 min) whereas no structure formation was detectable for the reduced C_H1 domain (dashed line). A

concentration of 10 µM was used for each protein. All measurements were carried out at 25°C in PBS in the presence of 50 µM TCEP.

Supplemental Figure 3 – C_L/C_H1 association coupled to intermolecular disulfide bridge formation. (A) The isolated C_L domain with the C-terminal cysteine residue shows an all-β far-UV CD spectrum (cyan), whereas the isolated C_H1 domain with the C-terminal cysteine residue displays a random coil spectrum (blue). The complex of both proteins is shown in green. The spectrum of the C_H1 domain, calculated from the individual spectra of C_L and the C_H1/C_L complex, reflects folding of the C_H1 domain in the presence of C_L (red). (B) Folding of the C_H1 domain, followed by far-UV CD spectroscopy at 205 nm, occurred with a time constant of τ = 53 ±5 min in the absence of CypB (red trace). It could be accelerated to τ = 38 ±4 min in the presence of 2 µM CypB (blue trace). The acceleration could be inhibited by 4 µM CspA (not shown). All measurements were carried out in PBS at 25°C with C_L respectively C_H1 containing their native C-terminal cysteine residue.

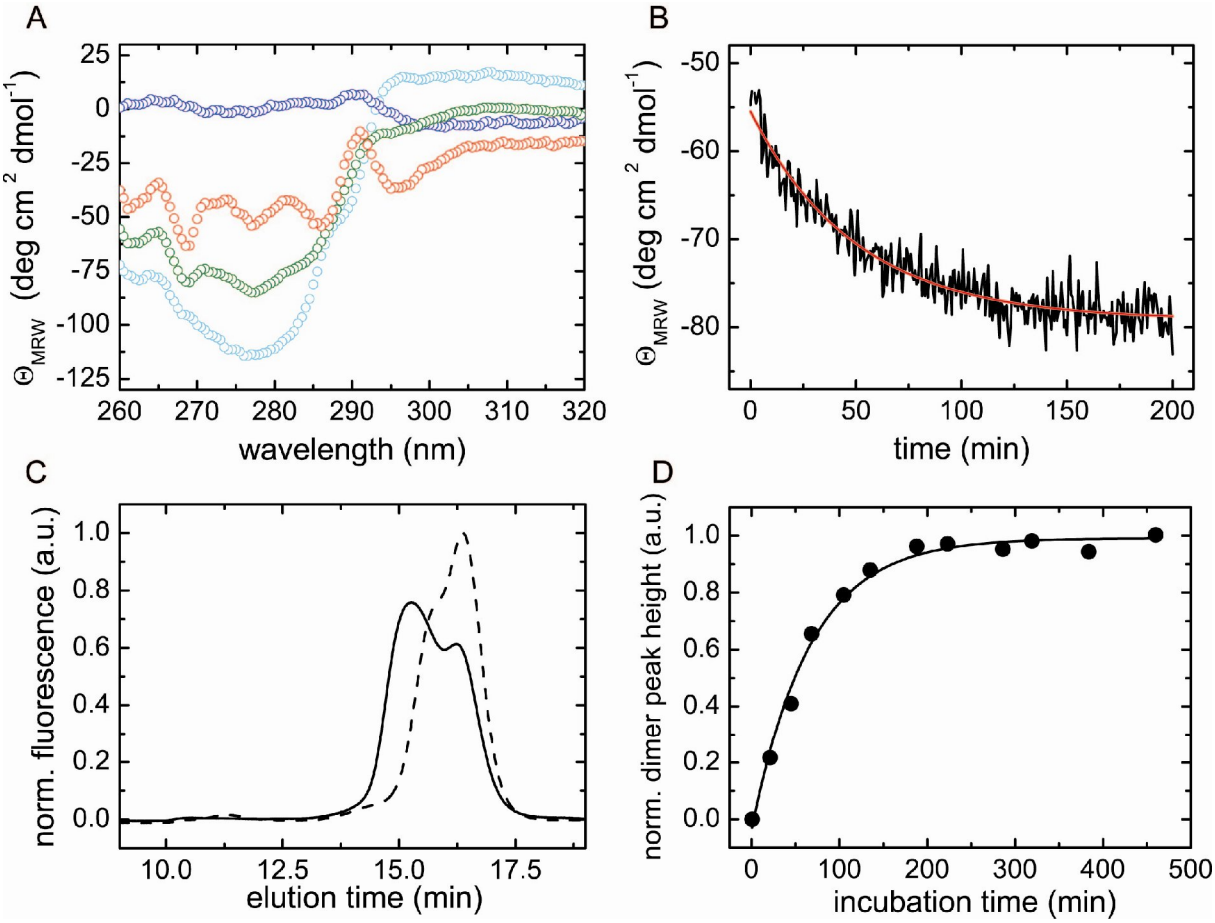
Supplemental Figure 4 – Assessment of the formation of triple complexes between BiP, C_H1 and C_L. To investigate the possibility that either C_L could associate with BiP bound C_H1 or if the C_L/C_H1 complex binds to BiP, 1 µM ATTO532 labeled C_L was incubated with 10 µM ATTO594 labeled BiP (A). The donor, ATTO532, was excited at 500 nm and the donor fluorescence was recorded at 550 nm (green line), the fluorescence of the acceptor, ATTO594, was recorded at 625 nm (red line). After 30 min, where no binding of C_L to BiP could be detected as expected (data not shown), 10 µM unlabeled C_H1 was added. Under these conditions, C_H1 binds to BiP within several minutes. If either C_L could bind stably to the BiP:C_H1

complex or if BiP could bind to the C_L/C_H1 complex, a FRET signal between BiP and C_L is expected. However, this was not observed arguing against the presence of BiP:C_H1:C_L triple complexes. As a control of the FRET system, the association between 10 µM ATTO594 labeled BiP and 1 µM ATTO532 labeled C_H1 was measured (B). The association reaction occurred with a rate of k_{obs} = 0.01 ±0.005 min^{-1}. In (C, left) a putative triple complex between BiP (grey, model based on the pdb code 3C7N, chain B), C_H1 (purple) and C_L (blue) is shown. The labels are schematically shown in green (ATTO532) respectively in red (ATTO594) and distances are indicated. The Förster radius of the dye pair is 6.6 nm (www.atto-tec.de). On the right, a putative complex between BiP and C_H1 is shown. All measurements were performed at 25°C in HKM buffer with 1 mM ADP and donor fluorescence was linearly corrected for bleaching.

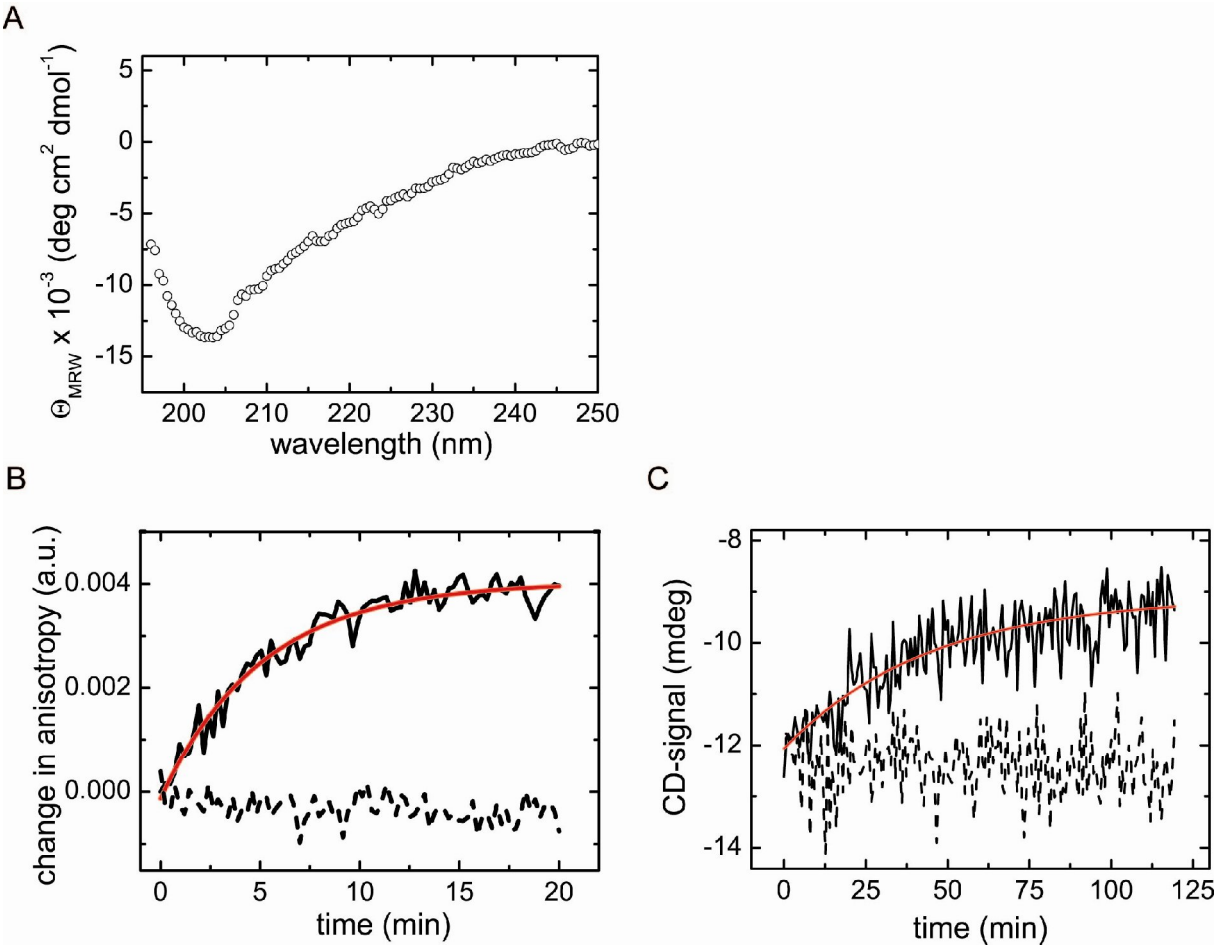
Supplemental Figure 5 – A folded C_L domain is required for the proper maturation and secretion of heavy chains from cells. COS-1 cells were co-transfected with vectors encoding BiP, the HA-tagged versions of either wild type light chain (LC_{wt}) or the mutant light chain containing an unfolded C_L domain (LC_{CLmut}) and either a humanized chimeric γ heavy chain or the MAK33 γ heavy chain. After 24 h, cells were metabolically labeled and both cell lysates (no subscript) and culture supernatants (subscript m) were immunoprecipitated with the indicated antisera. Precipitated proteins were separated by SDS-PAGE under non-reducing condition and visualized by autoradiography.

Supplemental Figures

Supplemental Figure 1



Supplemental Figure 2



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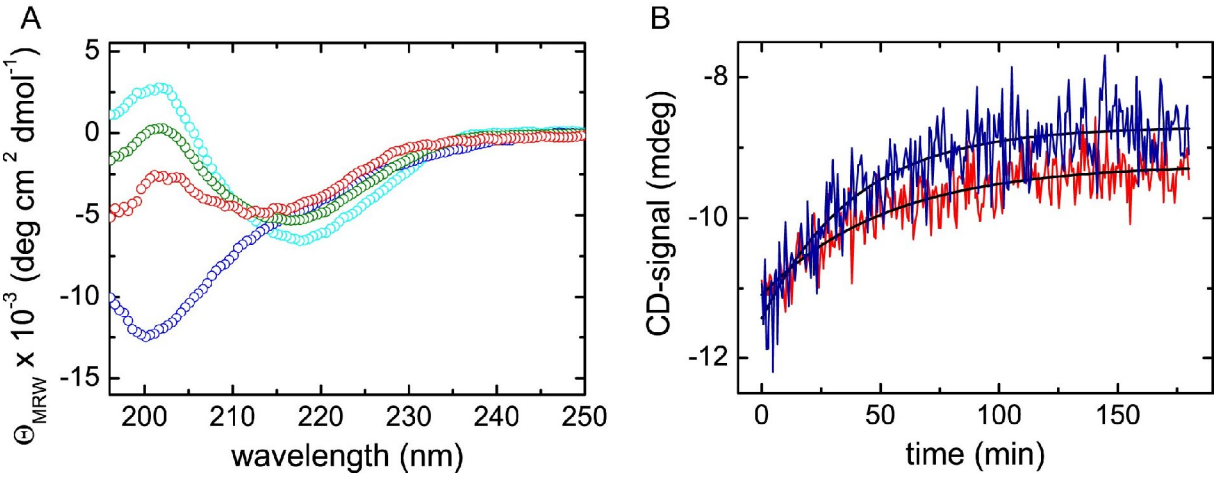
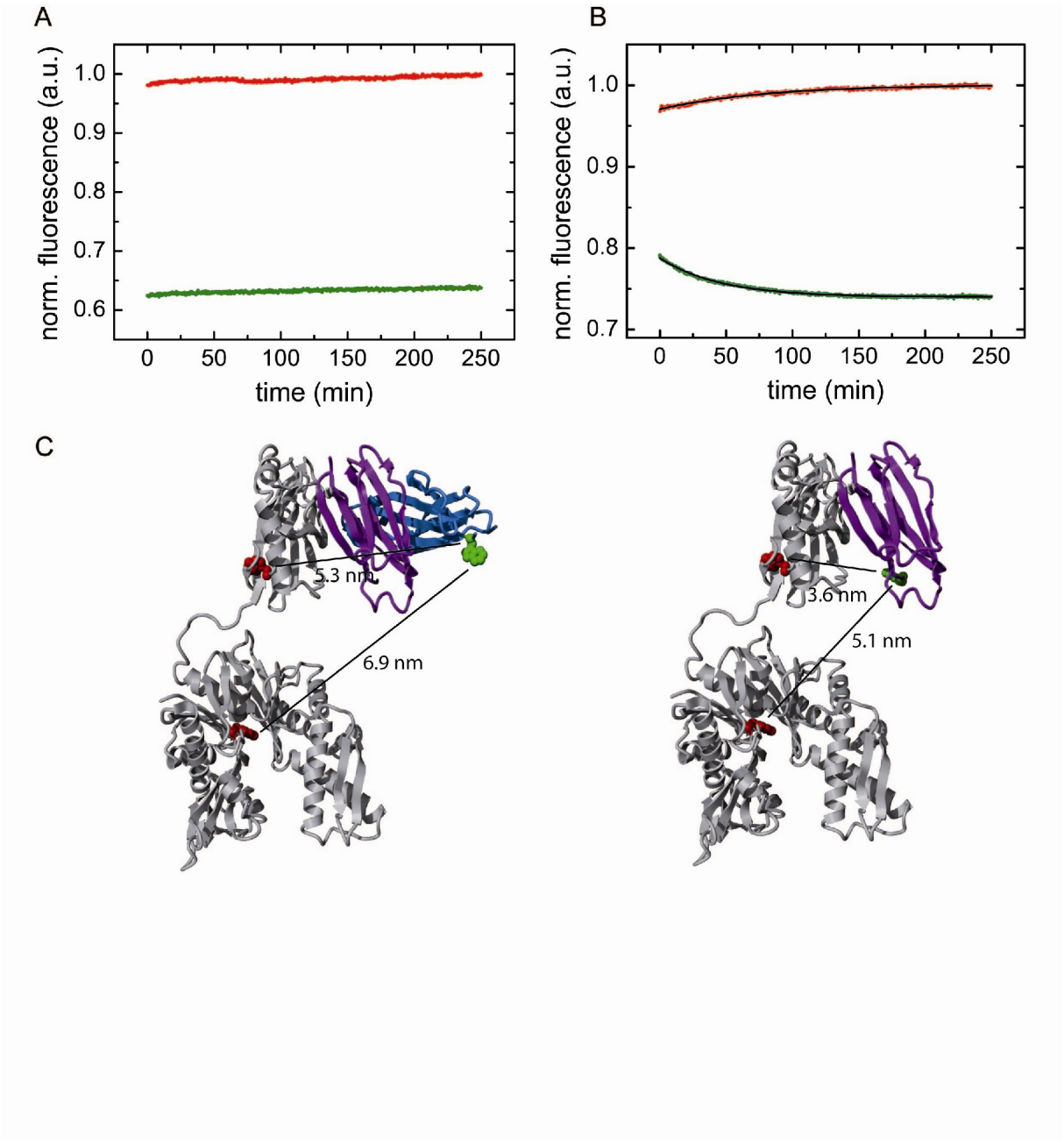


Supplemental Figure 4



Supplemental Figure 5