Supplementary Information for

Structural analysis of the regulatory GAF domains of cGMP phosphodiesterase elucidates the allosteric communication pathway

Richa Gupta, Yong Liu, Huanchen Wang, Christopher T. Nordyke, Ryan Z. Puterbaugh, Weijun Cui, Krisztina Varga, Feixia Chu, Hengming Ke, Harish Vashisth, and Rick H. Cote

Corresponding author: Rick H. Cote
Email: rick.cote@unh.edu

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Exp. m/z	z	Δ	aa1	aa2	Cross-linker
571.6118	3	0.57	72	151	Sulfo-SDA
979.5052	2	1	72	82	Sulfo-SDA
486.2567	4	-0.71	78	80	Sulfo-SDA
730.3591	2	0.82	78	79	Sulfo-SDA
687.1058	4	-0.056	78	122	BS3
515.7557	4	0.24	78	412	BS3
486.2567	4	-0.71	82	83	Sulfo-SDA
794.1235	3	-1.6	82	122	BS3
1018.5402	3	1.7	95	171	Sulfo-SDA
609.0884	4	2.9	109	150	Sulfo-SDA
711.3702	3	-2.9	122	160	BS3
625.6874	3	0.15	122	159	BS3
1045.5590	4	3.2	130	132	Sulfo-SDA
1024.5414	4	-15	130	214	Sulfo-SDA
983.8519	3	1.9	130	132	Sulfo-SDA
1019.1956	3	-0.25	132	171	BS3
825.4305	4	1.6	132	170	BS3
611.3123	3	-0.078	132	160	BS3
752.1084	3	2.2	135	406	Sulfo-SDA
815.7150	7	5.9	150	179	EDC
435.9857	4	0.091	151	210	Sulfo-SDA
533.2853	4	-1.7	151	168	Sulfo-SDA
481.5236	4	1.4	151	170	BS3
753.1982	5	0.38	151	171	BS3
760.4017	3	-1.1	151	160	BS3
599.6542	3	0.63	151	214	BS3
506.2894	4	-1.2	151	159	BS3
650.0952	4	3	157	160	EDC
404.7160	4	-1	159	170	BS3
397.2198	3	-1.2	159	412	BS3
514.6675	5	-0.061	160	163	Sulfo-SDA
562.5233	4	-0.091	160	368	Sulfo-SDA
497.0018	4	-1.1	160	293	Sulfo-SDA
778.8913	4	-0.26	160	171	BS3
1165.0922	2	-0.29	170	171	Sulfo-SDA
729.1262	4	3.2	171	412	BS3
1042.5483	3	0.27	188	189	Sulfo-SDA
782.1626	4	-0.29	188	190	Sulfo-SDA
721.7288	3	-0.52	199	200	Sulfo-SDA
738.4115	3	-0.76	211	215	Sulfo-SDA
554.0616	4	1.3	212	215	Sulfo-SDA
1084.5538	2	-2.4	249	250	Sulfo-SDA

Exp. m/z	z	Δ	aa1	aa2	Cross-linker
524.5365	4	1	252	263	Sulfo-SDA
652.8502	4	0.77	254	263	Sulfo-SDA
545.5443	4	0.33	258	319	Sulfo-SDA
473.2614	4	0.4	259	263	Sulfo-SDA
644.8098	4	14	263	450	Sulfo-SDA
516.0494	5	14	263	451	Sulfo-SDA
516.0497	5	14	263	452	Sulfo-SDA
601.3285	4	-0.72	263	446	Sulfo-SDA
439.7320	4	-0.2	263	439	Sulfo-SDA
630.6795	3	0.33	263	264	Sulfo-SDA
551.5588	4	5.1	263	323	Sulfo-SDA
660.3913	4	-0.35	263	396	BS3
610.5917	4	0.61	263	319	BS3
947.9791	4	1.7	283	288	EDC
710.1106	4	6.1	288	305	BS3
693.0577	3	0.28	288	395	BS3
738.3666	4	2.6	289	305	EDC
430.2311	3	0.31	304	308	Sulfo-SDA
644.8417	2	-1.7	305	306	Sulfo-SDA
812.9462	2	1.2	319	329	Sulfo-SDA
670.0311	3	-0.12	319	328	BS3
707.3931	3	0.45	327	332	Sulfo-SDA
482.2612	3	0.57	328	329	Sulfo-SDA
722.8885	2	1	328	330	Sulfo-SDA
707.3927	3	-0.11	328	333	Sulfo-SDA
591.1158	5	-0.51	329	409	Sulfo-SDA
574.6072	3	0.77	367	412	Sulfo-SDA
795.1028	3	1.3	368	396	Sulfo-SDA
715.8457	2	0.21	374	375	Sulfo-SDA
787.0561	3	1.9	374	378	Sulfo-SDA
836.1483	3	0.96	385	386	Sulfo-SDA
836.1470	3	-0.59	385	387	Sulfo-SDA
676.6324	4	-0.077	392	396	Sulfo-SDA
901.8438	3	3.3	395	396	Sulfo-SDA
868.4985	3	9.8	395	398	EDC
979.0488	4	3.8	395	397	EDC
708.6566	4	0.6	395	397	Sulfo-SDA
568.6570	3	-0.74	395	439	Sulfo-SDA
567.1266	5	0.4	396	398	Sulfo-SDA
901.8413	3	0.51	396	397	Sulfo-SDA
676.6335	4	1.6	396	399	Sulfo-SDA
516.0494	5	1.3	446	449	EDC

Table S1. Cross-links identified for the apo state of PDE6C GAFab.
Crosslinking conditions were followed as described in Fig. 3. Cross-linked peptides were identified following chemical cross-linking of 12 μM apo GAFab and analyzed as described in *Materials and Methods*. Exp. m/z is the experimentally measured mass-to-charge ratio, z is the charge state of the peptide, and Δ is the accuracy measured in parts per million. The crosslinked peptides are defined by amino acid residue number (aa1, aa2) identified using the indicated crosslinker.

Exp. m/z	z	Δ	aa1	aa2	Cross-linker
486.2574	4	0.73	78	80	Sulfo-SDA
730.3591	2	0.82	78	79	Sulfo-SDA
915.8051	3	-0.28	78	122	BS3
794.1254	3	0.75	82	122	BS3
1018.5391	3	0.58	95	171	Sulfo-SDA
674.8717	2	0.2	122	123	Sulfo-SDA
717.7296	3	0.29	122	151	BS3
975.0190	**2**	**2.1**	**122**	**412**	**BS3**
1075.8926	3	5.1	132	133	Sulfo-SDA
1045.5614	4	5.4	132	138	Sulfo-SDA
988.9919	2	-4.4	132	134	Sulfo-SDA
988.9966	2	0.39	132	135	Sulfo-SDA
1024.5280	3	0.49	132	171	BS3
611.3127	3	0.58	132	160	BS3
488.7177	4	-4.1	151	170	Sulfo-SDA
570.5550	4	2.2	151	160	BS3
779.6612	4	0.75	151	171	BS3
898.9781	2	1.1	151	214	BS3
650.0953	4	3.2	157	160	EDC
728.1359	4	4.6	159	180	BS3
680.8263	**4**	**-14**	**169**	**308**	**Sulfo-SDA**
1157.0958	2	0.62	170	171	Sulfo-SDA
1047.8823	3	2.5	188	189	Sulfo-SDA
723.3744	3	1.4	249	250	Sulfo-SDA
524.5358	4	-0.42	255	263	Sulfo-SDA
492.5123	4	2.1	258	319	Sulfo-SDA
648.6508	3	-0.35	258	374	Sulfo-SDA
473.2616	4	0.82	259	263	Sulfo-SDA
644.8104	4	15	263	450	Sulfo-SDA
516.0493	5	14	263	452	Sulfo-SDA
516.0497	5	14	263	451	Sulfo-SDA
630.6798	3	0.87	263	264	Sulfo-SDA
880.1890	3	3.1	263	396	BS3
517.0281	4	0.32	263	441	BS3
527.0438	4	-0.4	263	409	BS3

Exp. m/z	z	Δ	aa1	aa2	Cross-linker
610.5907	4	-1	263	319	BS3
552.5042	5	-0.28	263	328	BS3
495.0205	**4**	**0.46**	**263**	**412**	**BS3**
947.9793	4	1.9	283	288	EDC
907.1179	3	2.5	286	288	Sulfo-SDA
430.8685	3	-1.1	286	287	Sulfo-SDA
644.0007	**3**	**-0.13**	**286**	**319**	**BS3**
710.1086	4	3.3	288	305	BS3
738.3680	4	4.5	289	308	EDC
430.2317	3	1.7	304	308	Sulfo-SDA
644.8426	2	-0.33	305	306	Sulfo-SDA
672.8561	2	0.27	305	308	BS3
605.6481	**3**	**0.79**	**308**	**311**	**Sulfo-SDA**
605.6487	3	1.8	308	309	Sulfo-SDA
605.3184	3	0.11	319	441	BS3
670.0314	3	0.33	319	328	BS3
618.6730	3	-0.17	319	409	BS3
835.9720	2	0.22	328	329	Sulfo-SDA
1060.5872	2	1.6	328	330	Sulfo-SDA
707.3930	3	0.31	328	333	Sulfo-SDA
596.5784	4	0.42	367	396	Sulfo-SDA
574.6066	3	-0.28	367	412	Sulfo-SDA
596.5776	4	-0.92	368	396	Sulfo-SDA
715.8458	2	0.35	374	375	Sulfo-SDA
787.0552	3	0.76	374	378	Sulfo-SDA
720.3610	**3**	**-0.51**	**374**	**409**	**BS3**
707.0067	3	0.15	374	441	BS3
1063.0977	2	-1	385	386	Sulfo-SDA
901.8419	3	1.2	395	396	Sulfo-SDA
911.1931	3	5.2	395	398	EDC
901.8418	3	1.1	395	396	Sulfo-SDA
708.6564	4	0.29	396	400	Sulfo-SDA
632.3226	2	1.4	444	445	Sulfo-SDA
516.0505	5	3.4	446	449	EDC

Table S2. Cross-links identified for the cGMP-liganded state of PDE6C GAFab (GAFab·cGMP).

Crosslinking conditions were followed as described in Fig. 3 Cross-linked peptides were identified following chemical cross-linking of 12 μM GAFab pre-incubated with 10-fold molar excess cGMP and analyzed as described in *Materials and Methods*. Crosslinks found only in the cGMP liganded state are shown in bold. Abbreviations are defined in the legend to Table S1.

Exp. m/z	z	Δ	pep1	aa1	pep2	aa2	Cross-linker
929.4758	2	-2.4	GAFab	72	GAFab	82	EDC
687.1057	4	-0.2	GAFab	78	GAFab	122	BS3
730.3585	2	0.0019	GAFab	78	GAFab	79	Sulfo-SDA
547.8380	2	0.61	GAFab	82	GAFab	83	Sulfo-SDA
674.8718	2	0.34	GAFab	122	GAFab	123	Sulfo-SDA
760.0797	3	1.3	GAFab	122	GAFab	135	Sulfo-SDA
811.4542	3	0.47	GAFab	122	GAFab	189	EDC
769.7230	3	0.046	GAFab	122	GAFab	367	EDC
726.3870	3	-1.1	GAFab	122	GAFab	378	EDC
1045.5575	4	1.7	GAFab	130	GAFab	132	Sulfo-SDA
1024.5275	3	0.0031	GAFab	132	GAFab	171	BS3
611.3097	3	-4.3	GAFab	132	GAFab	160	BS3
988.9966	2	0.39	GAFab	132	GAFab	134	Sulfo-SDA
988.9964	2	0.19	GAFab	132	GAFab	135	Sulfo-SDA
752.1006	3	-8.2	GAFab	132	GAFab	386	Sulfo-SDA
745.1490	4	-1.3	GAFab	132	GAFab	133	EDC
699.0277	3	-1.6	GAFab	150	GAFab	170	Sulfo-SDA
464.6031	3	1	GAFab	151	GAFab	169	BS3
599.6564	3	4.3	GAFab	151	GAFab	214	BS3
646.8674	2	2	GAFab	151	GAFab	170	BS3
593.5720	4	0.23	GAFab	151	GAFab	209	Sulfo-SDA
902.1306	3	1.8	GAFab	156	GAFab	171	Sulfo-SDA
866.4572	3	2.3	GAFab	157	GAFab	160	EDC
1047.8821	3	2.3	GAFab	188	GAFab	189	Sulfo-SDA
786.1621	4	0.69	GAFab	188	GAFab	190	Sulfo-SDA
727.0612	3	0.53	GAFab	199	GAFab	200	Sulfo-SDA
705.0622	3	1.3	GAFab	210	GAFab	215	EDC
738.4125	3	0.59	GAFab	211	GAFab	215	Sulfo-SDA
1006.5064	2	0.51	GAFab	249	GAFab	250	Sulfo-SDA
872.4642	2	0.43	GAFab	253	GAFab	319	EDC
681.7285	3	-1.3	GAFab	255	GAFab	395	Sulfo-SDA
819.9417	2	1.4	GAFab	256	GAFab	319	Sulfo-SDA
580.0117	3	-0.92	GAFab	258	GAFab	395	EDC
597.3286	3	0.26	GAFab	258	GAFab	263	EDC
870.1294	3	-1.3	GAFab	263	GAFab	264	Sulfo-SDA
516.0483	5	12	GAFab	263	GAFab	452	Sulfo-SDA
517.0277	4	-0.45	GAFab	263	GAFab	441	BS3
947.9800	4	2.7	GAFab	283	GAFab	288	EDC
758.5859	5	3.3	GAFab	286	GAFab	289	EDC
993.9608	2	-0.52	GAFab	286	GAFab	314	EDC
641.0312	3	-0.15	GAFab	287	GAFab	395	EDC
481.0256	4	0.65	GAFab	287	GAFab	396	EDC
756.3618	2	-0.13	GAFab	288	GAFab	292	EDC
695.0173	3	-0.43	GAFab	289	GAFab	308	EDC
641.0309	3	-0.61	GAFab	289	GAFab	395	EDC
506.0449	4	1.8	GAFab	292	GAFab	395	EDC
546.5328	4	-1.7	GAFab	293	GAFab	308	EDC
430.2307	3	-0.62	GAFab	304	GAFab	308	Sulfo-SDA
644.8423	2	-0.79	GAFab	305	GAFab	306	Sulfo-SDA
743.8929	2	-0.18	GAFab	305	GAFab	308	Sulfo-SDA
605.6467	3	-1.5	GAFab	308	GAFab	311	Sulfo-SDA
723.0406	3	0.12	GAFab	308	GAFab	314	EDC
704.3428	3	0.86	GAFab	314	GAFab	396	EDC
605.3181	3	-0.39	GAFab	319	GAFab	441	BS3
707.3939	3	1.6	GAFab	327	GAFab	332	Sulfo-SDA
482.2609	3	-0.052	GAFab	328	GAFab	329	Sulfo-SDA
707.3934	3	0.88	GAFab	328	GAFab	330	Sulfo-SDA
707.3923	3	-0.68	GAFab	328	GAFab	333	Sulfo-SDA
579.6833	3	-1.6	GAFab	328	GAFab	378	EDC
839.4587	4	0.99	GAFab	328	GAFab	330	EDC
571.5649	4	-0.25	GAFab	367	GAFab	396	EDC
715.8451	2	-0.63	GAFab	374	GAFab	375	Sulfo-SDA
753.7033	3	-0.65	GAFab	374	GAFab	378	EDC
539.0643	4	1.2	GAFab	379	GAFab	396	EDC
751.7680	3	1.8	GAFab	385	GAFab	386	Sulfo-SDA
901.8422	3	1.5	GAFab	395	GAFab	396	Sulfo-SDA
868.4924	3	2.7	GAFab	395	GAFab	397	EDC
868.4937	3	4.2	GAFab	395	GAFab	398	EDC
722.6653	4	3.5	GAFab	395	GAFab	396	BS3
568.6575	3	0.078	GAFab	395	GAFab	439	Sulfo-SDA
92.6113	3	1.3	GAFab	395	GAFab	398	EDC
579.6833	3	-1.6	GAFab	395	GAFab	446	EDC
708.6545	4	-2.4	GAFab	396	GAFab	408	Sulfo-SDA
504.0407	4	-0.15	GAFab	396	GAFab	441	BS3
901.8417	3	0.95	GAFab	396	GAFab	397	Sulfo-SDA
708.6566	4	0.58	GAFab	396	GAFab	400	Sulfo-SDA
1092.6118	3	1.8	GAFab	396	GAFab	397	EDC
683.6432	4	0.17	GAFab	396	GAFab	398	EDC
819.7094	4	0.22	GAFab	409	GAFab	410	EDC
427.9638	4	-1.3	GAFab	441	GAFab	444	BS3
632.3215	2	-0.35	GAFab	441	GAFab	446	Sulfo-SDA
632.3214	2	-0.51	GAFab	444	GAFab	445	Sulfo-SDA
516.0495	5	1.5	GAFab	446	GAFab	449	EDC

Table S3. Cross-links identified for the Pγ-liganded state of PDE6C GAFab (Pγ-GAFab). Crosslinking conditions were followed as described in Fig. 3. Cross-linked peptides were identified following chemical cross-linking of 12 μM GAFab pre-incubated with 120 μM Pγ1-58 (or a mutant Pγ1-58; see *Materials and Methods*) and analyzed as described previously. Abbreviations are defined in the legend to Table S1.

Exp. m/z	z	Δ	pep1	aa1	pep2	aa2	Cross-linker
929.4771	2	-0.97	GAFab	72	GAFab	82	EDC
687.1059	4	0.09	GAFab	78	GAFab	122	BS3
730.3586	2	0.14	GAFab	78	GAFab	79	Sulfo-SDA
595.8455	4	0.13	GAFab	82	GAFab	122	BS3
547.8376	2	-0.12	GAFab	82	GAFab	83	Sulfo-SDA
1018.5392	3	0.68	GAFab	95	GAFab	171	Sulfo-SDA
766.4034	3	-1.5	GAFab	103	GAFab	171	Sulfo-SDA
674.8718	2	0.34	GAFab	122	GAFab	123	Sulfo-SDA
977.5193	3	1.2	GAFab	122	GAFab	126	Sulfo-SDA
811.4540	3	0.22	GAFab	122	GAFab	189	EDC
944.1684	3	1.2	GAFab	122	GAFab	124	EDC
988.9973	2	1.1	GAFab	130	GAFab	132	Sulfo-SDA
1024.5278	3	0.3	GAFab	132	GAFab	171	BS3
973.8683	3	1.3	GAFab	132	GAFab	216	Sulfo-SDA
988.9967	2	0.49	GAFab	132	GAFab	134	Sulfo-SDA
988.9957	2	-0.52	GAFab	132	GAFab	133	Sulfo-SDA
659.6673	3	1.1	GAFab	132	GAFab	135	Sulfo-SDA
944.1678	3	0.55	GAFab	132	GAFab	133	EDC
713.8801	8	11	GAFab	150	GAFab	179	EDC
464.6026	3	-0.048	GAFab	151	GAFab	159	BS3
599.6541	3	0.46	GAFab	151	GAFab	214	BS3
650.0968	4	5.5	GAFab	157	GAFab	160	EDC
514.6675	5	-0.061	GAFab	160	GAFab	163	Sulfo-SDA
363.8491	3	0.37	GAFab	161	GAFab	170	Sulfo-SDA
821.0855	3	2.7	GAFab	168	GAFab	171	Sulfo-SDA
797.6451	4	1.1	GAFab	169	GAFab	171	EDC
859.7726	3	-6.3	GAFab	171	GAFab	312	Sulfo-SDA
1047.8806	3	0.9	GAFab	188	GAFab	189	Sulfo-SDA
582.9872	3	0.67	GAFab	189	GAFab	412	EDC
705.0626	3	1.9	GAFab	210	GAFab	215	EDC
1084.5569	2	0.42	GAFab	249	GAFab	250	Sulfo-SDA
1006.5064	2	0.51	GAFab	249	GAFab	251	Sulfo-SDA
681.7287	3	-1	GAFab	255	GAFab	395	Sulfo-SDA
648.6512	3	0.26	GAFab	257	GAFab	374	Sulfo-SDA
819.9401	2	-0.5	GAFab	258	GAFab	319	Sulfo-SDA
413.8239	5	0.24	GAFab	263	GAFab	441	BS3
813.7858	3	-0.26	GAFab	263	GAFab	319	BS3
528.5142	5	-0.91	GAFab	263	GAFab	396	BS3
630.6799	3	1	GAFab	263	GAFab	264	Sulfo-SDA
947.9810	4	3.7	GAFab	283	GAFab	288	EDC
653.7973	2	0.14	GAFab	286	GAFab	288	Sulfo-SDA
993.9615	2	0.18	GAFab	286	GAFab	314	EDC
481.0217	4	-7.5	GAFab	287	GAFab	396	EDC
993.9622	2	0.89	GAFab	288	GAFab	314	EDC
738.3687	4	5.4	GAFab	289	GAFab	305	EDC
695.0166	3	-1.4	GAFab	289	GAFab	308	EDC
906.4911	3	-2.7	GAFab	289	GAFab	396	EDC
506.0467	4	5.3	GAFab	292	GAFab	396	EDC
607.9922	3	4.7	GAFab	292	GAFab	319	EDC
1011.0821	2	1.3	GAFab	293	GAFab	396	EDC
11.0825	2	1.7	GAFab	293	GAFab	395	EDC
644.8433	2	0.76	GAFab	305	GAFab	306	Sulfo-SDA
743.8934	2	0.5	GAFab	305	GAFab	308	Sulfo-SDA
542.5328	4	1.1	GAFab	308	GAFab	314	EDC
793.4263	3	5.9	GAFab	311	GAFab	319	EDC
1056.0089	2	-0.72	GAFab	314	GAFab	374	EDC
695.0345	3	-0.14	GAFab	319	GAFab	444	BS3
605.3187	3	0.61	GAFab	319	GAFab	441	BS3
707.3931	3	0.45	GAFab	327	GAFab	332	Sulfo-SDA
722.8879	2	0.21	GAFab	328	GAFab	329	Sulfo-SDA
361.9468	4	-2	GAFab	328	GAFab	330	Sulfo-SDA
1060.5868	2	1.2	GAFab	328	GAFab	333	Sulfo-SDA
839.4573	4	-0.68	GAFab	328	GAFab	330	EDC
758.3984	3	-0.25	GAFab	328	GAFab	378	EDC
574.6073	3	0.94	GAFab	367	GAFab	412	Sulfo-SDA
571.5655	4	0.8	GAFab	367	GAFab	396	EDC
787.0540	3	-0.77	GAFab	374	GAFab	378	Sulfo-SDA
787.0552	3	0.76	GAFab	374	GAFab	379	Sulfo-SDA
715.8455	2	-0.07	GAFab	374	GAFab	375	Sulfo-SDA
751.7666	3	-0.022	GAFab	385	GAFab	386	Sulfo-SDA
901.8412	3	0.4	GAFab	385	GAFab	387	Sulfo-SDA
676.6326	4	0.22	GAFab	393	GAFab	396	Sulfo-SDA
901.8425	3	1.8	GAFab	395	GAFab	396	Sulfo-SDA
568.6575	3	0.078	GAFab	395	GAFab	439	Sulfo-SDA
577.9899	3	1.4	GAFab	395	GAFab	415	Sulfo-SDA
868.4938	3	4.3	GAFab	395	GAFab	397	EDC
579.6843	3	0.13	GAFab	395	GAFab	446	EDC
546.8093	6	1.3	GAFab	395	GAFab	397	EDC
579.2989	4	-0.43	GAFab	396	GAFab	441	BS3
708.6578	4	2.3	GAFab	396	GAFab	397	Sulfo-SDA
622.3821	3	-0.72	GAFab	396	GAFab	446	EDC
819.7103	4	1.3	GAFab	409	GAFab	410	EDC
632.3220	2	0.44	GAFab	441	GAFab	445	Sulfo-SDA
601.9882	3	0.36	GAFab	441	GAFab	446	EDC
516.0496	5	1.7	GAFab	446	GAFab	449	EDC
519.2462	5	-2.9	GAFab	446	GAFab	449	EDC

Table S4. Cross-links identified for the Pγ- and cGMP-liganded state of PDE6C GAFab (Pγ-GAFab·cGMP).

Crosslinking conditions were followed as described in Fig. 3. Cross-linked peptides were identified following chemical cross-linking of 12 μM GAFab pre-incubated with 120 μM Pγ1-58 (or a mutant Pγ1-58; see *Materials and Methods*) and a 10-fold molar excess of cGMP. Abbreviations are defined in the legend to Table S1.

Exp. m/z	z	Δ	pep1	aa1	pep2	aa2	Cross-linker	Condition
802.9176	4	0.038	Pγ*	11	GAFab	131	EDC	+ cGMP
564.6147	3	1.2	Pγ*	11	GAFab	367	EDC	± cGMP
562.7966	2	2.3	Pγ*	11	GAFab	167	Sulfo-SDA	+ cGMP
883.1242	3	3	Pγ*	11	GAFab	132	BS3	± cGMP
750.6417	4	0.73	Pγ*	11	GAFab	171	BS3	± cGMP
759.8852	4	2	Pγ*	13	GAFab	171	EDC	+ cGMP
732.6146	4	-1.9	Pγ*	13	GAFab	132	EDC	± cGMP
794.3838	3	-1.7	Pγ*	13	GAFab	374	EDC	+ cGMP
538.6030	3	1.6	Pγ*	13	GAFab	412	EDC	± cGMP
767.0710	3	2.4	Pγ*	13	GAFab	122	EDC	- cGMP
436.2436	3	0.65	Pγ*	23	GAFab	314	EDC	+ cGMP
424.7423	4	2.7	Pγ	23	GAFab	300	EDC	+ cGMP
528.9405	3	0.81	Pγ*	23	GAFab	368	EDC	± cGMP
421.9698	4	-0.43	Pγ/Pγ*	23	GAFab	368	Sulfo-SDA	± cGMP
563.6599	3	1.8	Pγ/Pγ*	23	GAFab	319	BS3	± cGMP
511.7688	4	1.7	Pγ	23	GAFab	170	BS3	+ cGMP
463.7629	4	4.5	Pγ	23	GAFab	305	BS3	± cGMP
663.1042	4	-1.5	Pγ/Pγ*	23	GAFab	171	BS3	- cGMP
665.3478	3	0.95	Pγ/Pγ*	23	GAFab	374	BS3	- cGMP
396.9566	4	-0.69	Pγ*	23	GAFab	367	EDC	- cGMP
562.2911	3	0.38	Pγ/Pγ*	23	GAFab	369	Sulfo-SDA	- cGMP
437.7418	4	-2.1	Pγ*	29	GAFab	300	EDC	+ cGMP
876.1541	3	14	Pγ	29	GAFab	396	BS3	+ cGMP
546.2759	3	0.81	Pγ*	29	GAFab	368	EDC	+ cGMP
682.6824	3	-0.22	Pγ/Pγ*	29	GAFab	374	BS3	- cGMP
676.1063	4	-0.67	Pγ/Pγ*	29	GAFab	171	BS3	- cGMP
494.2818	3	-0.17	Pγ/Pγ*	29	GAFab	151	BS3	- cGMP
752.9006	4	0.14	Pγ*	37	GAFab	249	BS3	+ cGMP
581.1049	4	0.97	Pγ/Pγ*	37	GAFab	396	BS3	+ cGMP
617.3434	4	0.57	Pγ*	37	GAFab	398	EDC	- cGMP
494.0759	5	0.016	Pγ*	37	GAFab	397	EDC	- cGMP
480.2988	4	-0.099	Pγ/Pγ*	39	GAFab	396	BS3	+ cGMP
869.1240	3	-0.29	Pγ/Pγ*	39	GAFab	249	BS3	+ cGMP
573.6627	3	0.47	Pγ/Pγ*	39	GAFab	319	BS3	± cGMP
493.2857	4	-0.62	Pγ*	39	GAFab	263	BS3	- cGMP
378.9750	4	-0.18	Pγ/Pγ*	42	GAFab	409	BS3	± cGMP
616.3616	3	1.4	Pγ*	42	GAFab	319	BS3	+ cGMP
428.5677	3	0.29	Pγ	43	GAFab	288	BS3	+ cGMP
377.8853	3	0.45	Pγ*	43	GAFab	412	BS3	+ cGMP
449.0372	4	0.61	Pγ/Pγ*	43	GAFab	396	BS3	± cGMP
827.4419	3	0.25	Pγ*	43	GAFab	249	BS3	+ cGMP
420.5837	3	0.59	Pγ/Pγ*	43	GAFab	409	BS3	± cGMP
797.4665	2	0.42	Pγ/Pγ*	43	GAFab	319	BS3	± cGMP
499.6087	3	0.53	Pγ*	46	GAFab	264	Sulfo-SDA	+ cGMP
619.0176	3	1	Pγ/Pγ*	46	GAFab	319	BS3	± cGMP
475.7602	4	-2.1	Pγ	49	GAFab	263	Sulfo-SDA	± cGMP
739.0413	3	-0.17	Pγ*	51	GAFab	396	EDC	- cGMP

Table S5. Inter-molecular cross-links identified for the Pγ-liganded state of PDE6C GAFab in the presence or absence of cGMP.

Crosslinking conditions were followed as described in Fig. 3. Inter-molecular cross-linked peptides were identified following chemical cross-linking of 12 μM GAFab (in the presence or absence of cGMP) pre-incubated with 120 μM Pγ1-58 or a mutant Pγ1-58 containing two lysine substitutions: Thr7Lys and Thr11Lys (denoted in table with *). Cross-links identified in the absence (- cGMP), presence (+ cGMP), or in both conditions (± cGMP) are indicated. Samples were prepared and analyzed as described in *Materials and Methods*. Abbreviations are defined in the legend to Table S1.

Table S6. Parameters for MD simulations

#	Atoms	Water	Length	Runs	Box size
X-ray	113990	33856	500 ns	3	87 Å × 131 Å × 107 Å
apo state	104728	30533	360 ns	3	88 Å × 120 Å × 107 Å
cGMP bound	104792	30531	360 ns	3	88 Å × 120 Å × 107 Å

Details of MD simulations on three different systems are highlighted including the numbers of total and water atoms in each simulation, the length of each trajectory (3 independent simulations for each system) and the dimensions of each simulation domain.

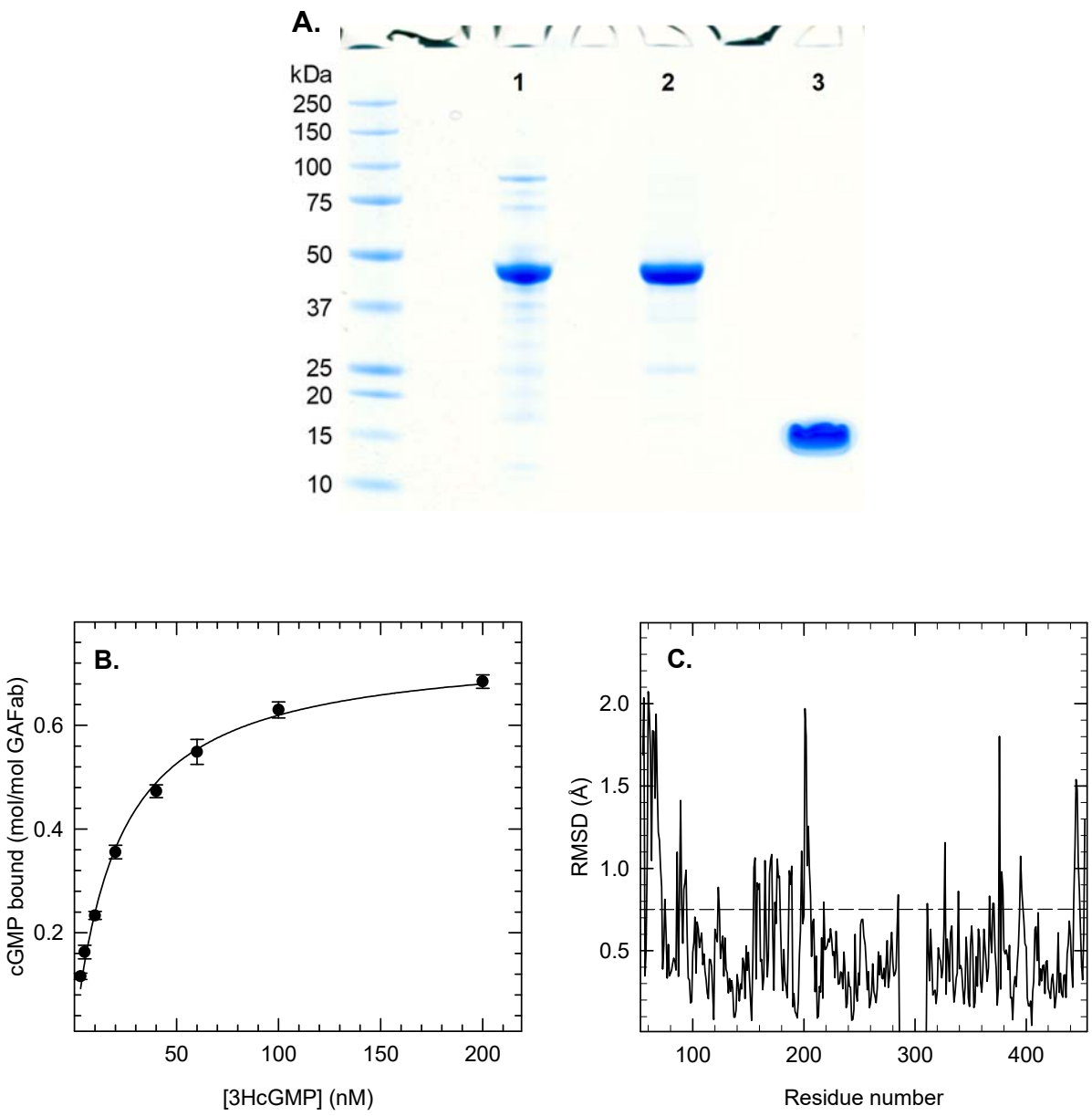

Fig S1. Purification, cGMP binding properties, and characterization of structural asymmetry of chain A and chain B of the x-ray structure. A. SDS-PAGE of purified, recombinant chicken cone PDE6 GAFab (PDE6C, residues 42-458) and chicken cone Pγ1-58. Lane 1, affinity-purified GAFab; Lane 2, gel filtration-purified GAFab; Lane 3, HPLC-purified Pγ1-58. **B.** Binding curve of 1.5 nM GAFab incubated with increasing concentrations of [^{3}H]cGMP; the *solid line* represents fitting the data to a hyperbolic curve, with a K_D = 22 ± 2.2 nM (n=3). **C**. RMSD analysis of chain A versus chain B of the chicken cone PDE6 GAFab x-ray structure was calculated using VMD; average RMSD value was 0.74 Å (*dotted line*).

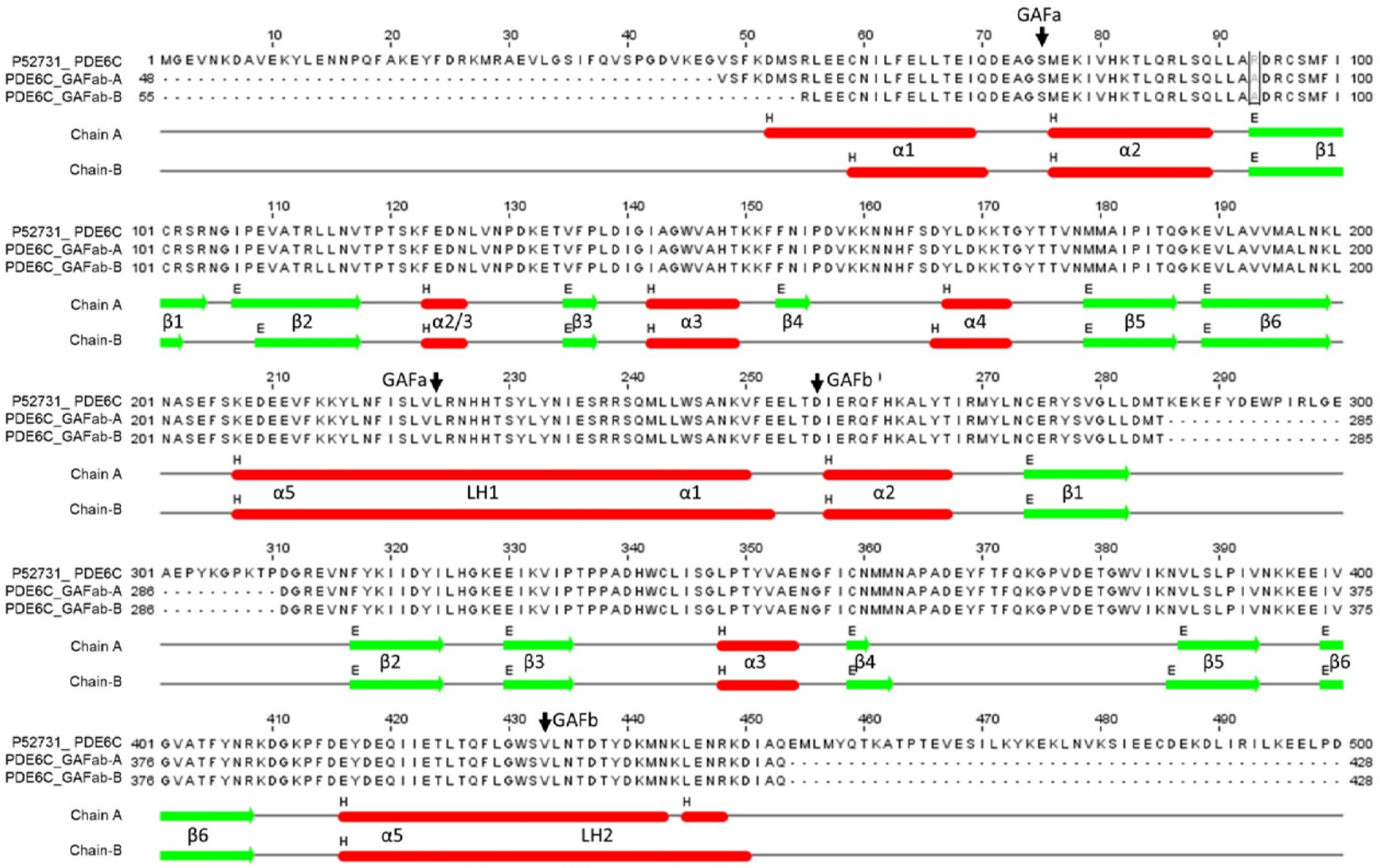

Fig. S2. Structural alignment of cone PDE6C regulatory domains. The chicken cone PDE6 amino acid sequence (PDE6C_CHICK, P52731) was aligned with the two subunits of the crystal structure (Fig. 2A) with secondary structure elements highlighted in red (α-helix) or green (β-strand). The boundaries for the GAFa (residues 75-224) and GAFb (residues 256-433) domains (black arrows) are delimited according to their Pfam (PF01590) entry.

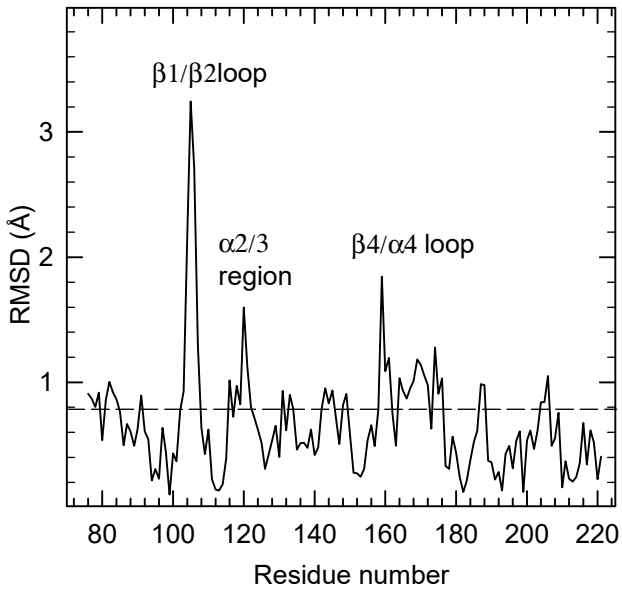

Fig. S3. Comparison of the x-ray structures of the liganded and apo states of the PDE6C GAFa domain. The GAFa domain x-ray structures for the unliganded (Fig. 2) and cGMP-liganded (3DBA; [1] states were aligned, and the RMSD values for each residue were determined using VMD. The overall average RMSD = 0.8 Å is represented by the dashed line.

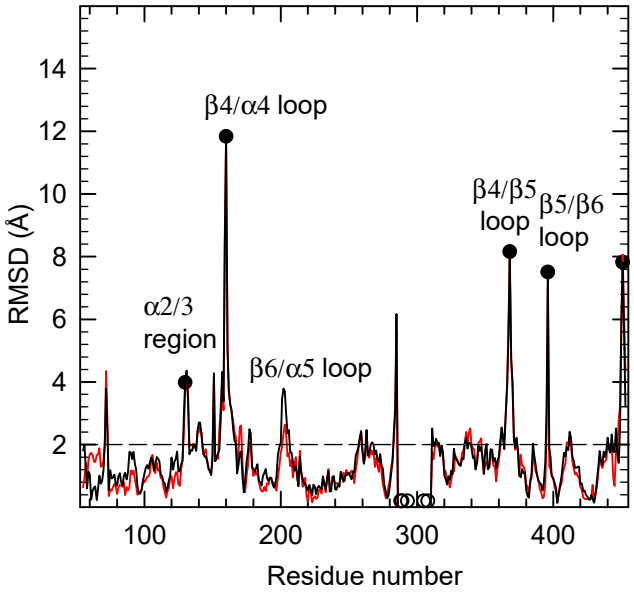

Fig. S4. RMSD analysis of the chicken cone GAFab x-ray structure versus the cross link-refined apo GAFab structure. RMSD per residue was calculated using VMD after aligning the GAFab X-ray structure with crosslink-refined apo structure. The black and red lines represent RMSD values for the A and B chains of GAFab, respectively. Black circles identify intra-molecular crosslinked residues of GAFab, and the open circles represent crosslinks obtained in the region missing in the x-ray structure (residues 285 to 311); only those cross-links that were at positions with RMSD values 2-fold or greater above the average are shown in this figure. The overall average RMSD = 2.0 Å is represented by the dashed line.

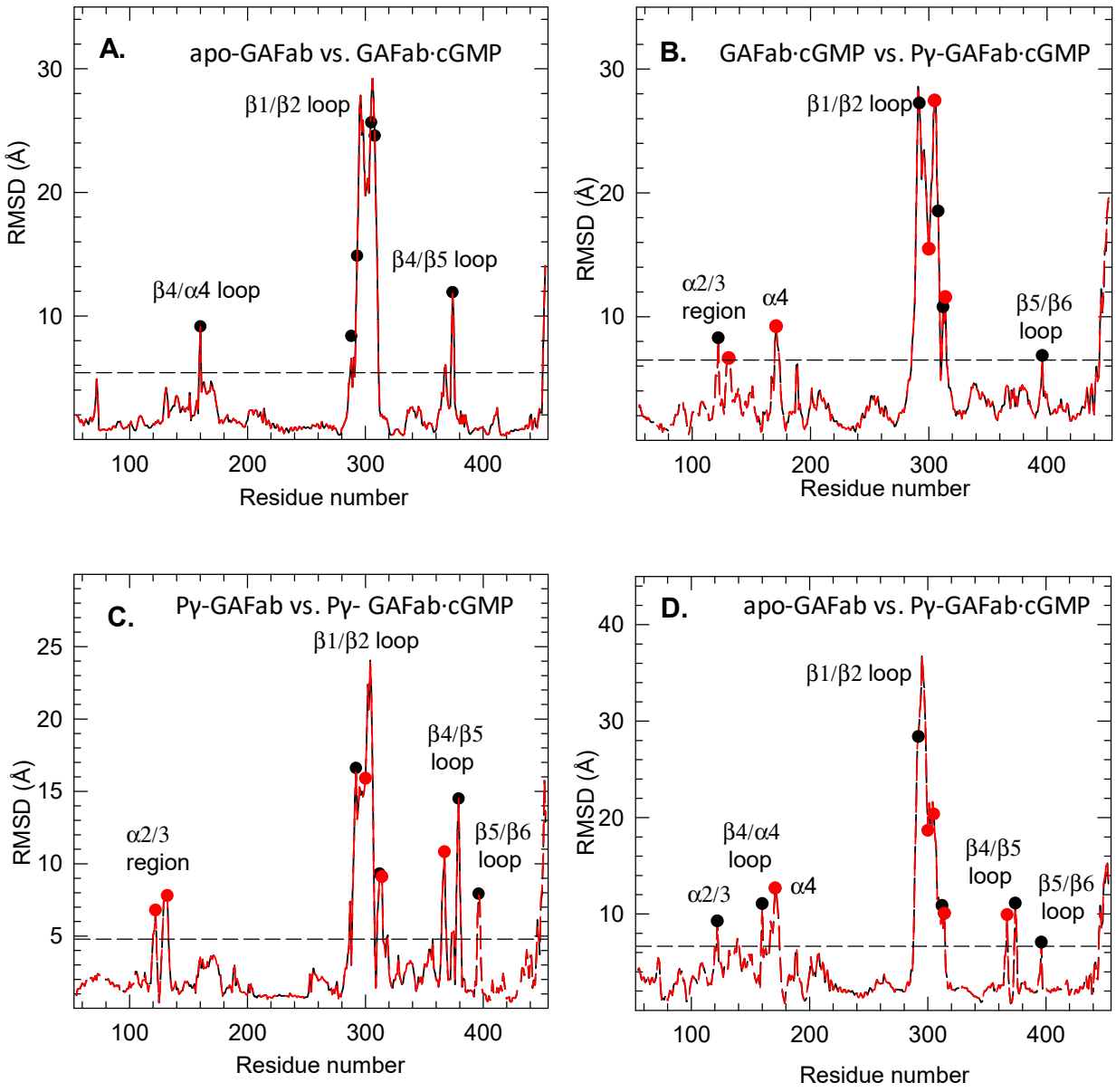

Fig. S5. RMSD plots for various liganded states of GAFab. RMSD per residue was calculated using VMD after aligning the apo GAFab structure with other liganded states: **A.** apo state versus GAFab·cGMP. **B.** GAFab·cGMP versus Pγ-GAFab·cGMP. **C.** Pγ-GAFab versus Pγ-GAFab·cGMP. **D.** apo state versus Pγ-GAFab·cGMP. The black and red lines represent RMSD values for the A and B chains of GAFab, respectively. Black and red circles represent intra-molecular GAFab and inter-molecular Pγ-GAFab crosslinked residues, respectively, for each liganded state; for clarity, only those cross-links whose RMSD exceed the average value are shown. Dashed lines represent the average RMSD.

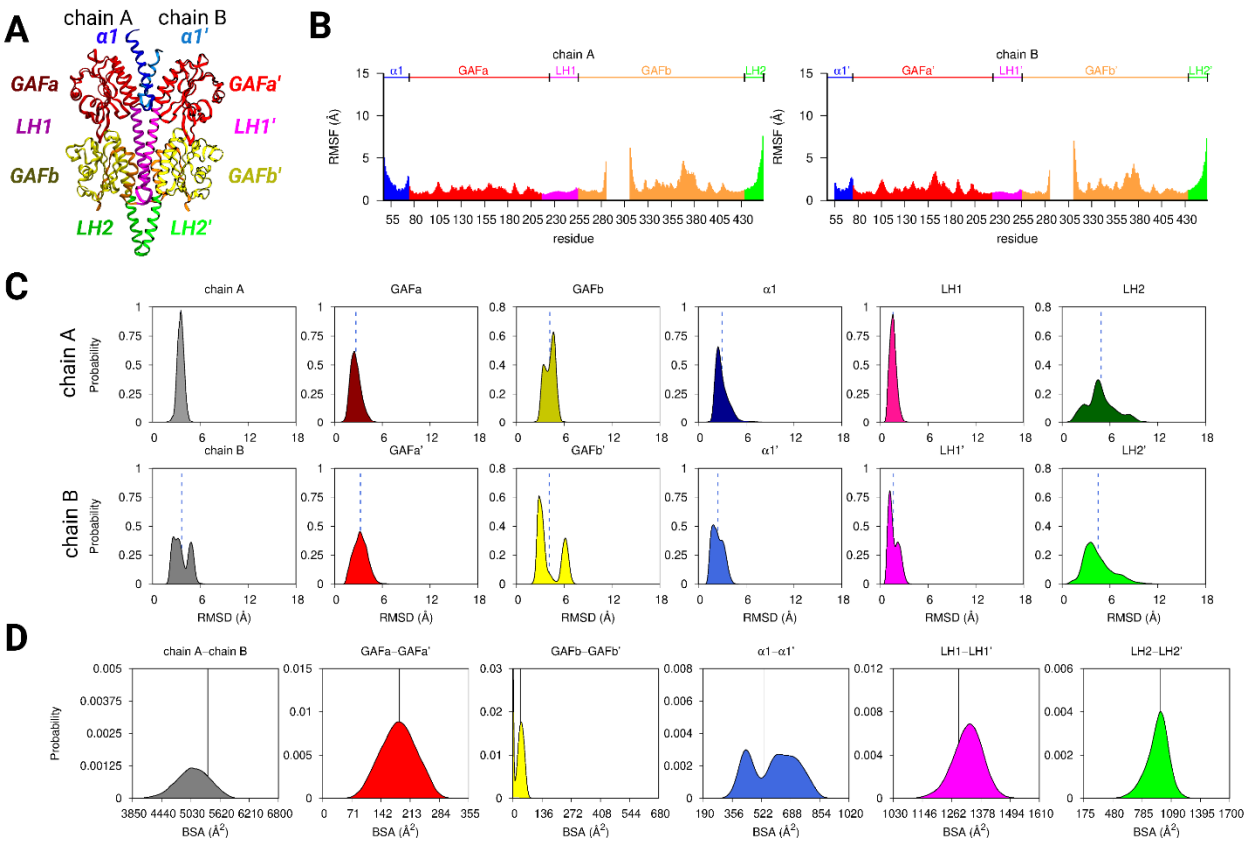

Fig. S6. Dynamics and stability of the crystal structure of the GAFab homodimer as characterized by various conformational metrics. A. Cartoon representation of the x-ray structure with color scheme for the subdomains characterized in panels B-D; note that chain B subdomains are denoted with an apostrophe for clarity. **B.** Root mean squared fluctuation (RMSF) per residue for each subunit, color-coded to correspond with the representation in panel A. **C.** Probability distributions of the root mean squared deviation (RMSD) for individual domains of each subunit. **D.** Distributions of the buried surface area (BSA) between various domains of the homodimer. Vertical lines marked on distributions correspond to mean values of the RMSD data from simulations (panel C) and the BSA values (panel D) observed in the crystal structure.

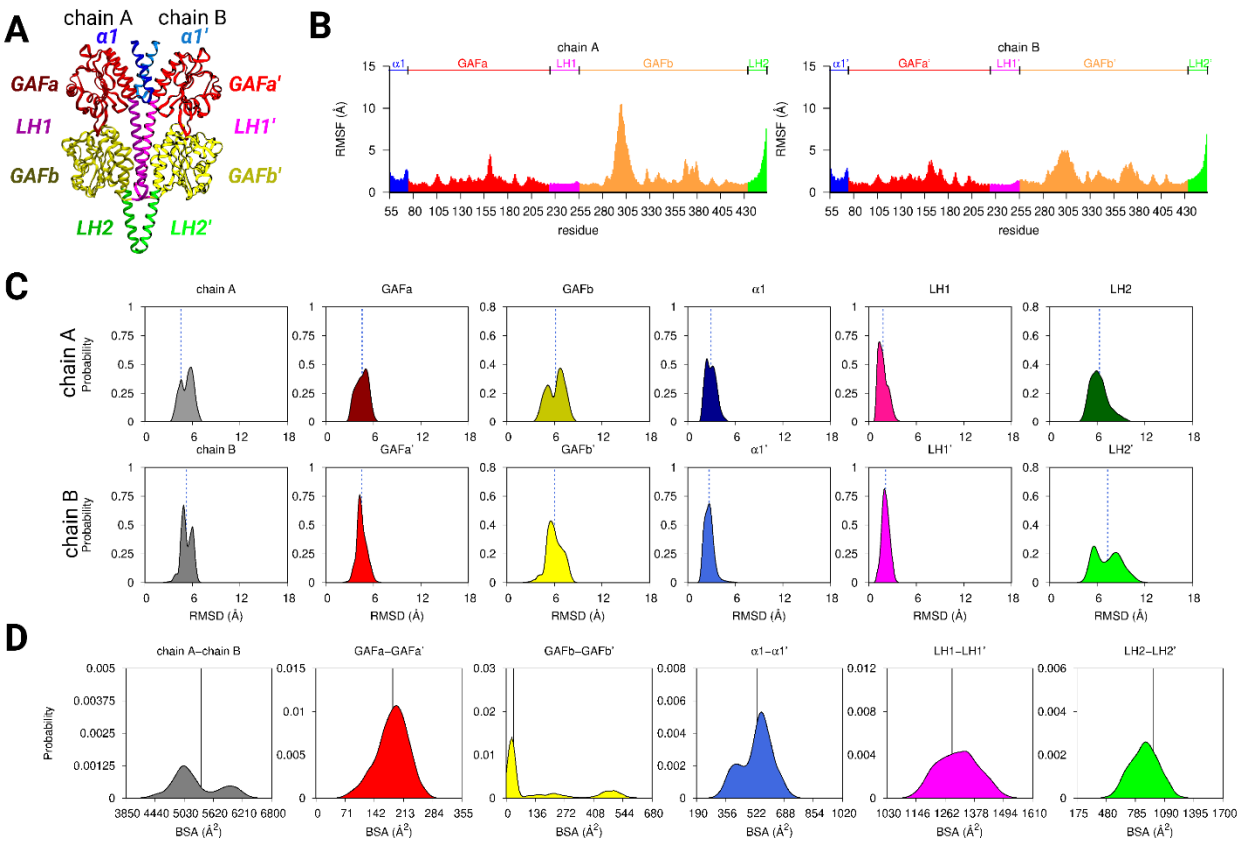

Fig. S7. Conformational metrics highlighting dynamics of the apo state of the GAFab homodimer. For the apo-state of GAFab (panel A), RMSF (panel B), RMSD probabilities (panel C) and BSA probabilities (panel D) are presented as described in the legend to Fig. S6.

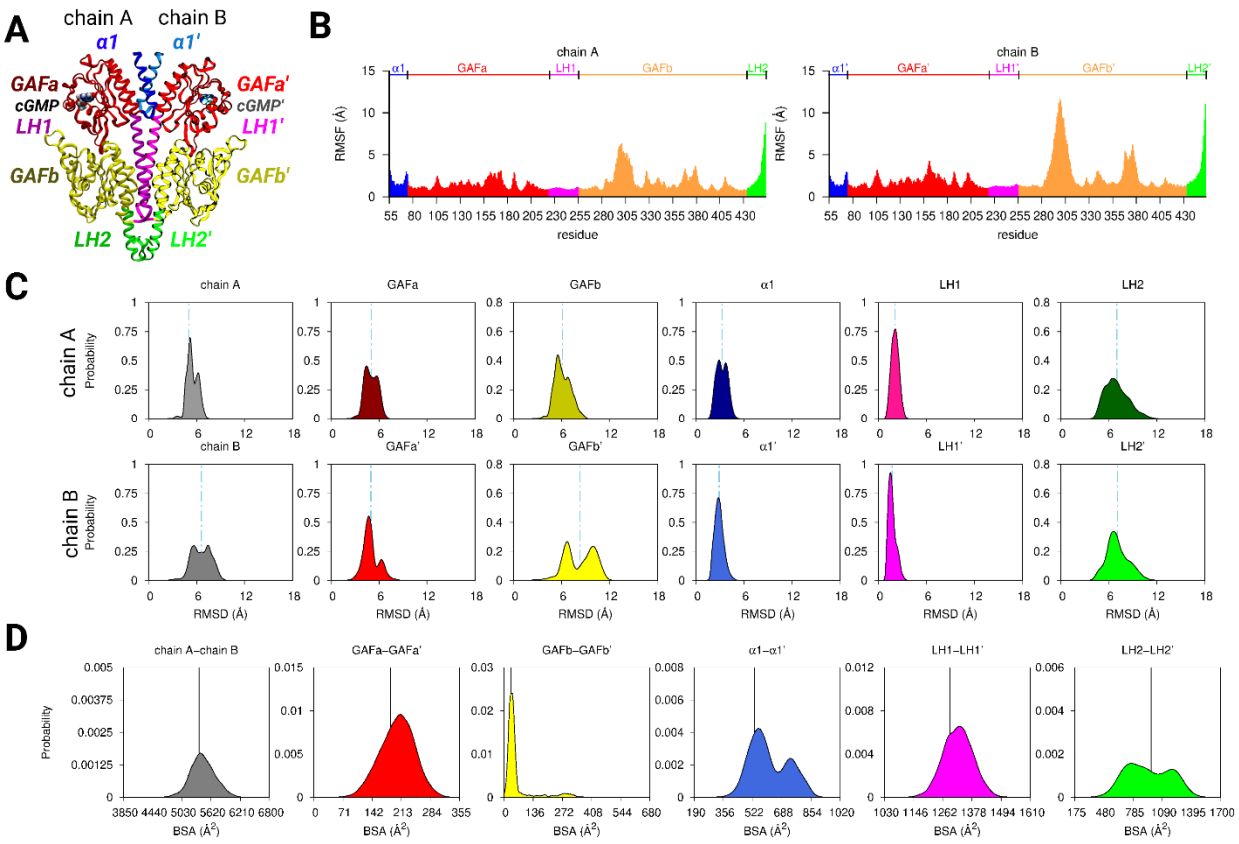

Fig. S8. Conformational metrics highlighting the dynamics of the cGMP-bound state of the GAFab homodimer. For the GAFab·cGMP state (panel A), RMSF (panel B), RMSD probabilities (panel C) and BSA probabilities (panel D) are presented as described in the legend to Fig. S6.

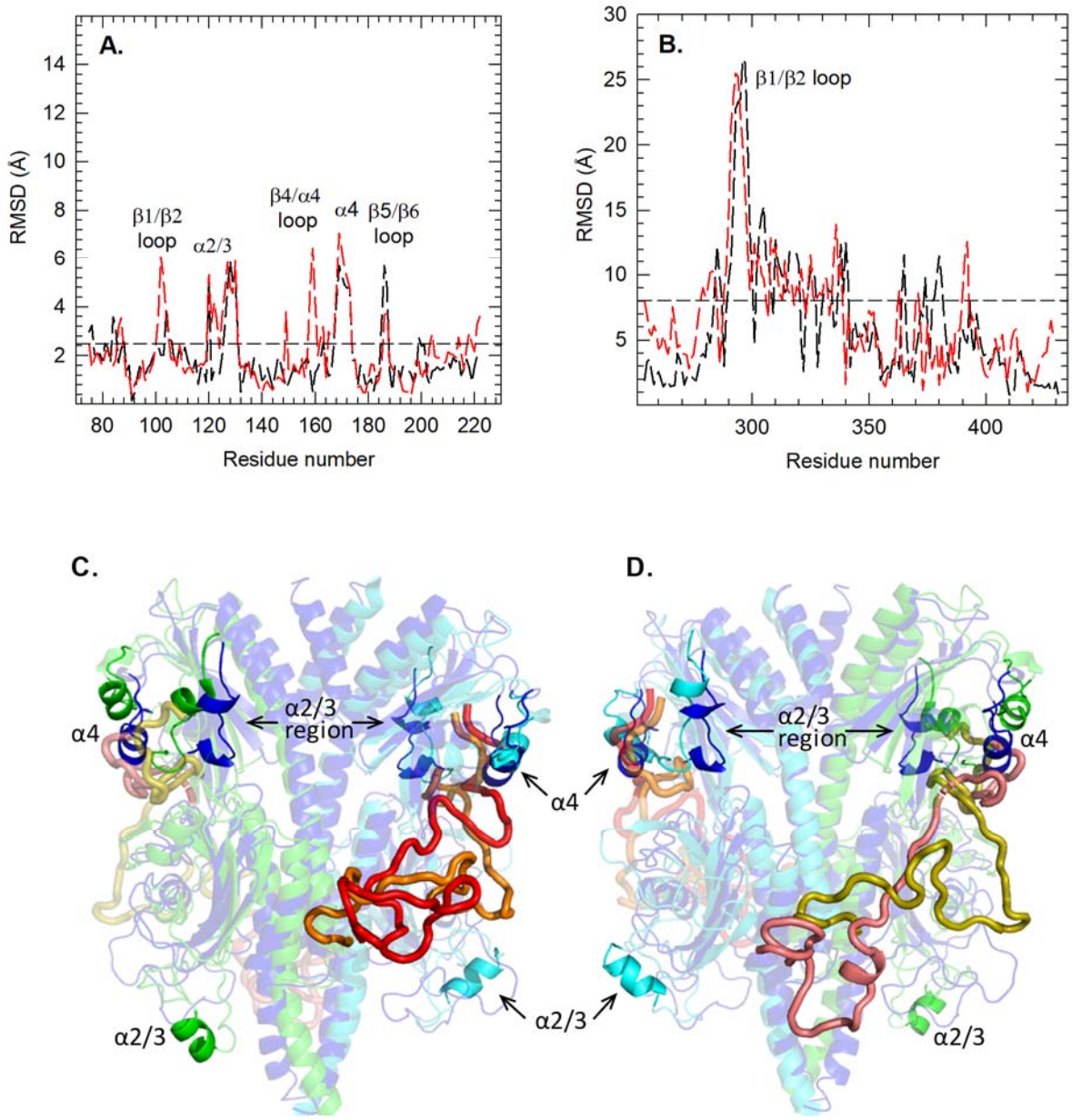

Fig. S9. Structural comparison of chicken cone PDE6 GAFab with bovine rod PDE6 GAFab. The GAFa domain (residues 77-224) and GAFb domain (residues 256-433) of chicken cone Pγ-GAFab·cGMP were structurally aligned with the corresponding residues of bovine rod PDE6 [containing Pγ and bound cGMP; [2]]. **A.** RMSD analysis for the GAFa domains of PDE6A (black line) and PDE6B (red line) compared to cone GAFab with an overall average RMSD = 2.8 Å (dashed line). **B.** RMSD analysis for the GAFb domain with the overall average RMSD = 7.8 Å (dashed line). **C.** Alignment of PDE6A (cyan; associated rod Pγ in red) with chain A of cone Pγ-GAFab·cGMP (blue; associated cone Pγ in orange). **D.** Alignment of PDE6B (green; rod Pγ in salmon) with chain B of cone Pγ-GAFab·cGMP (blue; cone Pγ colored olive). The GAFb α2/3 helix identified in rod PDE6 [2] is disordered in cone GAFb.

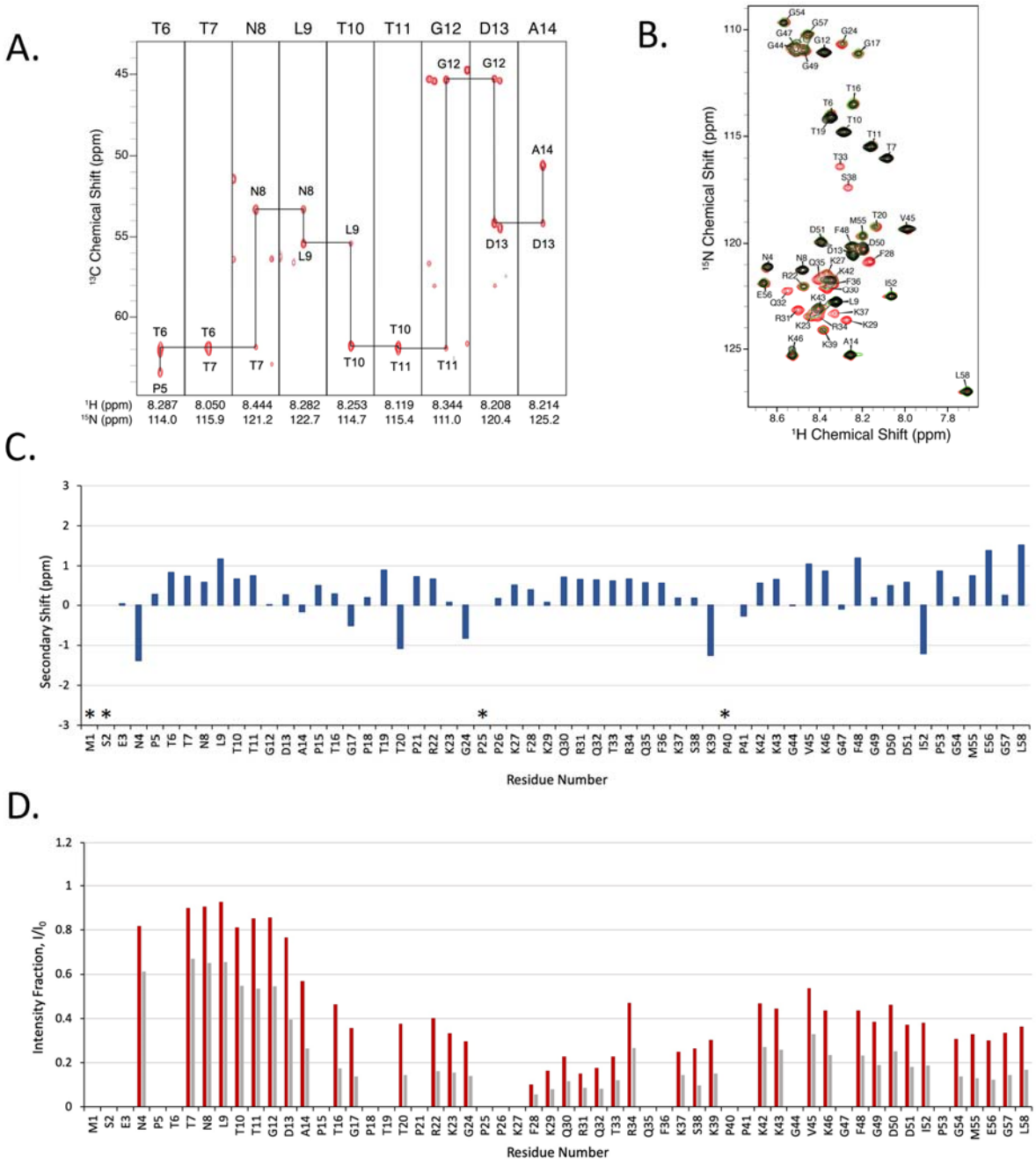

Fig. S10. Solution NMR spectroscopy of cone Pγ1-58. **A.** Representative 3D HNCA NMR strip plot of Pγ1-58 for residues T6-A14. The Cα peaks of the *i* and *i*-1 residues are labeled and connected to guide the eye. **B.** Overlay of the 2D ^{1}H-^{15}N HSQC NMR spectra of unbound Pγ (red spectrum) and Pγ bound to GAFab without cGMP (green spectrum) and in the presence of cGMP (black spectrum). **C.** Secondary chemical shifts of Pγ1-58, defined as the observed ^{13}C chemical shifts relative to tabulated random coil shifts. Combined ^{13}Cα and ^{13}Cβ secondary chemical shifts ($\Delta\delta^{13}C\alpha$- $\Delta\delta^{13}C\beta$) of Pγ1-58 were plotted, and missing chemical shifts are noted with an asterisk. **D.** Peak intensities of Pγ residues expressed as the ratio between peak volume after (I) and before (I_0) addition of GAFab (red) or after subsequent addition of cGMP to the Pγ-GAFab complex (grey). Some peaks were not analyzed due to signal overlap or missing because they are prolines.

References

[1] Martinez SE, Heikaus CC, Klevit RE, Beavo JA. The structure of the GAF A domain from phosphodiesterase 6C reveals determinants of cGMP binding, a conserved binding surface, and a large cGMP-dependent conformational change. J Biol Chem. 2008;283:25913-9.
[2] Irwin MJ, Gupta R, Gao XZ, Cahill KB, Chu F, Cote RH. The molecular architecture of photoreceptor phosphodiesterase 6 (PDE6) with activated G protein elucidates the mechanism of visual excitation. J Biol Chem. 2019;294:19486-97.