Selective Precipitation and Purification of Monovalent Proteins Using Oligovalent Ligands and Ammonium Sulfate

SUPPORTING INFORMATION

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General Methods. All chemicals were obtained from Sigma-Aldrich (Atlanta, GA) and used without further purification, unless otherwise noted. DNSA was recrystallized from ethanol prior to use. Fluorescence measurements were performed on a Molecular Devises SpectraMax Gemini XS instrument. Varian Inova spectrometer operating at 600 MHz (^{1}H) was used for NMR experiments. UV-Vis spectroscopy was conducted on either a Hewlett-Packard 8453 spectrophotometer (Palo Alto, CA) for quantifying concentration of BCA (ε_{280} = 57 000 M^{-1} cm^{-1}) and DNSA (ε_{326} = 4640 M^{-1} cm^{-1}) or on NanoDrop ND-1000 Spectrophotometer for measuring the concentration of precipitated protein. Mass spectrometry was carried out on Applied Biosystems Voyager DE-Pro MALDI-TOF Mass Spectrometer or Waters Micromass LCT Mass Spectrometer. Concentration of monovalent and oligovalent ligands was quantified by ^{1}H NMR spectroscopy. Analytical HPLC was performed using a Varian Prostar system equipped with a C18 column, using a linear gradient of water with 0.1% TFA (A) and acetonitrile containing 0.08% TFA (B) at a flow rate of 1.2 mL/min. Preparative reverse-phase HPLC was run on a Varian instrument with a C18 column at a flow rate of 15 mL/min.

Synthesis of Oligovalent Ligands. Monovalent, bivalent, and trivalent ligands were prepared using condensation reactions of nucleophilic amines with activated carboxylates. O, O-bis(2-aminoethyl)octadecaethylene glycol was purchased from Sigma-Aldrich (Atlanta, GA; product # 06703-1G-F). Tris-succinimidyl aminotriacetate (TSAT) was purchased from Pierce (Rockford, IL; product # 33063). 3-carboxybenzenesulfonamide was purchased from Matrix Scientific (www.matrixscientific.com; product # 033834). NHS-activated 4-carboxybenzenesulfonamide was synthesized according to the general procedure of Jain et al.[1] Procedures below outline specific examples for synthesizing each ligand.

Synthesis of L_1. NHS-activated 4-carboxybenzenesulfonamide (100 mg, 0.335 mmol) dissolved in 5.2 mL of acetone was added to a reaction flask containing a solution of O, O-bis(2-aminoethyl)octadecaethylene glycol (600 mg, 0.670 mmol) in 10.3 mL of 0.5 M KH_2PO_4 buffer pH 8.0. The reaction mixture was allowed to stir at ambient temperature for 16 hours. The solvent was removed under reduced pressure, the reaction mixture was resuspended in acetonitrile, and the product was purified using reverse-phase HPLC. ^{1}H NMR in DMSO-d6: δ 3.40 – 3.65 (m), 7.48 (s, 2H), 7.89 (d, 2H), 7.99 (d, 2H), 8.70 (t, 1H). HRMS: found 1080.5691 for $[C_{47}H_{89}N_3O_{22}S]^+$.

Synthesis of L_2. NHS-activated 4-carboxybenzenesulfonamide (46.5 mg, 0.156 mmol) dissolved in 0.43 mL of acetone was added to a reaction flask containing a solution of O, O-bis(2-aminoethyl)octadecaethylene glycol (50 mg, 0.056 mmol) in 0.86 mL of 0.5 M KH_2PO_4 buffer pH 8.0. The reaction mixture was stirred at ambient temperature for 16 hours. The solvent was removed under reduced pressure, the reaction mixture was resuspended in acetonitrile, and the product was purified using reverse-phase HPLC. ^{1}H NMR in DMSO-d6: δ 3.40 – 3.65 (m), 7.48 (s, 4H), 7.89 (d, 4H), 7.99 (d, 4H), 8.70 (t, 2H). HRMS: found 1284.2159 for $C_{54}H_{94}N_4O_{25}S_2 + Na^+$

Synthesis of $L_{2\text{-weak}}$. In a reaction flask, combined O, O-bis(2-aminoethyl)octadecaethylene glycol (100 mg, 0.11 mmol), 3-carboxybenzenesulfonamide (46 mg, 0.23 mmol), diisopropylethylamine (DIEA, 40 μL, 0.23 mmol), (HBTU, 87 mg, 0.23 mmol), and N,N-dimethylformamide (DMF, 222 μL). The reaction mixture was allowed to stir at ambient temperature for 18 hours, concentrated under reduced pressure, resuspended in

acetonitrile, and the product was purified using reverse-phase HPLC. ^{1}H NMR in DMSO-d6: δ 3.20 – 3.80 (m), 7.44 (s, 4H), 7.67 (t, 2H), 7.95 (d, 2H), 8.04 (d, 2H), 8.31 (s, 2H), 8.77 (t, 2H). HRMS: found 1264.5574 for $C_{54}H_{94}N_4O_{25}S_2 + H^+$

Synthesis of L_3. In a reaction flask, combined TSAT (7.4 mg, 0.015 mmol), purified L_1 (50 mg, 0.046 mmol), DIEA (8 μL, 0.046 mmol), and DMF (600 μL). The reaction mixture was allowed to stir at ambient temperature for 16 hours, concentrated under reduced pressure, resuspended in acetonitrile, and the product was purified using reverse-phase HPLC. ^{1}H NMR in DMSO-d6: δ 3.40 – 3.65 (m), 7.48 (s, 6H), 7.89 (d, 6H), 7.99 (d, 6H), 8.71 (t, 3H). MALDI MS: found 3376.802 for $C_{147}H_{270}N_{10}O_{69}S_3 + H^+$.

Synthesis of analogs of L_2 with different OEG linkers. Analogs of L_2 were synthesized using a procedure completely analogous to the synthesis of L_2 starting from NHS-activated 4-carboxybenzenesulfonamide and commercially available starting materials.

Determination of K_d for L_1, L_2, L_2-weak and L_3. We used a fluorescence-based competition assay of CA with dansyl amide (DNSA) to measure the K_d of L_1, L_2, and L_3. To the wells of a black microwell plate were added dilutions of L (two-fold dilutions across the plate), BCA (final [BCA] = 50 nM per well), DNSA (final [DNSA] = 5 μM per well), in a final volume of 200 μL of 20 mM sodium phosphate pH 7.5. The plate was allowed to incubate at 25°C for 15 minutes, and then its fluorescence was measured (excitation wavelength = 280 nm and emission wavelength = 460 nm, with a 455 nm cut-off filter). Wells were read 3 times. Fluorescence intensities (F) were fit to eq S.1(a-b).[2, 3]

$$F = F_{\min} + (F_{\max} - F_{\min})\left(\frac{-A + \sqrt{A^2 + 4(K_d^{DNSA} + (K_d^{DNSA})^2/[DNSA]_{total})[CA]_{total}K_d^L[L]_{total}}}{2[CA]_{total}(K_d^{DNSA} + (K_d^{DNSA})^2/[DNSA]_{total})} \right) \quad \text{(S1a)}$$

$$A = K_d^{DNSA}[L]_{total} - K_d^{DNSA}[CA]_{total} + K_d^L[DNSA]_{total} + K_d^{DNSA}K_d^L \quad \text{(S2b)}$$

In this equation, $[CA]_{total}$ and $[DNSA]_{total}$ are the total concentrations of carbonic anhydrase (CA) and dansyl amide (DNSA), respectively. $F_{\min}$ is a constant that takes into account background fluorescence (close to zero in our experiments), and $F_{\max}$ is the maximum fluorescence at total saturation of CA by DNSA. K_d^{DNSA} and K_d^L are the dissociation constants of the CA-DNSA and CA-L complexes, respectively; $[L]_{total}$ is the total concentration of the ligand (L_1, L_2, L_3, etc.). We constrained the values of $[DNSA]_{total}$ and K_d^{DNSA} to their known values, and allowed the other parameters to vary in order to optimize the non-linear least squares fit in Origin7.

This equation makes the assumption that the concentration of free DNSA (that is not bound to CA) is equal to that of total DNSA (a reasonable assumption given that $[DNSA]_{total} >> [CA]_{total}$). In our analysis, we also assumed independent and equivalent binding of each benzene sulfonamide to BCA; that is we assumed that each benzene sulfonamide on the oligovalent scaffold binds independently, and does not inhibit or enhance the binding of CA to other benzenesulfonamides on the scaffold.

Using this model yielded the following monovalent dissociation constants from BCA for each of the benzene sulfonamides: $K_d(L_1) = 0.5 \pm 0.1$ μM, $K_d(L_2) = 0.4 \pm 0.1$ μM, $K_d(L_3) = 0.6 \pm 0.6$ μM.

Precipitation of BCA from Ammonium Sulfate. We dissolved $(NH_4)_2SO_4$ in Tris-Gly to obtain a saturated solution. Precipitation experiments were performed in triplicate for each ligand type and each concentration of $(NH_4)_2SO_4$. The experiments were carried out in 1.7 mL polypropylene microtubes with a total sample volume of 250 μL and final concentration of BCA of 15 μM. To each microtube we added saturated ammonium sulfate, appropriate amount of Tris-Gly buffer required to dilute ammonium sulfate to a desired concentration, and an aliquot of ligand stock in deuterated DMSO (0.750 μL of 10.00 mM Bivalent ligand stock, 0.822 μL of 9.125 mM trivalent ligand stock, 2.25 μL of 10.00 mM monovalent ligand stock, and 2.25 μL of deuterated DMSO for the control experiments with no ligand). We agitated the samples by vortexing each tube for 10 s and then added 25 μL of 150 μM BCA stock solution in Tris-Gly to each vial to obtain a total sample volume of 250 μL and final BCA concentration of 15 μM. We then gently agitated the vials on a rocker in the coldroom at 4 °C for 4 hours. We centrifuged all vials at 4 °C for 30 minutes at 14,000 rpm, withdrew the supernatant and re-suspended the pellet in 50 μL of Tris-Gly buffer. The amount of recovered protein in the pellet was analyzed using UV-Vis.

Optimization of Ligand Concentration for Maximizing the Yield of Precipitated Protein. We empirically optimized the amount of ligand required to maximize the yield of precipitated BCA from a solution of ammonium sulfate in Tris-Gly buffer. We chose to carry out this optimization in 60 % AMS (w/v) solution in Tris-Gly (i.e., 2.36 M AMS) because this concentration did not cause precipitation of BCA alone, or BCA-L_1, but yielded a significant amount of precipitated BCA upon addition of L_2 and L_3. For each precipitation experiment, we used a conical plastic vial filled 250 μL of 15 μM solution of BCA in 2.36 M AMS (60 % AMS

w/v). We then added a variable amount of ligand from a 10.00 mM stock of L_1, 10.00 mM stock of L_2, and a 9.13 mM stock of L_3 in DMSO to each vial. The volume of total DMSO added did not exceed 5 μL. We vortexed each vial for ~ 10 s to ensure mixing of the ligand with the protein, and then gently agitated the vials on a rocker in the coldroom at 4 °C for 4 hours. We centrifuged all vials at 4 °C for 30 minutes at 14,000 rpm, withdrew the supernatant and re-suspended the pellet in 50 μL of Tris-Gly buffer. The amount of recovered protein in the pellet was analyzed using UV-Vis.

Effect of Buffer and pH on the Yield of Precipitation of BCA in the presence of L_2.

To test the effect of buffer type on the yield of precipitation of BCA alone and in the presence of L_2, we two types of concentrated AMS solutions: one in 20 mM sodium phosphate buffer at pH 7.5 and another one in Tris-Gly buffer at pH 7.5. We then used analogous procedure to that described in section "Precipitation of BCA from Ammonium Sulfate" to precipitate BCA from AMS solutions at each pH. We quantified the amount of precipitated protein using UV-Vis.

To test the effect of pH on the yield of precipitation of BCA in the presence of L_2, we prepared concentrated AMS solution in 20 mM sodium phosphate buffer at pH of 6.0, 6.5, 7.0, 7.5. We then used analogous procedure to that described in section "Precipitation of BCA from Ammonium Sulfate" to precipitate BCA from AMS solutions at each pH.

Purification of CA from Cellular Lysate.

Bacterial strains, culture, and HCA expression. The *E. coli* strains used in this work – BL21(DE3)pLysS competent cells (Promega, Madison, WI) – were transformed, with the HCA

II containing pACA plasmid[4,5], using the standard procedure provided by the manufacturer. The *E. coli* were cultured in rich media and HCA expression was initiated with isopropyl β-D-1-thiogalactopyranoside (IPTG), in the presence of 200 mM $ZnSO_4$.[5] Each culture was pelleted (20 min, 5000x*g*, 4°C), washed with and then resuspended in lysis buffer, lysed with in a French press and then cellular debris removed with centrifugation (20 min, 14000x*g*, 4°C). The cell lysate was flash frozen, in aliquots, with liquid nitrogen and stored at -80°C until needed. The lysis buffer was composed of 50 mM Tris sulfate buffer (pH = 8.0), 50 mM NaCl, 200 mM $ZnSO_4$, 10 mM EDTA, 1 mM DTT, 1 μg/mL TAME, and 10 μg/mL PMSF.

Salt-induced precipitation. We added ammonium sulfate salt (AMS, 1.6 – 3.2 M in 0.2 M increments) to 1 mL aliquots of crude lysate, gently mixed on a rocker for 4 hours at 4°C, and then centrifuged for 15 minutes at 14000x*g* at 4°C. The supernatant was decanted and each pellet was resuspended with 1 mL of phosphate buffered saline (1X concentration). We analyzed each supernatant and precipitated sample with SDS PAGE, to obtain a qualitative view of which AMS concentration removed the largest number of proteins from the crude lysate while not precipitating the HCA. **Figure S7** contains a gel of the precipitated samples (**S7a**) and the supernatant samples (**S7b**) with increasing amount of AMS.

We prepared the samples for SDS PAGE by: mixing the samples with Laemmli sample buffer (BioRad, Hercules, CA) in a 1:1 vol/vol ratio and incubating the mixtures at 95°C for 5 minutes. We loaded the denatured samples into a 12% Tris-HCl gels (BioRad) and ran the gel at constant voltage (110 V) in 1X Tris/glycine/SDS buffer (25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS, pH = 8.3). We stained each gel with Coomassie and imaged on a light table.

We determined the amount of protein present in each sample spectrophotometrically with a Coomassie dye-based assay (i.e., a Bradford assay) and compared each sample with a calibration curve constructed from known concentrations of bovine serum albumin (BSA).

Ligand-induced precipitation. We added the trivalent ligand (dissolved in DMSO) to the supernatant containing HCA, mixed thoroughly by vortexing, and then placed on a rocker for 4 hours and 4^{o}C. The protein-ligand aggregates were precipitated via centrifugation (15 minutes at 14000x*g*). A number of centrifugation times and speeds were tested to determine the optimal time needed to remove the HCA aggregates but not to precipitate extra proteins from solution. Time periods longer than 15 minutes resulted in a large fraction of unwanted proteins precipitating, while shorter time periods did not completely remove all the HCA aggregates. We chose fifteen minutes as this duration of centrifugation removed the majority of the HCA and resulted in a small fraction of extra proteins being precipitated. We washed the protein pellets with 2.4 M AMS (0.1X the original supernatant volume) to remove the excess proteins that were precipitated during centrifugation.

We analyzed the HCA-ligand aggregates, as well as the remaining supernatant, with SDS PAGE and quantified the amount of protein using the Bradford assay mentioned above.

Figure S1. Optimization of ligand concentration for maximizing the yield of precipitated BCA from 60 % w/v (2.36 M) AMS in Tris-Gly. Error bars represent maximum deviation from the average based on three measurements.

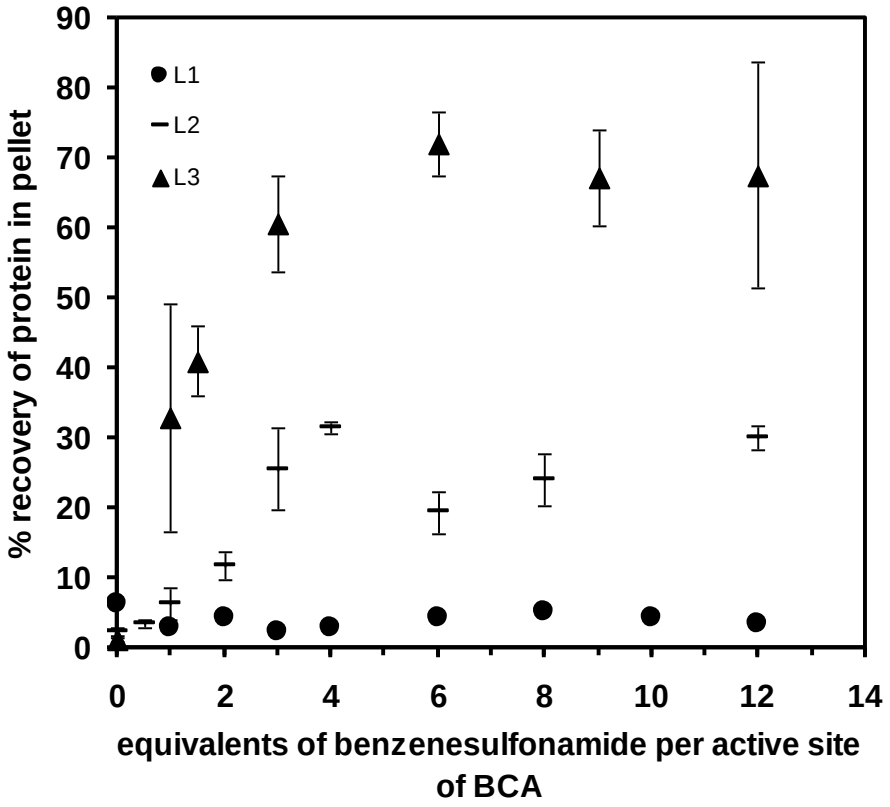


Figure S2. Effect of buffer type on the yield of precipitated protein from ammonium sulfate.

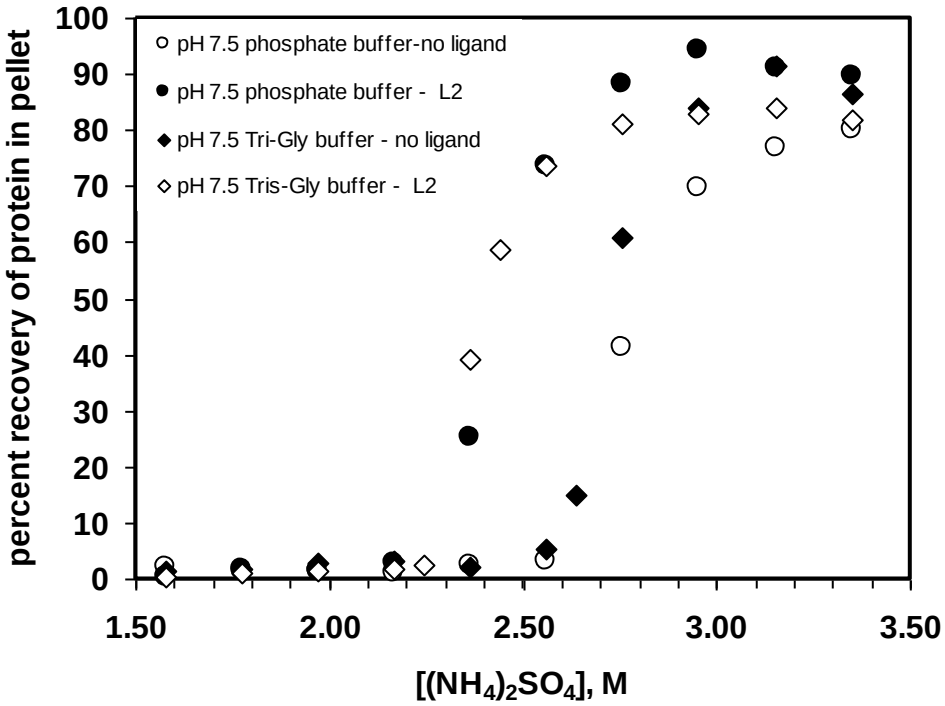


Figure S3. Effect of pH on the yield of precipitated protein from solutions of ammonium sulfate in phosphate buffer in the presence of L_2.

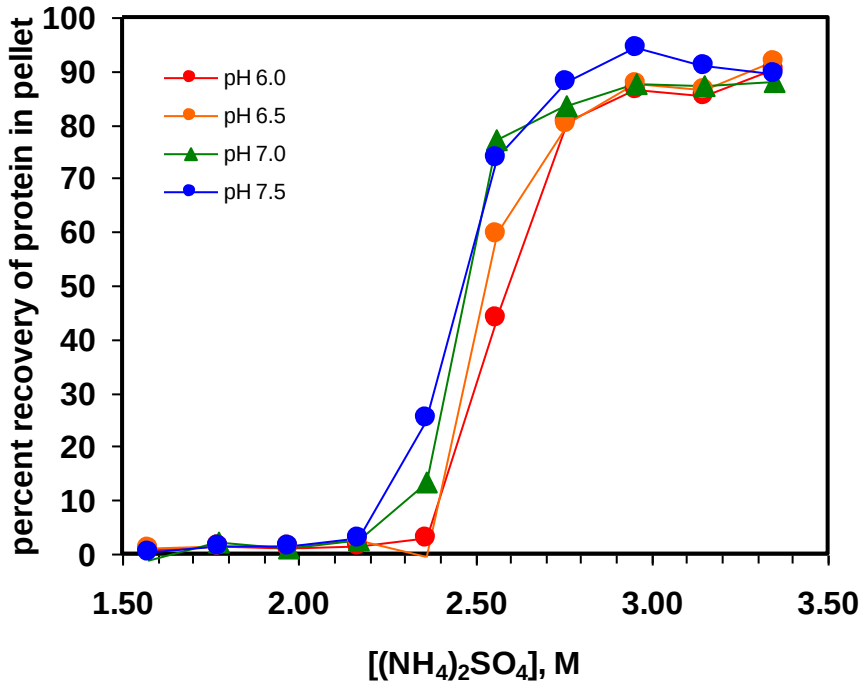


Figure S4. Effect of the length of OEG linker on precipitation efficiency of BCA from solutions of AMS. Error bars represent the maximum deviation from the average based on three independent measurements.

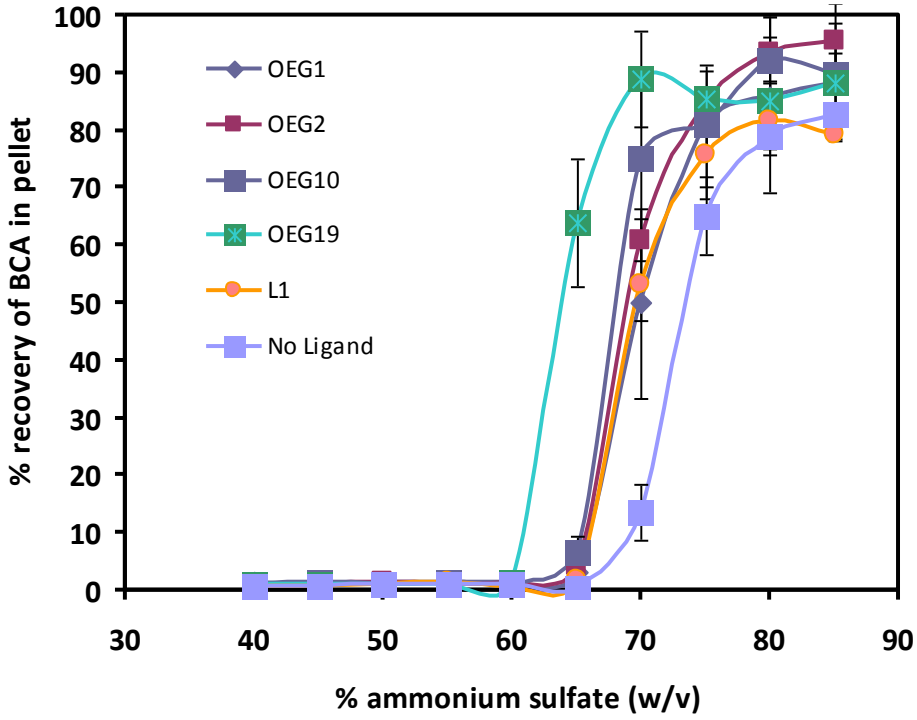


Figure S5. Models of selected analogs of L_2 with varying length of oligoethyleneglycol linkers [OEG2 (top) and OEG19 (bottom)] binding to carbonic anhydrase. OEG2 is not long enough to allow binding of two CA's simultaneously. OEG19 is sufficiently long to enable bivalent binding.

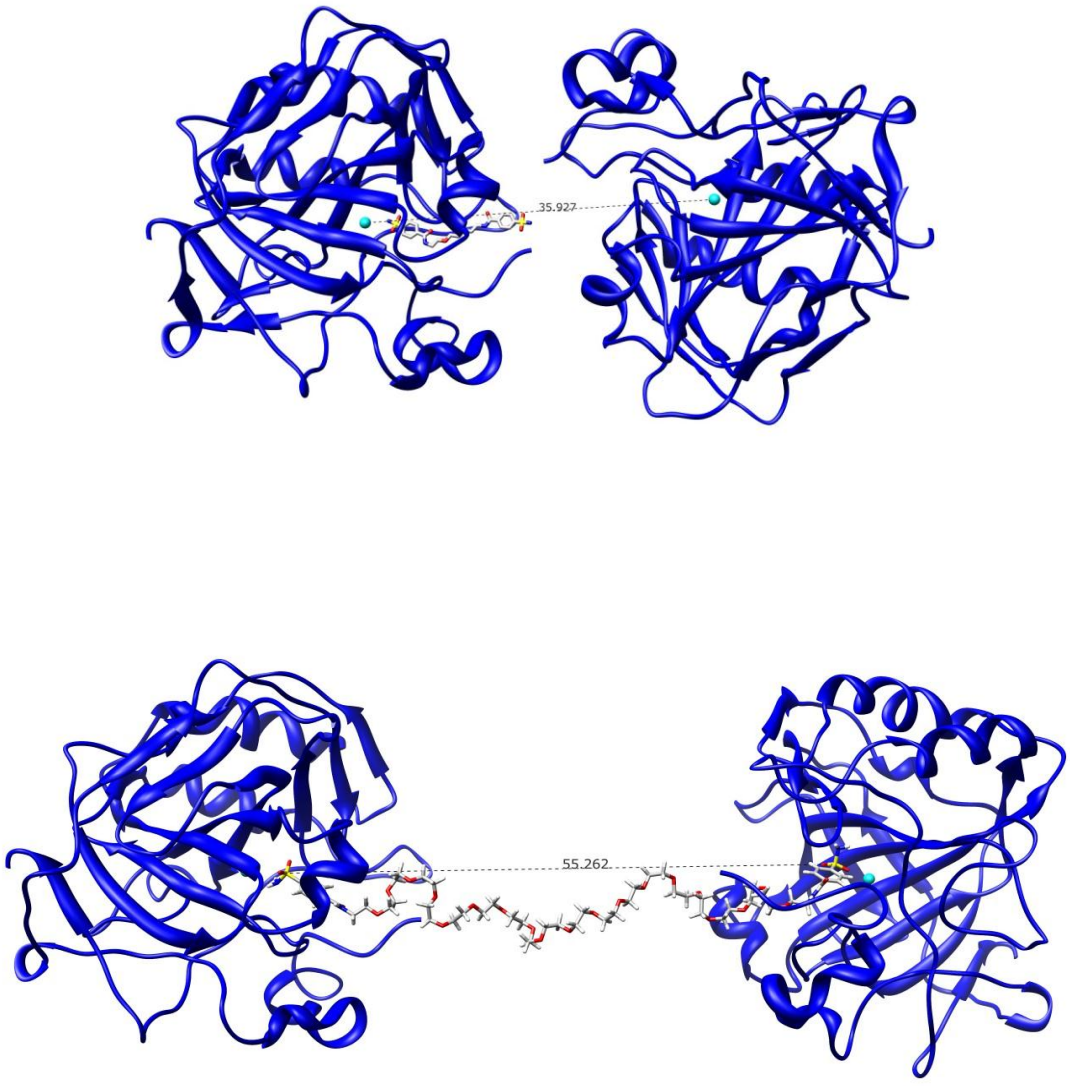
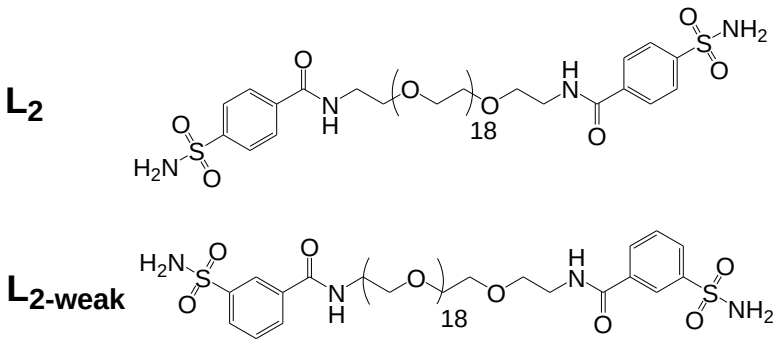


Figure S6. A) Structures of L_2 and $L_{2\text{-weak}}$. B) Effect of [P] and K_d on the efficiency of precipitation of BCA from AMS. BCA was dissolved in 60 % w/v (2.36 M) AMS in Tris-Gly and the ligand was added from a concentrated stock in DMSO. $K_d(L_2) = 0.4 \pm 0.1$ μM and $K_d(L_{2\text{-weak}}) = 10.8 \pm 0.7$ μM)

A)



B)

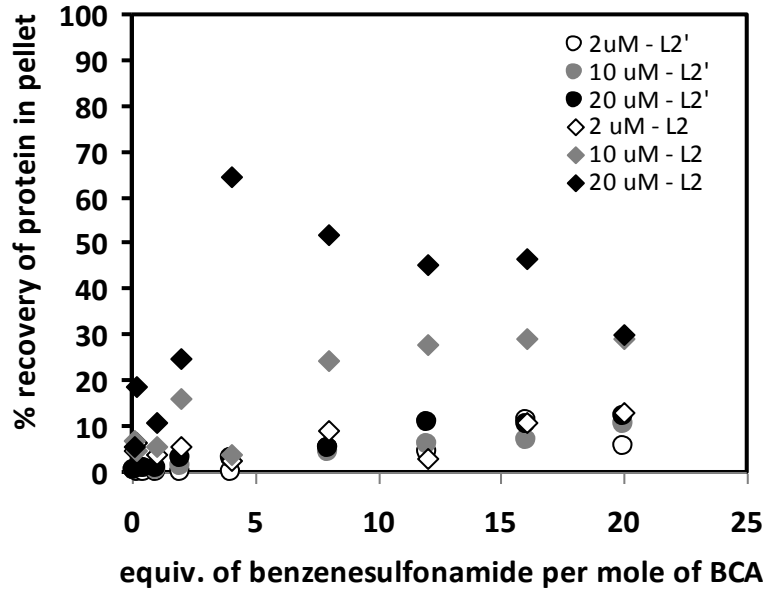


Figure S7. Total protein precipitated from crude *E. coli* lysate (over-expressing human carbonic anhydrase) with increasing concentrations of AMS. Protein concentrations were determined with a colorimetric, Bradford, assay. The dashed line represents the total protein concentration in a sample of cellular lysate (1914 ± 65 μg/mL), ■ is the concentration of protein remaining in the supernatant after salt precipitation, ● is the concentration of precipitated protein, and ▲ is the protein concentration (precipitate + supernatant) to show that no appreciable sample loss that occurred during the procedure. Each point on the graph is the average and standard deviation of n = 10 samples.

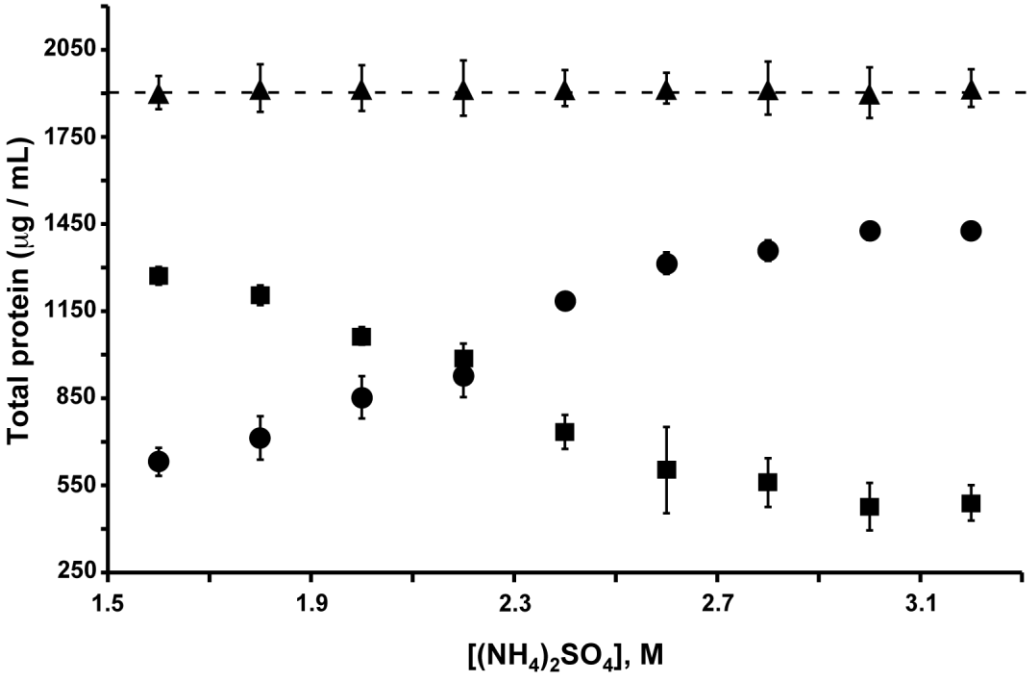
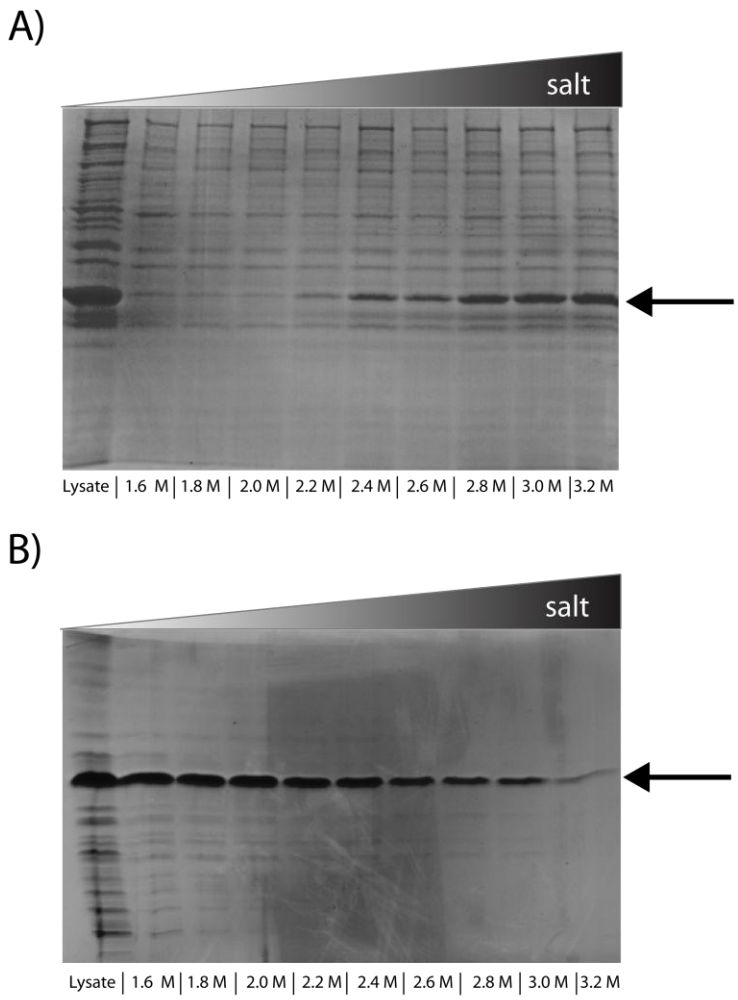


Figure S8. PAGE gels of cellular lysate fractions, where a known amount of ammonium salt was added to crude cellular lysate over-expressing HCA, incubated, and then centrifuged. The arrow on each gel designates the HCA band. A) Protein precipitates as a function of increasing ammonium sulfate concentrations. B) Protein remaining in solution as a function of ammonium sulfate concentration.



Supporting References:

[1] A. Jain, S. G. Huang, G. M. Whitesides, *J. Am. Chem. Soc.* **1994**, *116*, 5057-5062.

[2] V. M. Krishnamurthy, V. Semetey, P. J. Bracher, N. Shen, G. M. Whitesides, *J. Am. Chem. Soc.* **2007**, *129*, 1312-1320.

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[4] The pACA plasmid used in this work was, kindly, provided by the laboratory of Professor Carol Fierke, University of Michigan Department of Chemistry.

[5] S.K. Nair, T.L. Calderone, D.W. Christianson, C.A. Fierke, *J. Biol. Chem.* **1991**, 266, 17320-17325.