Supporting Information (13 pages) for:

NMR Assignments for a Helical 40 kDa Membrane Protein

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Labeling and purification of diacylglycerol kinase.

Plasmid pSD005 containing a synthetic IPTG-inducible gene for wt- or s-DAGK[1] was used to transform *E. coli* BL21. The constructs included an added purification tag (MGHHHHHHEL-) in place of the N-terminal Met of native DAGK. Transformed BL21 was adapted for growth in perdeuterated medium by successively growing 50 ml cultures in Luria broth/H_2O, then minimal medium/H_2O, then minimal medium/70% D_2O, and finally ^{15}N-enriched minimal medium with perdeuterated $^{13}C_6$-glucose and 99% D_2O. 1 ml of each culture was used to inoculate the succeeding culture. The final 50 ml culture was grown with shaking to OD_{600} = 0.5 at 37° C and 5 ml was used to inoculate a fresh 500 ml of the same triple-labeled medium. This culture was grown under the same conditions to OD_{600} = 1.0 (ca. 20 hours) and protein expression was induced using 0.2 g/liter IPTG, followed by incubation for another 8-14 hours, and cell harvesting by centrifugation. The minimal medium used in this protocol was supplemented with the aqueous extract from a multiple vitamin (2 ml per liter). This extract was prepared by crushing a 1.5 gram Centrum vitamin (Wyeth Pharmaceuticals, Inc.), which was mixed vigorously with 20 ml water followed by centrifugation and sterile filtration of the supernatant.

Cells harboring recombinant DAGK were suspended in pH 7.7 buffer (75 mM Tris, 0.3 M, 0.2 mM EDTA and 10 micromolar β-hydroxyltoluene-- a free-radical scavenger) and lysed using lysozyme, DNase, and sonication. Following low speed centrifugation to remove suspended material, the detergent Empigen (dodecyl-N,N-dimethylglycine, Calbiochem, San Diego, CA) was added to the supernatant to a concentration of 3%. To the mixture was added Ni(II)-agarose resin (Qiagen, Valencia, CA; 1 gram of wet resin was added for every gram of E. coli paste originally used). The resin was then transferred to a column and step-eluted by first washing all non-DAGK protein from the resin with a buffer containing 40 mM imidazole and 1.5% Empigen until A_{280} returned to baseline. This was followed by re-equilibrating the resin with 8 X 1 column volumes of a solution containing 0.5% dodecylphosphocholine (DPC, Anatrace, Maumee, OH) and 50 mM sodium phosphate, pH 7.0. DAGK was then eluted using 0.5% DPC plus 0.25 M imidazole pH 7.8 solution. At this stage, DAGK was either concentrated for NMR (next paragraph) or was first unfolding/refolded/re-purified and then concentrated for NMR. Yields of DAGK were in the range of 10-30 mgs of pure protein per liter of culture, with yields from perdeuterated medium typically being higher than from non-deuterated medium. Prior to the work of this study early work with s-DAGK was plagued by a spectroscopically-visible impurity which was originally identified as misfolded s-DAGK[2]. This contaminant was later shown to be the *E. coli* YodA protein (Sanders et. al, correction in *Biochemistry* in press). None of the samples used in the present study were contaminated by a second protein, as was evident from the 2-D TROSY spectra.

To prepare purified DAGK in DPC micelles (plus 250 mM imidazole) for NMR, EDTA was added to 0.5 mM and D_2O to a concentration of 10%. The pH was adjusted to 6.5 using acetic acid and ammonium hydroxide and the solution was then concentrated to a DAGK homotrimer concentration of 0.4 to 1.1 mM by centrifugal ultrafiltration using a Centricon Plus-20 PL-10 filter cartridge (Millipore, Bedford, MA; 10 kDa molecular weight cut-off). By this operation both DAGK *and* the detergent DPC were concentrated (since DPC has a relatively low critical micelle concentration). Samples were then transferred to 5 mm NMR tubes.

Unfolding/refolding method for effecting amide deuterium → hydrogen back-exchange in perdeuterated wt-DAGK.

In this case, wild type DAGK was purified using a slight modification of the procedure described above. After eluting non-DAGK proteins from the Ni(II)-agarose resin using 1.5% Empigen plus 40 mM imidazole, the resin was rinsed with 3 bed volumes of water. The perdeuterated DAGK was then eluted from the column using 250mM imidazole, pH 7.8, 0.5% sodium dodecylsulfate (SDS). At this stage, DAGK is at least partially unfolded and is susceptible to back exchange of amide deuterons for protons. Samples were allowed to incubate overnight at room temperature and DAGK was then refolded by adapting a procedure known as "reconstitutive refolding"[3]. Briefly, to the DAGK/SDS solution was added a DPC/1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC, Avanti Polar Lipids, Alabaster, AL) solution to make the DAGK:POPC mol:mol ratio = 1:100. The protein solution was transferred to dialysis membrane (Spectra-Por 1.1 or 2.1) and dialyzed exhaustively against 5 changes of buffer over a period of 5 days to remove SDS and DPC. The dialysis buffer contained 10 mM imidazole, 0.5 mM EDTA, 0.2 mM dithiothreitol buffer at pH 6.5 (dithiothreitol was omitted in the final round of dialysis). Following this refolding process, the DAGK-containing vesicles were re-dissolved using 0.5% DPC solution, followed by addition of Ni(II)-agarose resin to the solution. POPC was removed by washing the resin with 0.5% DPC, followed by elution of DAGK with 0.5% DPC plus 0.25 M imidazole, pH 7.8. Measurement of enzyme activity was used to verify that DAGK was correctly refolded[4]. DAGK was then concentrated for NMR as described in the above section.

Direct Determination of the Moles of DPC bound per DAGK Trimer.

Pure DAGK bound to Ni(II)-agarose resin was equilibrated with a salt-free solution of 0.5% DPC and then eluted with 0.5% DPC plus 0.5 M ammonium hydroxide. The resulting DAGK pool was weighed and the DAGK concentration was determined spectrophotometrically at 280 nm. The solution was then free-dried. The resulting powder was weighed to give the total DPC+DAGK weight. From this, the known weight of the DAGK present and the weight of the free DPC in the original solution (ml of solution X 5 mg/ml) was subtracted to give the weight of the DAGK-associated DPC present in the original solution. Control experiments indicated that virtually all of the ammonium hydroxide was removed during the freeze-drying process (in the form of ammonia), except for that which serves as counterions to charged DAGK side chains. This procedure was conducted three times on different days and using different batches of DAGK, leading to determination of 151 ± 22 molecules of DPC associated with each 43,120 Da DAGK trimer. This corresponds to an aggregate molecular weight of 96 ± 14 kDa.

Determination of the Overall Rotational Correlation Time for DAGK.

2.2 mM of uniformly ^{15}N labeled s-DAGK was used for these experiments. ^{15}N T_1 and T_2 measurement were carried out at 45 degrees C on a Bruker DRX-600 spectrometer operating at ^1H resonance frequency of 600 MHz equipped with a triple resonance cryoprobe. The pulse sequences described in Farrow *et al.* (1994)[5] were used to collect ^{15}N T_1, and T_2 data sets in which 64 scans were acquired for each t_1 increment. 128 × 1024 complex points were acquired in the $t_1 \times t_2$ dimensions. For T_1 measurements a total of 5 data sets were collected with T_1 relaxation delays of 5, 800, 1500, 2000, 2500 ms, while for T_2 measurement, with T_2 relaxation delays of 6.4, 12.8, 19.2, 25.6, 32 ms were used. A 1.5 s relaxation delay was used between scans. The T_1 and T_2 rates were obtained by non-linear least square fitting of single exponential decays to the experimental data. The rotational correlation time, t_m, was estimated for each resonance by solving equation 8 from Kay *et al.* (1989)[6] using a MatLab-based (MathWorks, Natick, MA) program "calctaum_v2" kindly provided to us by Lewis Kay and Peter Hwang of the University of Toronto. Measured correlation times from backbone amide ^{15}N of transmembrane segments were averaged to estimate an estimated overall correlation time and associated standard deviation of 35.5 ± 7 nsec. Based on the Stokes-Einstein relationship which assumes a spherical aggregate, this corresponds to an aggregate DAGK + detergent molecular weight of 101 ± 20 kDa.

Determination of the Aggregate Molecular Weight of DAGK in DPC Micelles Using Light Scattering

An estimate of the aggregate molecular weight of DAGK in DPC micelles at room temperature was very generously conducted by Dr. Micelle H. Chen of Wyatt Technology Corporation (Santa Barbara, CA) using size exclusion chromatography coupled with in-line light scattering, ultraviolet absorption and refraction index detectors, essentially according the method of Yernool et al. (2003)[7]. An aggregate molecular weight of 90 ± 12 kDa was determined, with the primary sources of uncertainty being the facts that an estimated 280 nm extinction coefficient was used for DAGK and that an estimated derivative of the refraction index with respect to concentration was used for DPC.

Estimate of DAGK/DPC Aggregate Size From Diffusion Coefficients

In Vinogradova et al. (1998)[8] diffusion coefficients were measured using NMR methods for DAGK in a variety of different micelle types and also for a variety of protein-free micelles of know aggregate sizes. The measured diffusion coefficient for DAGK in DPC micelles was (4.7 ± 0.9) X 10^{-7} cm^2/sec. This diffusion coefficient can be compared to those measured in that same study for free Triton X-100 and lyso-1-myristoyl-*sn*-glycero-3-phosphocholine micelles (both thought to have aggregate molecular weight of ca. 90 kDa) of (6.8 ± 0.9) X 10^{-7} cm^2/sec and (5.9 ± 0.3) X 10^{-7} cm^2/sec, respectively.

NMR Data Processing

Multidimensional NMR spectra were processing using NMRView[8] and NMRPipe[10] software.

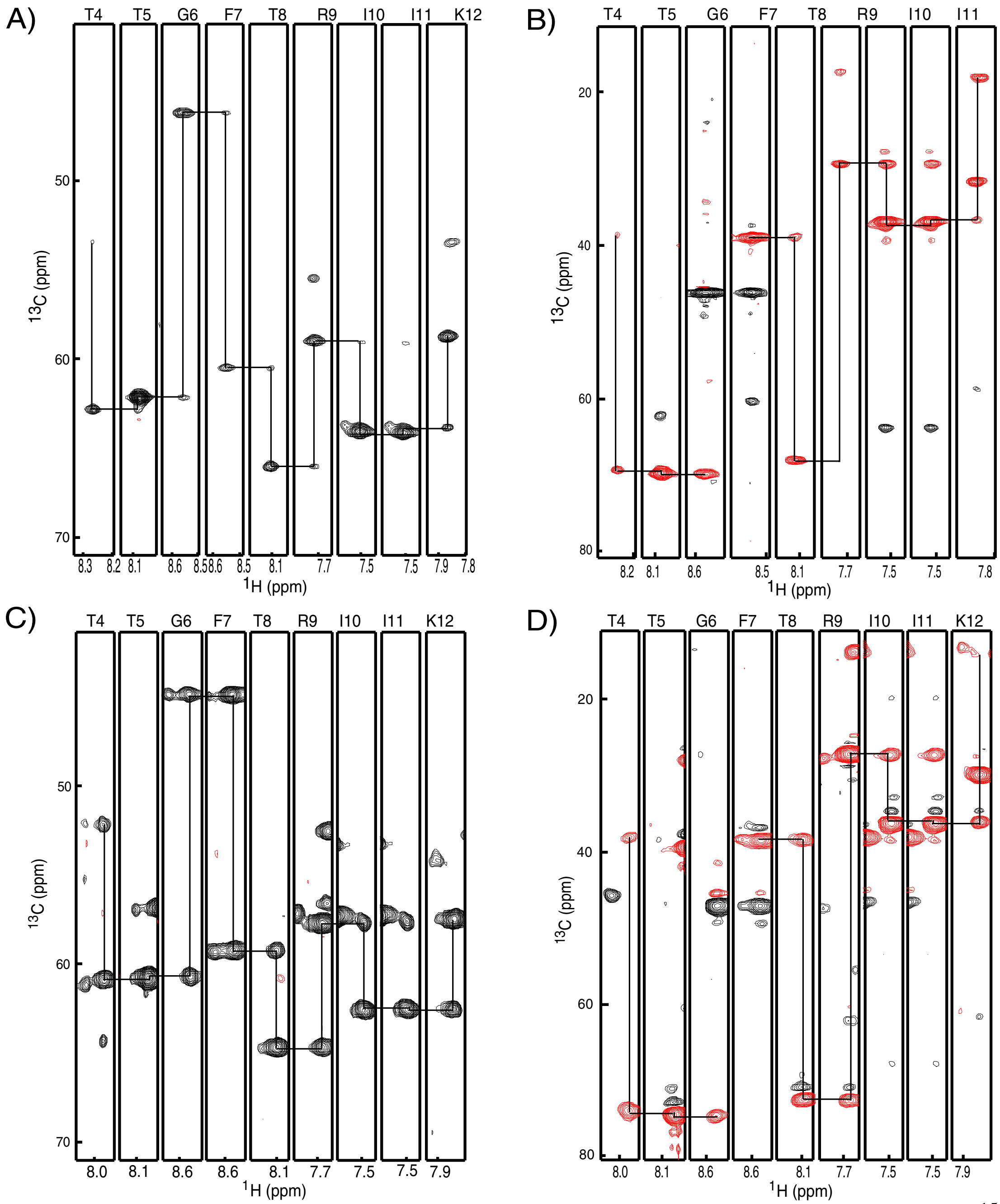

Figure S1. Strip plots corresponding to resonances from residues 4-12 in 800 MHz [^{15}N-^{1}H]-TROSY-HNCA and HNCACB experiments of uniformly ^{2}H, ^{13}C, ^{15}N-labeled s-DAGK (A and B) and wt-DAGK (C and D) in DPC micelles at 45 °C. Residues are labeled and numbered on the top of each strip. The lines indicate the connectivities established by intra-residual and sequential peaks detected by each experiment.

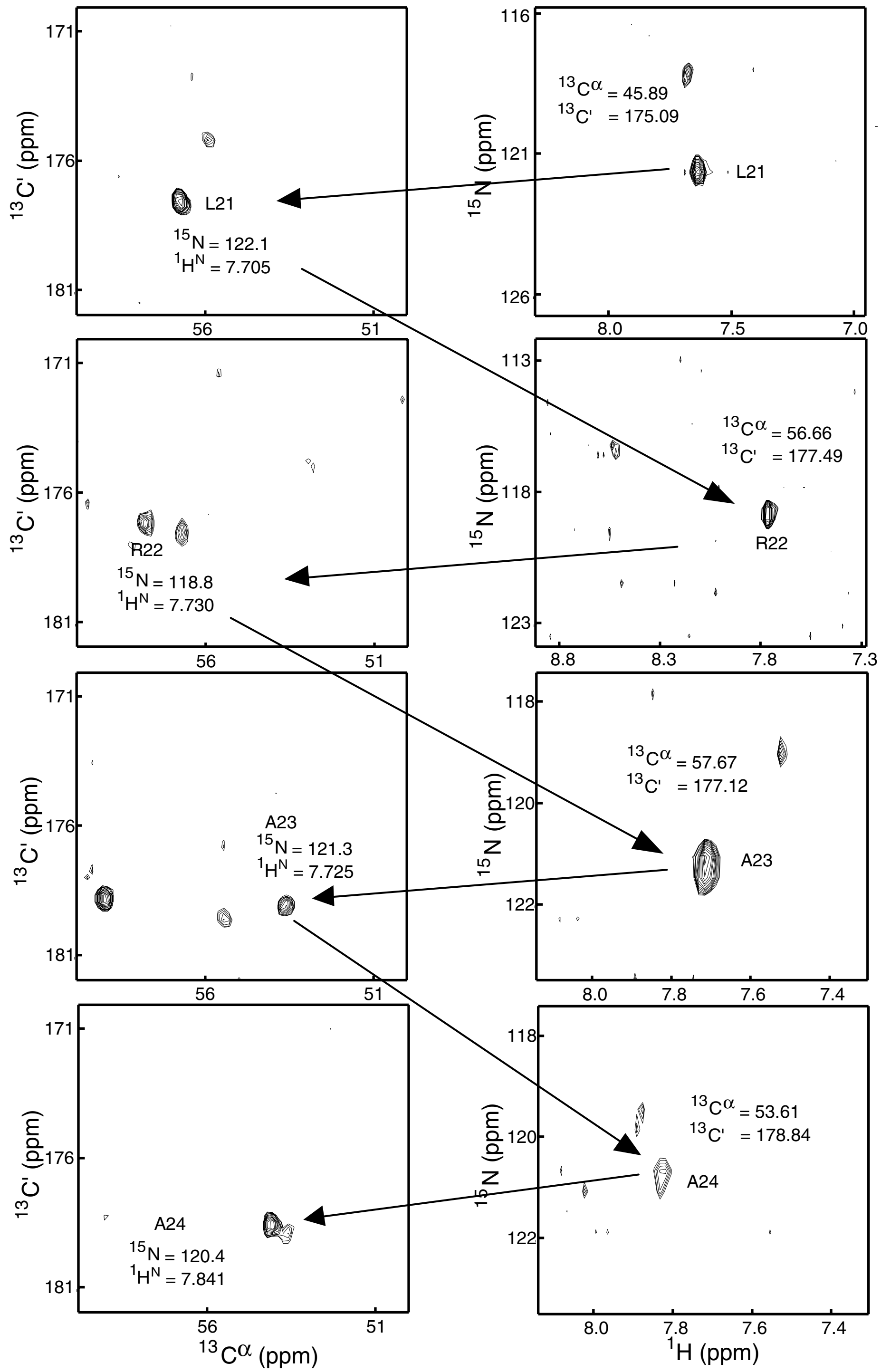

Figure S2. Representative 2D $^{13}C^{\alpha}$-$^{13}C'$ and ^{15}N-$^{1}H^{N}$ planes from 800 MHz 4D HNCACO and 4D HNCOCA data sets of 3 mM s-DAGK in micelles at 45° C. Illustrated are 2D planes showing resonances and connectivities for residues 21-24. Chemical shifts and cross-peaks are labeled on each plane.

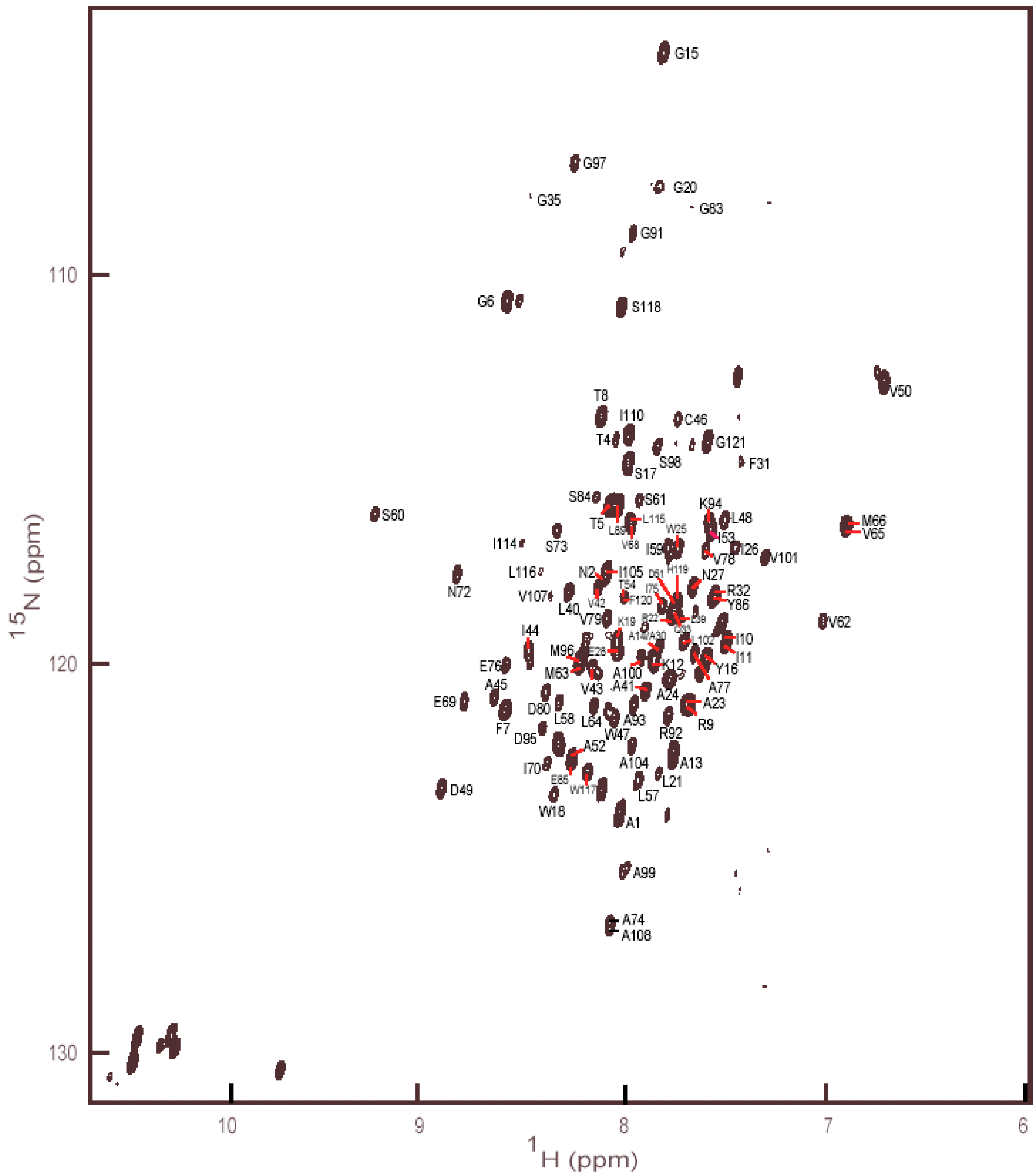

Figure S3. 800 MHz TROSY spectrum of wild type DAGK in DPC micelles at 45° C showing assignments for the amide resonances.

Table 1. Backbone chemical shift assignment of s-DAGK

Residue	H^N	N	C^α	C^β	C'
A1	8.013	123.663	52.468	18.557	177.57
N2	8.081	117.438	53.282	38.431	
N3			53.43	38.713	176.12
T4	8.266	114.458	62.832	69.299	175.36
T5	8.078	115.986	62.136	69.299	175.626
G6	8.567	110.703	46.228		175.126
F7	8.558	121.071	60.496	38.867	177.375
T8	8.113	113.603	66.04	67.977	176.31
R9	7.725	121.093	58.976	29.315	178.745
I10	7.517	119.314	63.897	36.831	177.123
I11	7.517	119.314	63.897	36.831	178.231
K12	7.871	119.919	58.743	31.597	179.305
A13	7.759	122.274	54.056	17.914	179.07
A14	7.854	119.688	53.399	18.011	178.152
G15	7.829	104.301	45.967		175.017
Y16	7.616	120.008	58.677	38.533	176.054
S17	7.995	115.025	58.615	64.686	174.889
W18	8.258	123.253	58.668	29.472	177.191
K19	7.994	119.601	58.723	31.522	178.781
G20	7.846	107.73	45.894		175.095
L21	7.705	122.16	56.664	41.278	177.495
R22	7.73	118.885	57.673	29.4	177.127
A23	7.725	121.349	53.617	17.9	178.848
A24	7.841	120.485	54.063	18.11	178.519
W25	7.717	117.038	58.738	29.18	176.968
I26	7.408	117.032	62.352	37.319	176.342
N27	7.69	117.825	54.162	39.225	175.911
E28			56.527	30.662	178.837
A29	8.212	119.876	53.827	18.134	178.154
A30	8.212	119.876	53.827	18.134	178.154
F31	7.385	115.0	58.583	38.503	176.297
R32	7.565	118.86	57.669	29.898	
Q33			60.03		177.983
E34	8.808	120.689	59.381	28.238	177.83
G35	8.343	106.873	46.769		175.098
V36	7.721	120.542	66.387	30.591	177.037
A37	7.921	121.501	55.469	17.745	178.782
V38	7.721	115.813	66.345	30.72	177.239
L39	7.734	119.266	58.044	40.862	178.644
L40	8.389	118.047	57.798	40.686	178.256
A41	8.083	120.733	55.476	18.223	179.39
V42	8.08	116.871	66.981	30.805	178.353
V43	8.08	116.871	66.981	30.805	178.353
I44	8.579	119.266	65.794	37.15	179.199
A45	8.537	120.079	54.938	18.779	179.434

C46	7.795	113.375	63.137	27.907	173.643
W47	8.092	121.704	58.876	30.949	177.171
L48	7.5	116.016	54.863	43.615	176.609
D49	8.829	122.966	52.813	38.933	175.218
V50	6.747	112.817	57.962	34.555	174.907
D51	7.813	118.491	52.936	41.255	175.48
A52	8.29	121.889	55.879	18.708	179.332
C53	8.07	115.293	63.541	27.186	178.151
T54	7.574	119.063	63.91		
R55					
V56					
L57					
L58					
I59					
S60					
S61					
V62					
M63					
L64					
V65					
M66					
I67					
V68					
E69					
L70					178.557
L71	8.256	119.063	57.858		179.101
N72	8.88	117.641	57.082	38.949	177.397
S73	8.521	116.422	62.49	63.366	177.351
A74	8.104	126.512	55.492	17.897	
I75			65.056	36.634	178.077
E76	8.654	120.204	60.031	28.98	177.628
A77	7.659	119.764	54.751	16.896	179.809
V78	7.67	116.942	66.122	30.613	177.777
V79	8.197	119.266	66.538	30.553	178.954
D80	8.377	120.854	56.631	39.608	
R81					
I82			63.494		177.286
G83	7.536	107.686	45.57		174.255
S84	8.21	115.813	59.194	63.818	175.48
E85	8.346	122.455	56.688	29.185	176.549
Y86	8.18	121.908	58.525		
H87					
E88					
L89					
S90					
G91	7.887	108.702	46.821		175.517
R92	7.785	121.551	58.921	29.512	178.2

A93	7.857	120.485	55.687	18.222	179.67
K94	7.505	115.406	59.397	31.524	180.009
D95	8.473	121.908	57.396	39.819	179.465
M96	8.274	120.079	58.507	32.019	178.431
G97	8.007	106.597	47.186		176.247
S98	8.047	115.406	61.918	62.877	177.739
A99	7.97	125.147	54.82	17.2	178.622
A100	7.762	121.053	55.491	17.316	179.414
V101	7.454	117.628	66.499	30.888	177.813
L102	7.758	119.876	58.207	40.331	
I103					
A104					
I105					
I106					
D107					
A108					
V109					
I110			62.475		175.798
T111	8.227	116.016	62.854	69.625	
W112					
C113	8.725	116.014	63.365	26.8	176.583
I114	7.926	118.251	67.281		179.178
L115	7.965	118.86	57.707		180
L116	8.486	117.641	56.892	40.288	178.966
W117	8.334	122.553	60.638	29.441	178.701
S118	7.923	110.734	60.648	63.091	175.869
H119	7.695	118.657	58.268	29.968	175.881
F120	8.01	115.629	58.148	39.743	175.104
G121	7.608	114.308	46.067		

Table 2. Backbone chemical shift assignment of wt-DAGK

Residue	H^N	N	C^α	C^β	C'
A1	8.041	123.268	52.446	18.08	177.65
N2	8.107	117.691	53.24	38.292	
N3			53.736	38.388	175.594
T4	7.994	114.112	62.236	69.259	175.117
T5	8.089	115.936	62.099	69.731	175.613
G6	8.588	110.688	46.24		175.387
F7	8.597	121.189	60.554	38.624	177.409
T8	8.13	113.67	66.081	67.962	176.35
R9	7.713	121.08	59.018	28.976	178.816
I10	7.518	119.484	63.995	36.805	179.01
I11	7.518	119.484	63.932	36.596	178.31
K12	7.873	119.98	58.769	31.28	179.431
A13	7.777	122.297	54.098	17.408	
A14	7.856	119.666	53.404	17.68	178.16
G15	7.821	104.326	45.95		175.065
Y16	7.619	119.919	58.677	38.265	176.023
S17	7.909	114.862	58.493	64.394	174.912
W18	8.363	123.394	58.795	28.873	178.869
K19	8.053	119.371	58.823	31.17	178.979
G20	7.853	107.77	45.974		175.473
L21	7.85	122.836	56.913	41.067	177.694
R22	7.773	118.716	58.047	29.497	177.308
A23	7.694	121.072	53.748	17.4	179.025
A24	7.795	120.42	53.98	17.606	178.604
W25	7.755	117.083	58.733	28.981	176.99
I26	7.472	117.073	62.303	36.934	176.347
N27	7.682	118.07	54.112	38.993	175.896
E28	8.052	119.755	56.928	28.852	
A29					178.31
A30	7.856	119.666	53.404	17.68	178.244
F31	7.442	114.892	58.539	38.226	176.147
R32	7.567	118.243	57.26	29.739	177.697
Q33	7.773	118.716	56.82	29.499	179.369
E34	8.23	119.819	58.051	31.844	177.876
G35	8.26	107.15	47.384		175.117
V36	7.647	120.277	66.323	30.455	
A37			55.388	17.54	178.807
V38	7.579	115.497	66.384	30.418	177.694
L39	7.729	118.831	57.939	40.586	178.723
L40	8.287	118.16	57.841	40.439	178.316
A41	7.912	120.741	55.321	17.347	179.583
V42	8.144	118.039	67.39	30.35	178.09
V43	8.171	120.104	67.503	30.265	178.692
I44	8.484	119.752	65.894	36.929	178.47
A45	8.656	120.965	54.925	18.03	179.431

C46	7.752	113.711	63.27	27.435	173.343
W47	8.066	121.423	58.666	30.415	177.085
L48	7.526	116.327	55.037	43.769	176.818
D49	8.911	123.257	52.926	38.868	175.217
V50	6.745	112.811	58.00	34.488	174.82
D51	7.766	118.463	53.083	41.36	175.219
A52	8.342	122.0	55.68	18.211	178.925
I53	7.591	116.586	65.107	36.21	177.853
T54	8.021	118.33	67.693	66.792	
R55					178.873
V56	6.936	116.478	66.789	30.45	179.034
L57	7.947	123.026	57.842	41.496	180.378
L58	8.336	121.062	57.874	39.664	179.657
I59	7.804	117.048	65.461	38.404	178.054
S60	9.237	116.271	62.653	62.646	177.379
S61	7.935	115.872	61.645	62.646	177.519
V62	7.046	118.906	65.155	30.532	178.109
M63	8.242	119.989	57.165	30.314	179.397
L64	8.165	121.108	57.938	39.536	178.837
V65	6.936	116.478	66.789	30.345	177.474
M66	6.931	116.516	58.072	32.406	
I67					178.304
V68	7.982	116.436	66.92	30.382	177.866
E69	8.801	121.043	59.076	28.066	179.876
I70	8.395	122.605	65.642	36.767	178.721
L71	8.084	120.736	58.113	41.072	179.186
N72	8.831	117.737	56.928	38.464	177.431
S73	8.344	116.626	62.289	62.646	177.244
A74	8.089	126.737	55.334	17.275	178.806
I75	7.832	118.51	65.318	36.804	178.251
E76	8.595	120.065	59.843	28.73	178.35
A77	7.612	119.968	54.454	17.281	180.115
V78	7.62	117.128	66.077	30.376	177.775
V79	8.102	118.797	66.25	30.396	178.923
D80	8.4	120.764	56.613	39.678	177.922
R81	7.529	117.67	57.018	30.315	
I82					177.385
G83	7.684	108.223	45.703		174.504
S84	8.152	115.757	59.291	63.199	175.513
E85	8.328	122.181	56.823	28.927	178.119
Y86	7.591	118.4	63.284	37.721	
H87					
E88					175.813
L89	8.044	115.928	58.093	39.483	
S90					176.064
G91	7.975	108.959	46.629		175.434
R92	7.802	121.364	58.448	29.416	178.014

A93	7.971	121.056	55.282	18.038	179.514
K94	7.601	116.324	59.049	31.41	179.515
D95	8.415	121.633	56.833	39.891	179.369
M96	8.23	119.819	58.051	31.889	177.876
G97	8.26	107.15	47.384		176.279
S98	7.854	114.455	61.723	62.684	177.324
A99	8.028	125.426	54.092	16.887	179.055
A100	7.936	119.884	55.465	16.699	179.263
V101	7.33	117.291	66.396	30.82	177.755
L102	7.726	119.481	58.148	40.396	179.012
I103	8.266	117.18	64.822	35.818	177.518
A104	7.977	122.179	55.865	16.432	179.941
I105	8.103	117.18	63.933	35.891	179.141
I106	7.609	117.891	66.611	36.671	177.417
V107	8.376	118.308	66.912	30.368	179.144
A108	8.082	126.871	56.161	15.939	
V109					
I110	7.997	114.189	65.724	36.391	177.852
T111	8.302	116.899	68.42		
W112					178.547
C113	8.699	115.842	64.883	26.785	176.983
I114	8.519	116.935	65.887	36.772	178.932
L115	7.992	116.267	57.341	40.295	180.297
L116	8.422	117.641	56.532	40.368	178.82
W117	8.198	122.812	60.641	28.878	178.654
S118	8.031	110.86	60.268	62.647	176.008
H119	7.776	118.762	57.233	29.499	175.392
F120	8.014	118.159	58.246	39.512	175.089
G121	7.61	114.282	46.049		

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