Supplemental material

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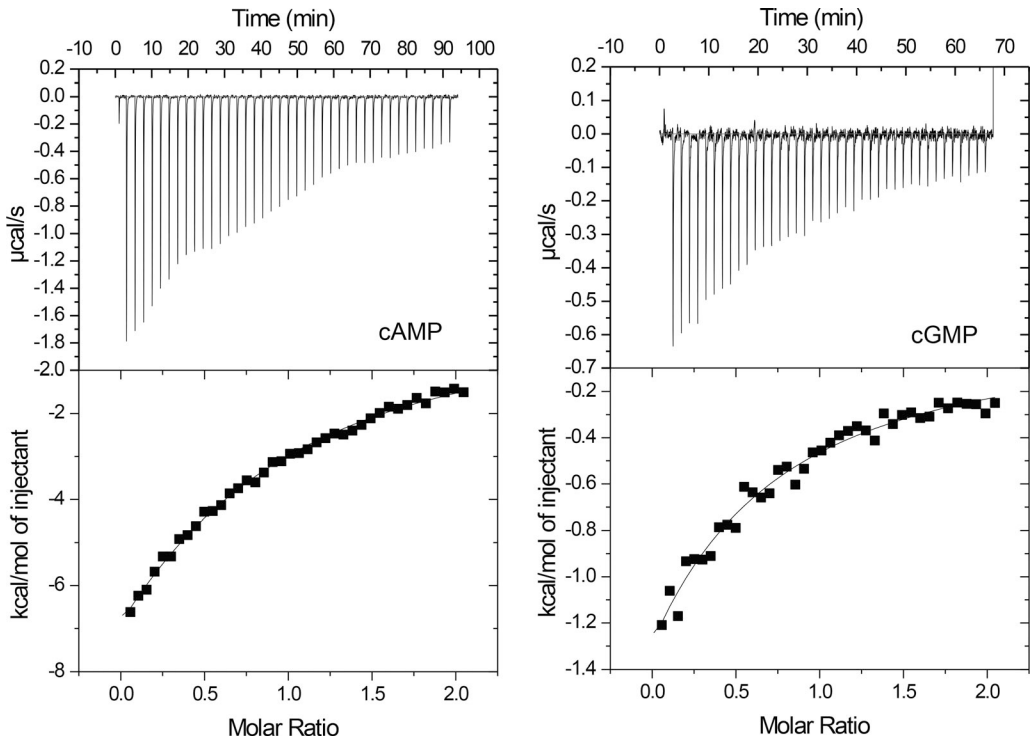

Figure S1. **cAMP and cGMP bind to the E582A mutant with reduced affinity compared with their binding to the wild-type HCN2 C-terminus fragment.** Plots of heat produced upon progressive injections of cAMP (left; 200 mg) or cGMP (right; 400 mg) in the mutant HCN2 C-terminus, measured by ITC. The inflections in the top plot arise from injections of ligand, where each inverted peak shows the heat difference between the sample and reference compartment. The peaks decrease in magnitude as binding sites become saturated. The lower plot shows values determined by integration of the area under the peaks from the upper plot versus the ratio of injected ligand to protein. The solid line through the values represents a single binding site model, which yielded values for affinity and energetics. Values for binding affinity were 335 mM for cAMP (two trials) and 690 mM for cGMP (one trial).

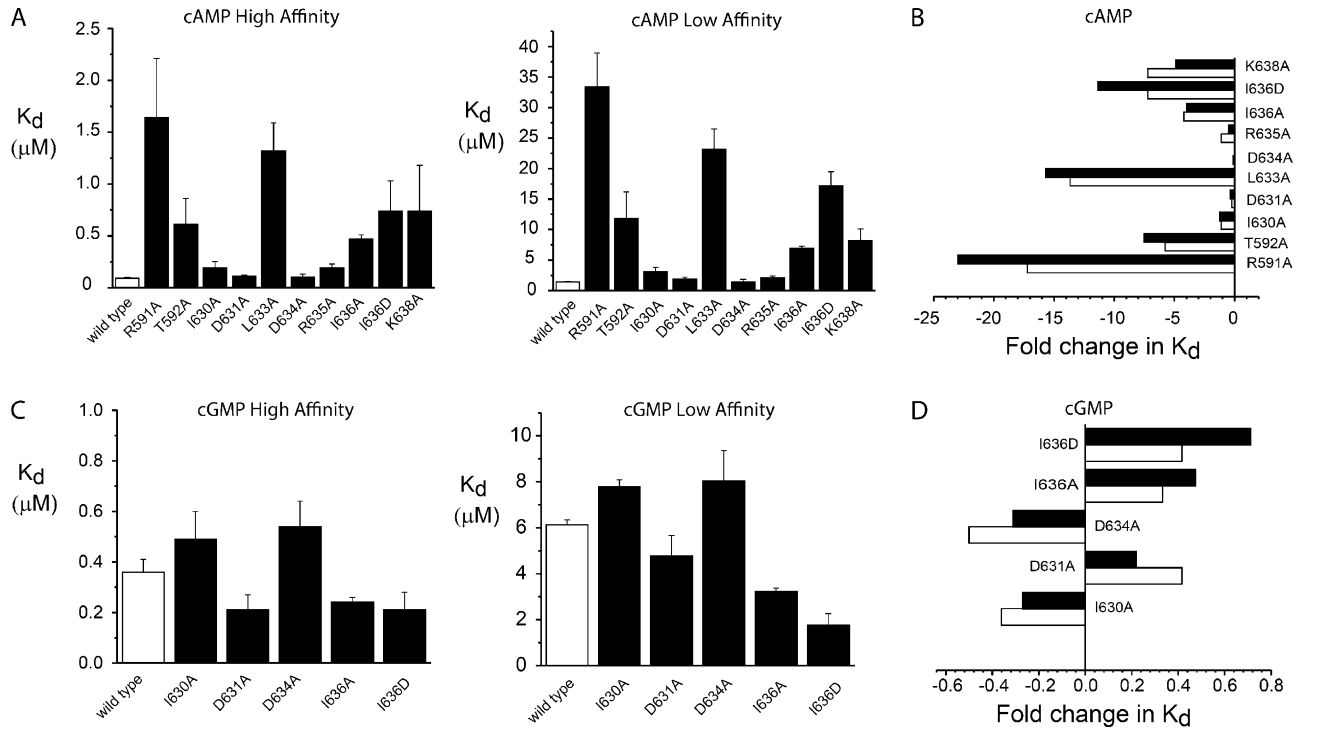

Figure S2. **The effect of single mutations of residues of the HCN2 cyclic nucleotide binding region on high and low binding affinity. (A)** A bar plot of values for high- and low-affinity binding of cAMP to the tetrameric HCN2 C-terminal wild-type protein and mutants containing single mutations. Values from Table 1. **(B)** Plot of fold-change in K_d values for high-affinity (white bars) and low-affinity (black bars) for binding of cAMP to single substitution tetrameric HCN2 mutant C-terminal protein compared with the wild-type values. Calculated from the values in Table 1. Fold-change = (wild type K_d – mutant K_d)/wild-type K_d. **(C)** A bar plot of values for high- and low-affinity binding of cGMP to the tetrameric HCN2 C-terminal wild-type protein and mutants containing single substitutions. Values from Table 1. **(D)** Plot of fold-change in K_d values for high-affinity (white bars) and low-affinity (black bars) for binding of cGMP to single substitution tetrameric HCN2 mutant C-terminal protein compared with the wild-type values. Calculated from the values in Table 1. Fold change = (wild type K_d – mutant K_d)/wild type K_d. Values in A and C represent means ± SEM. Each mean was determined from independent ITC binding experiments.

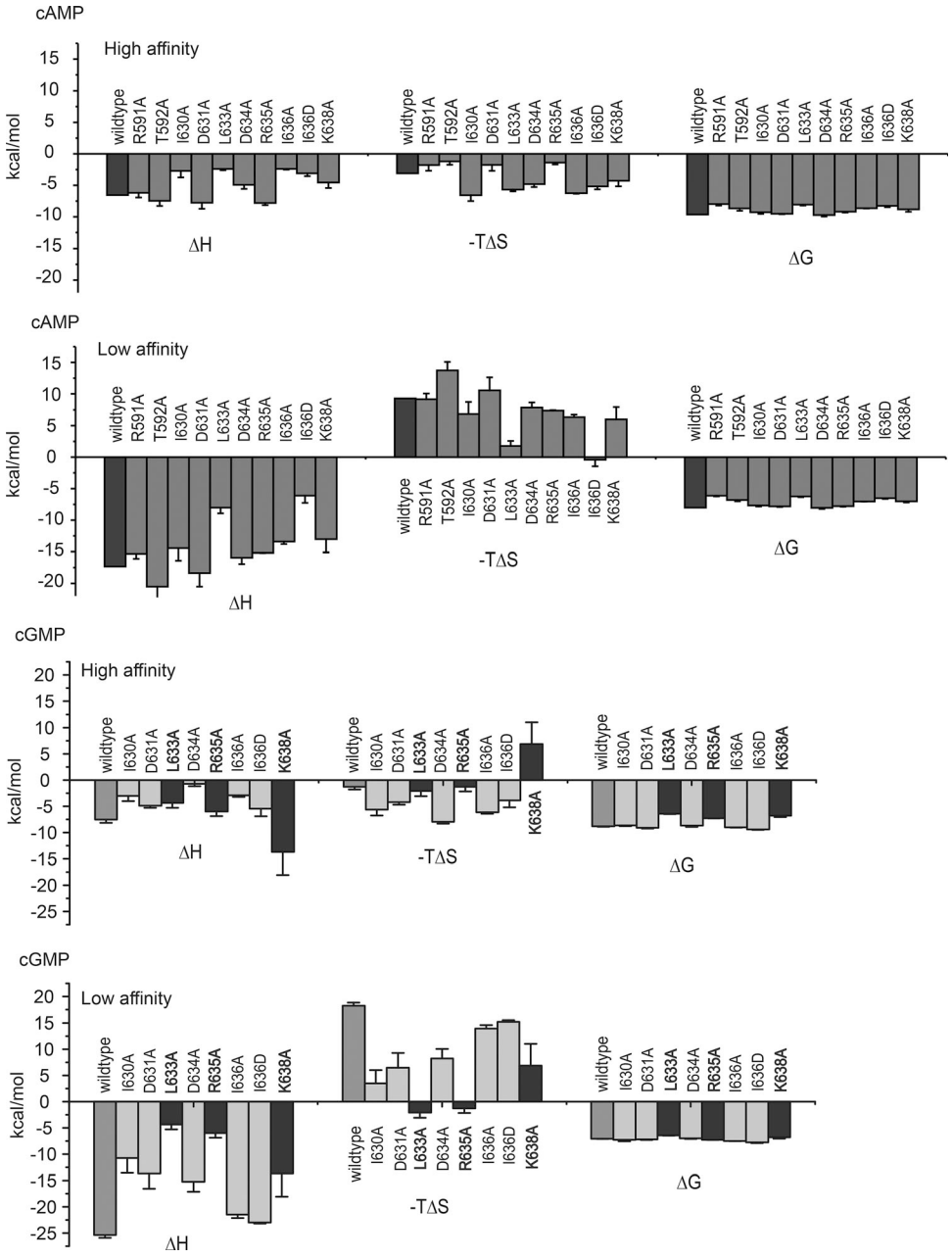

Figure S3. **The thermodynamics of cAMP and cGMP binding to the wild-type and mutant HCN2 C-terminus.** Bar graphs of the thermodynamics of binding, ΔG, ΔH, and −TΔS, which were derived from the fits of the heat generated from ligand binding shown in the figures for the corresponding constructs and ligand. Values of thermodynamics for mutants with only one binding event for cGMP are presented on both plots for comparison (purple). Values plotted represent the means ± SEM. The thermodynamics for the binding of cGMP to the R591A and T592A mutants are not shown on the graph. R591A values for ΔH = −427,700 kcal/mol, −TΔS = 426,140 kcal/mol, ΔG= −1,560 kcal/mol. T592A values for ΔH = −20.71 kcal/mol, −TΔS = 11.98 kcal/mol, ΔG = −5.10 kcal/mol.