Supplementary Methods

Determination of the Excess Heat: When analyzing the ITC data we found a small excess heat that is not accounted for by the separate heats of dilution of salt and protein (**Supplementary Fig.** 6a). When we compare the heat released by KCl injections into a chamber containing the Y445A mutant (black squares) –where no detectable binding is taking place– to the dilution heats of KCl alone (blue triangle) and Y445A protein alone (green circles). The sum of the dilution heats (red diamonds) does not account for the total heat measured. This excess heat is also present in protein samples that show strong binding (**Supplementary Fig. 6b, c**) but is much smaller than the heat resulting from the specific ion binding processes taking place inside the transport pathway.

The excess heat appears to be independent of the salt used, the mutant protein analyzed and any combination of the two (**Supplementary Fig. 6d**). This suggests that the excess heat is due to ions interacting with low affinity and non-specific binding sites on the surface of CLC-ec1. Consistent with this hypothesis we have crystallographically observed weak anomalous Bromine electron density peaks on the water-exposed surface of the protein, suggesting the existence of weak sites (A. Accardi and C. Miller, unpublished observations). Furthermore, electrophysiological studies of some eukaryotic CLCs have suggested the existence of an additional binding site outside of the permeation pathway [39,40,56].

The non-specific nature of this excess heat differentiates it from the specific heats resulting from ion binding to the sites in the translocation pathway of CLC-ec1. Thus, we estimated the excess heat from the last 3-5 injections and subtracted it. In all cases this correction leads to a small effect on the K_d (<3-fold in all cases). The estimate of the ΔH, on the other hand, is more sensitive to this subtraction. For reactions where ions bind more tightly the correction is

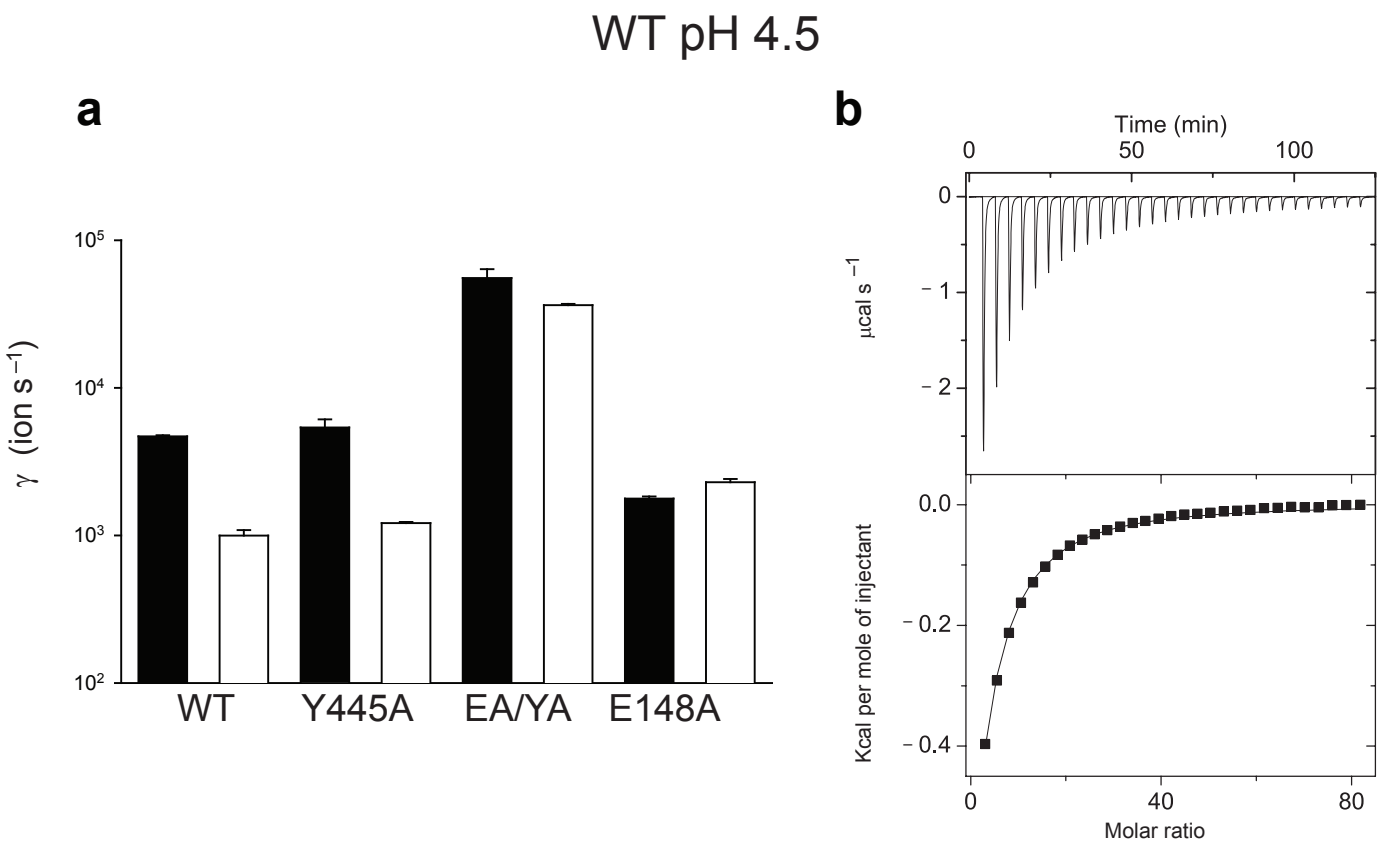
small (**Supplementary Fig. 7a**), while in weaker binding cases, such as NO_3^- binding to the WT or SCN^- binding in all cases (**Supplementary Fig. 7b**) the effect is more pronounced. Thus, the enthalpy and entropy determinations for weakly binding anions are affected by a large error and as such are not reliable.

Protein	Salt	K_d (μM)	ΔH (Kcal Mol^{-1})	TΔS (Kcal Mol^{-1})	ΔG (Kcal Mol^{-1})
WT	*KCl*	707	-4.18	0.12	-4.29
		671	-5.63	-1.30	-4.32
		518	-5.41	-0.92	-4.48
		699	-4.60	-0.30	-4.30
		1067	-6.30	-2.24	-4.05
		628	-5.57	-1.20	-4.36
		720±80	**-5.3±0.3**	**-1.0±0.3**	**-4.3±0.1**
	KBr	2907	-4.55	-1.09	-3.45
		3225	-4.25	-0.85	-3.39
		1515	-6.19	-2.35	-3.84
		2550±530	**-5.0±0.6**	**-1.4±0.5**	**-3.6±0.1**
	KNO_3	10235	-5.30	-2.54	-2.71
		11614	-5.09	-2.45	-2.63
		11223	-5.62	-2.95	-2.65
		12820	-6.95	-4.38	-2.57
		18761	-11.4	-9.02	-2.35
		13000±1500	**-6.9±1.2**	**-4.3±1.2**	**-2.6±0.1**
	KSCN	9708	-15.7	-12.96	-2.74
		9345	-15.6	-12.84	-2.76
		8403	-15.4	-12.52	-2.83
		9200±400	**-15.6±0.1**	**-12.8±0.1**	**-2.78±0.03**
WT pH 4.5	*KCl*	885	-7.88	-3.70	-4.16
		658	-5.97	-1.63	-4.33
		654	-5.95	-1.61	-4.34
		730 ±80	**-6.6±0.6**	**-2.3±0.7**	**-4.3 ±0.1**
E148A	*KCl*	18.28	-6.14	0.32	-6.45
		8.40	-7.16	-0.24	-6.91
		13.12	-4.19	2.47	-6.65
		9.71	-7.22	-0.38	-6.83
		11.89	-5.50	1.22	-6.71
		12.3±1.7	**-6.0±0.6**	**0.7±0.5**	**-6.7±0.1**
	KBr	78	-3.75	1.86	-5.59
		114	-4.7	0.66	-5.37
		61	-1.6	4.11	-5.74
		84±16	**-3.4±0.9**	**2.2±1.0**	**-5.6±0.5**
	KNO_3	1876	-6.20	-2.44	-3.71
		1348	-4.42	-0.50	-3.91
		933	-4.78	-0.65	-4.13

		1400±300	**-5.1±0.5**	**-1.2±0.6**	**-3.9±0.1**
	KSCN	8264	-5.15	-2.31	-2.84
		4201	-10.7	-7.48	-3.24
		5000	-7.39	-4.26	-3.13
		5800±1200	**-7.7±1.6**	**-4.7±1.5**	**-3.1±0.1**
E148Q	*KCl*	62.11	-0.39	5.36	-5.73
		86.21	-0.67	4.86	-5.54
		51.55	-0.38	5.48	-5.84
		66.6±10.3	**-0.48±0.09**	**5.23±0.2**	**-5.7±0.1**
Y445A	*KSCN*	6757	-21	-18.21	-2.96
		6289	-20	-17.14	-2.99
		7042	-22	-19.37	-2.93
		6700±200	**-21±1**	**-18.2±0.6**	**-2.96±0.02**
Y445L	*KCl*	3774	-3.88	-0.56	-3.30
		5780	-4.44	-1.38	-3.05
		2141	-1.75	1.89	-3.64
		3900±1100	**-3.4±0.8**	**0±1**	**-3.3±0.2**
SG/EA/YA	*KCl*	758	-5.33	-1.09	-4.25
		998	-5.11	-1.00	-4.09
		787	-5.17	-0.94	-4.23
		898	-4.18	-0.01	-4.15
		860±180	**-4.9±0.3**	**-0.8±0.3**	**-4.18±0.04**
EA/YA	*KCl*	1515	-2.10	1.75	-3.84
		1483	-2.47	1.39	-3.85
		1392	-1.69	2.20	-3.89
		1706	-2.08	1.69	-3.77
		1520±70	**-2.1±0.2**	**1.7±0.2**	**-3.84±0.03**
S107P	*KNO_3*	5376	-1.52	1.58	-3.09
		3278	-3.73	-0.33	-3.38
		2638	-3.33	0.19	-3.51
		3800±800	**-2.9±0.7**	**0.5±0.6**	**-3.3±0.1**
	KSCN	8264	-12.2	-9.27	-2.84
		5291	-15.2	-12.07	-3.10
		10526	-18.0	-15.35	-2.69
		8000±1500	**-15.1±1.7**	**-12.2±1.8**	**-2.9±0.1**

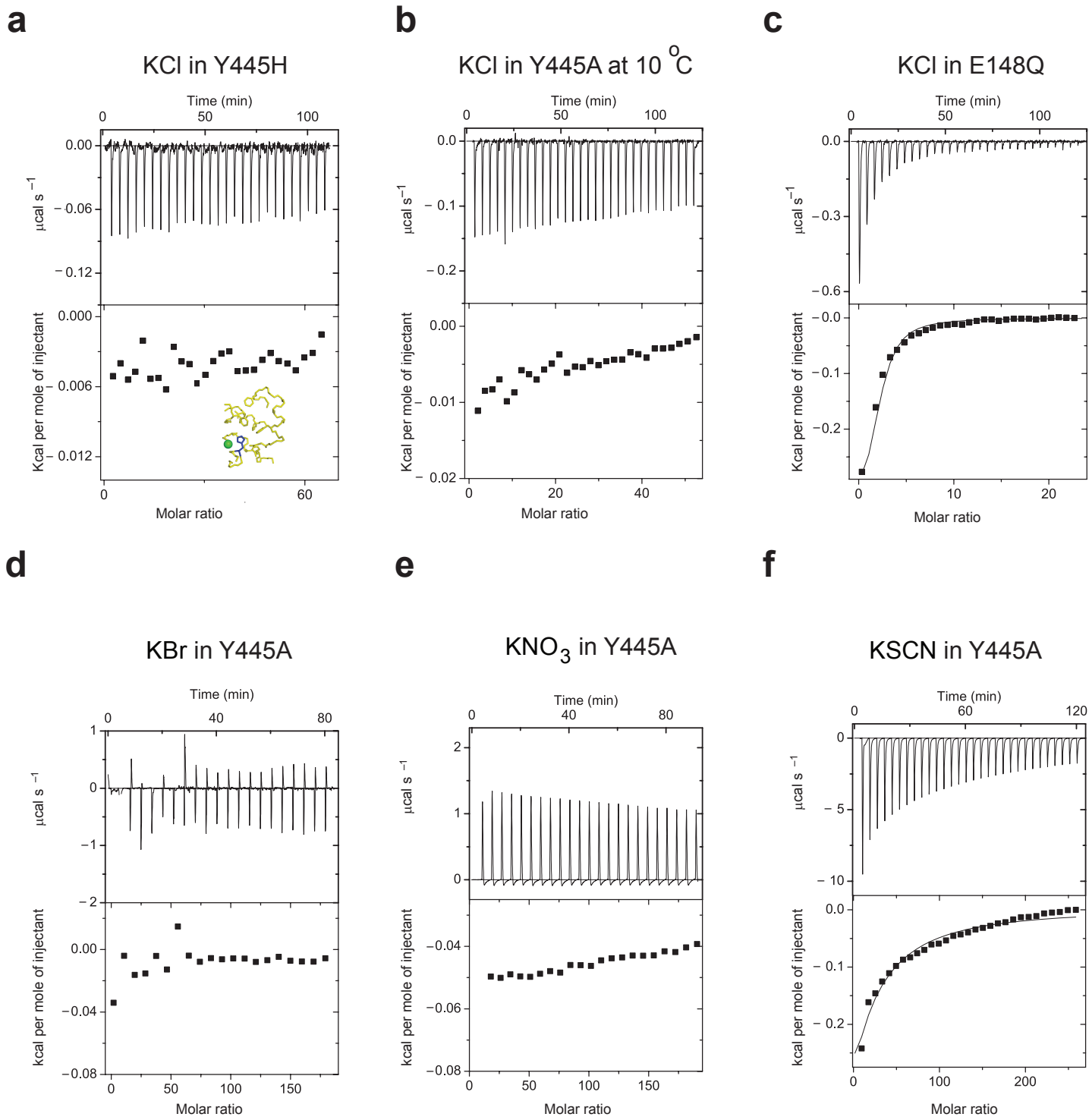
Supplementary Table 1 Thermodynamic parameters of individual experiments measuring anion binding to WT and mutant CLC-ec1. K_d and ΔH^o were obtained from a fit to a single binding isotherm, while ΔG^o and $T\Delta S^o$ were calculated from $\Delta G^o = RT \ln K_d$ and $T\Delta S^o = \Delta H^o - \Delta G^o$. The averages are shown in bold.

Supplementary Figure 1



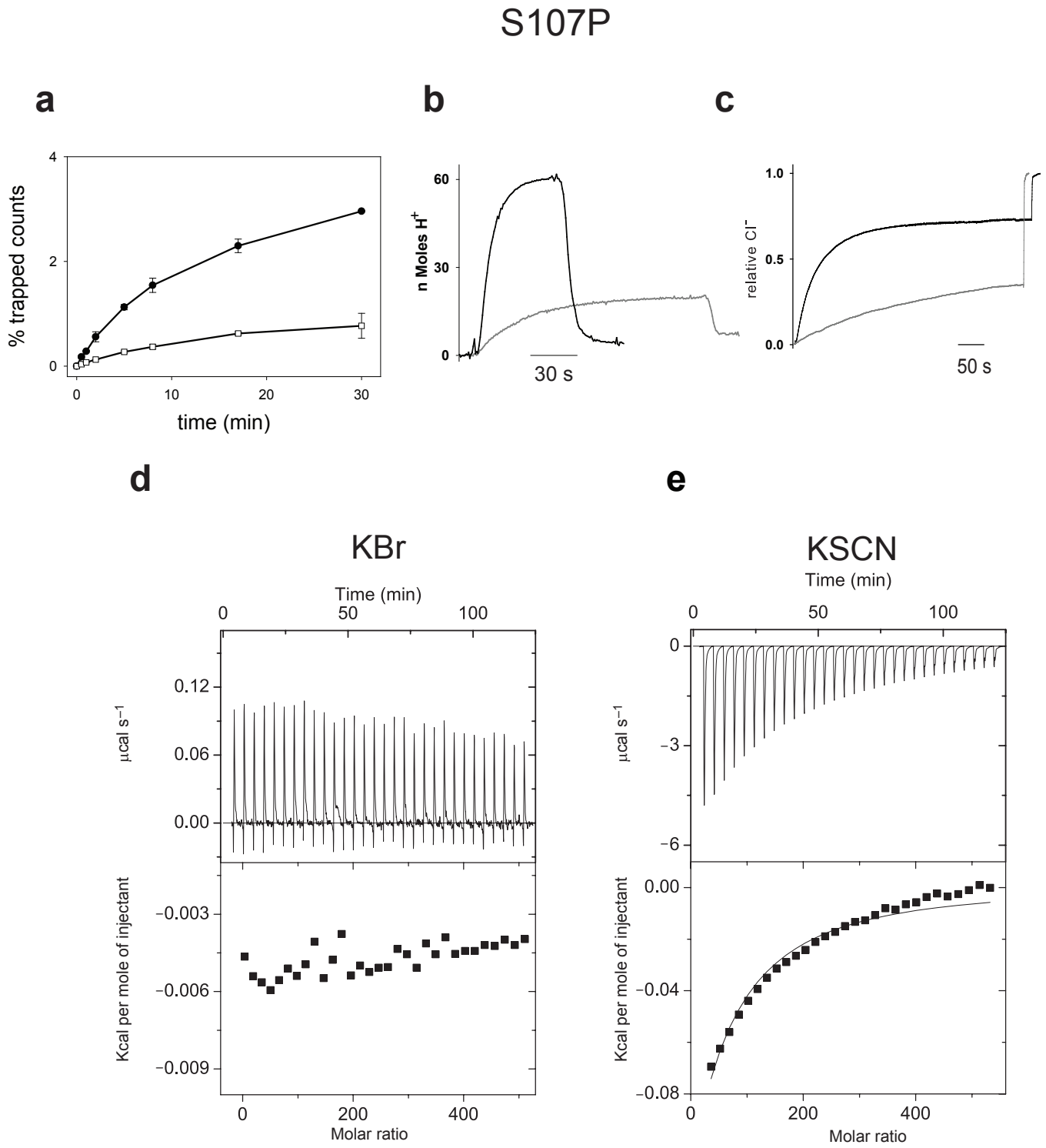
Supplementary Figure 1 pH dependence of Cl^- transport and binding. a) Cl^- transport rates at pH 7.5 (white) and pH 4.5 (black) for WT CLC-ec1, Y445A, E148A/Y445A and E148A. b) Cl^- binding to WT CLC-ec1 at pH 4.5. Top panel: heat liberated when KCl is titrated into the experimental chamber containing buffer and protein. The concentration of the KCl stock used was 25 mM. Each downward deflection corresponds to one injection. The area underneath each deflection n is integrated and represents the total heat exchanged and is shown in the bottom panel (squares). Solid line represents the fit to a single-site binding isotherm. The averaged thermodynamic parameters are reported in Supplementary Table 1.

Supplementary Figure 2



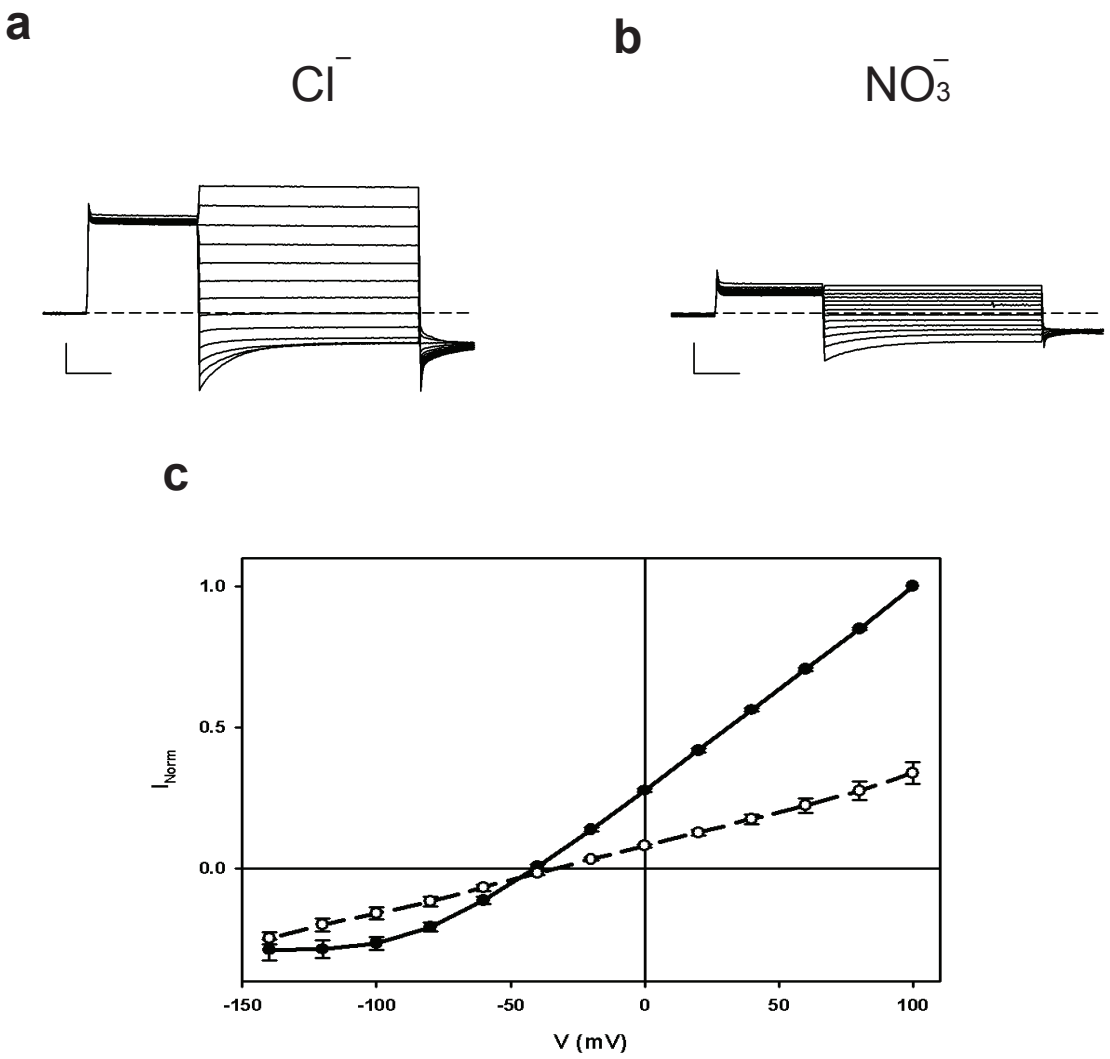
Supplementary Figure 2 Anion binding to CLC-ec1 mutants. Top panel: heat liberated when salt is titrated into the experimental chamber containing buffer and protein. The concentration of the KCl stock used was 25 mM for the Y445A and Y445H mutants and 15-20 mM for the E148Q mutant. 100 mM stock solution of KBr, KNO_3 and KSCN were used for the Y445A mutant. Each downward deflection corresponds to one injection. The area underneath each deflection is integrated and represents the total heat exchanged and is shown in the bottom panel (squares). (a) Cl^- binding to the Y445H mutant. (b) Cl^- binding to the Y445A mutant at 10 °C. (c) Cl^- binding to the E148Q CLC-ec1 mutant. The solid line in the bottom panel is the fit to a binding isotherm with 2 identical and independent sites. The averaged thermodynamic parameters are reported in Table 1. (d-f) Binding selectivity of the Y445A mutant. (d) KBr, (e) KNO_3 and (f) KSCN Solid lines are fits to a 1-site binding isotherm. The averaged thermodynamic parameters are reported in Table 2.

Supplementary Figure 3



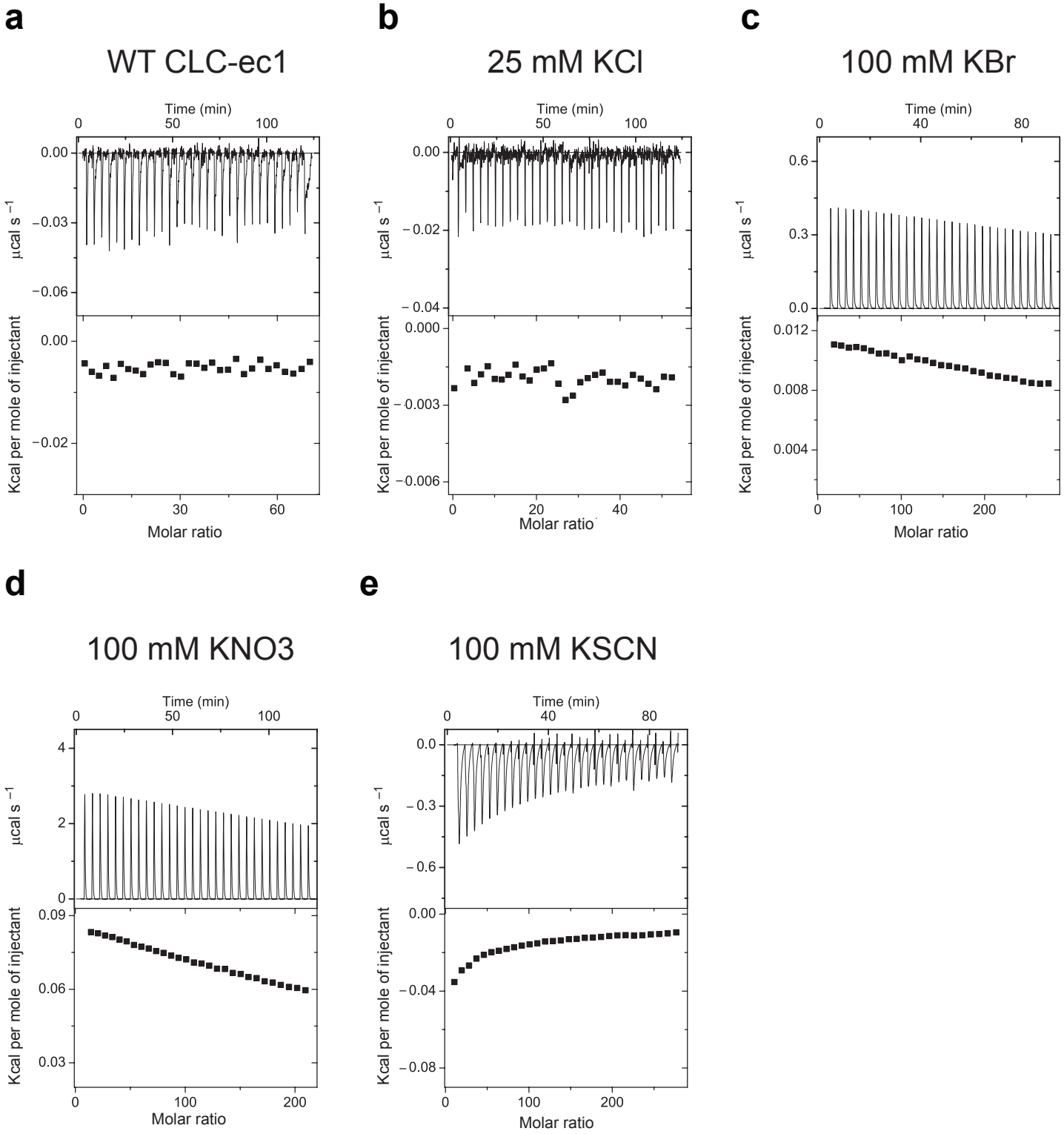
Supplementary Figure 3 Functional properties of the S107P mutant. a) $^{36}Cl^-$ uptake mediated by the S107P mutant at pH 7 (empty squares) and 4.5 (filled circles). Solid lines hold no theoretical meaning. b) Cl^--driven H^+ uptake and (c) Time course of Cl^- efflux mediated by WT CLC-ec1 (black line) and S107P mutant (grey line) d-e) Br^- and SCN^- binding to the S107P mutant. Panels as in Figure 2. 100 mM KBr and 75-150 mM KSCN stock solutions were used in these experiments. Solid line in the bottom panel represents the best fit to a single-site binding isotherm.

Supplementary Figure 4



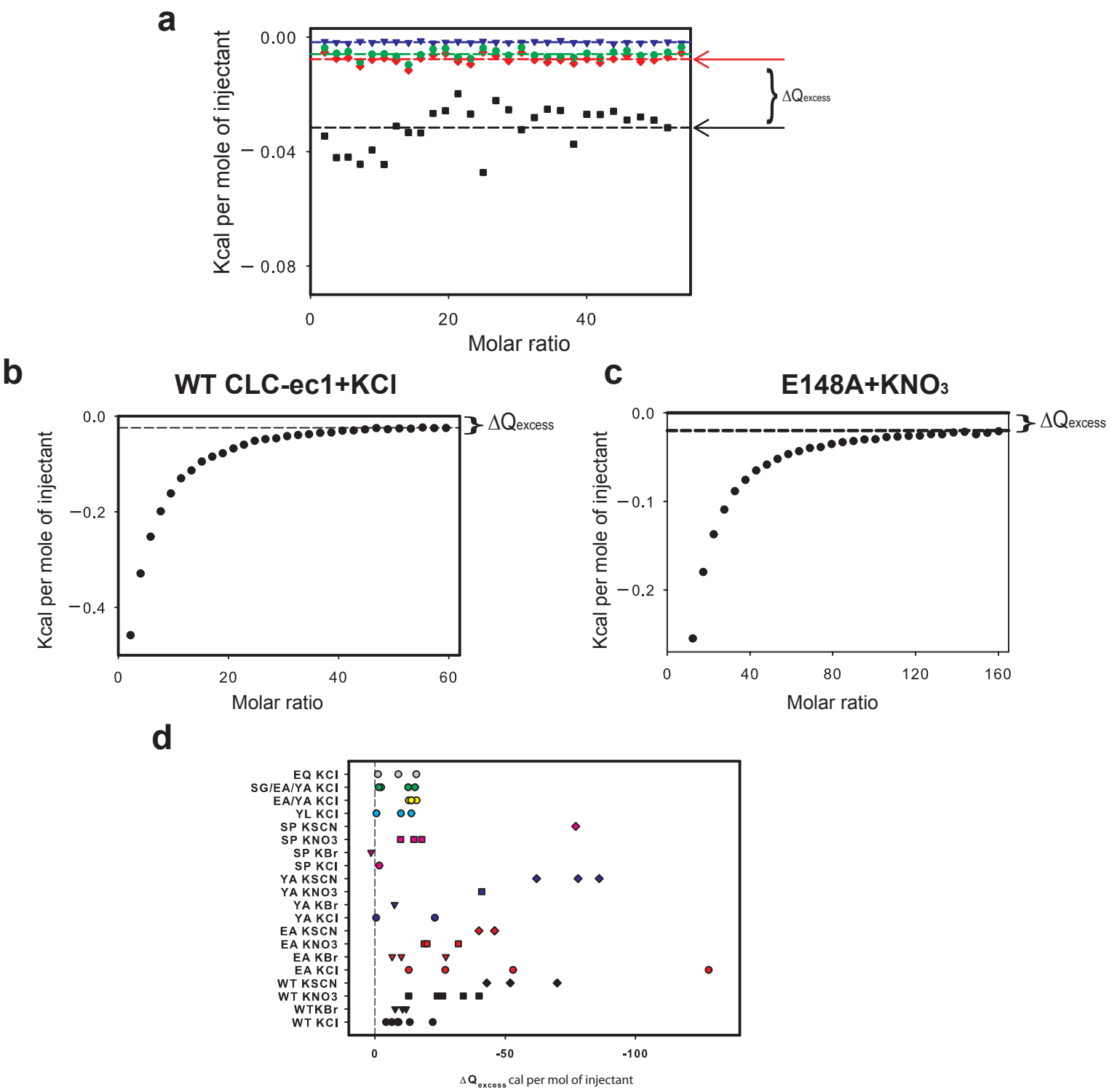
Supplementary Figure 4 Selectivity of the CLC-0 channel. a) ionic currents mediated by WT CLC-0 in 100 mM $[Cl^-]_{ex}$ and (b) 100 mM $[NO_3^-]_{ex}$. c) Normalized steady state current-voltage relationship in Cl^- (filled circles) and NO_3^- (empty circles). Each point represents the average of 3-7 independent oocytes.

Supplementary Figure 5



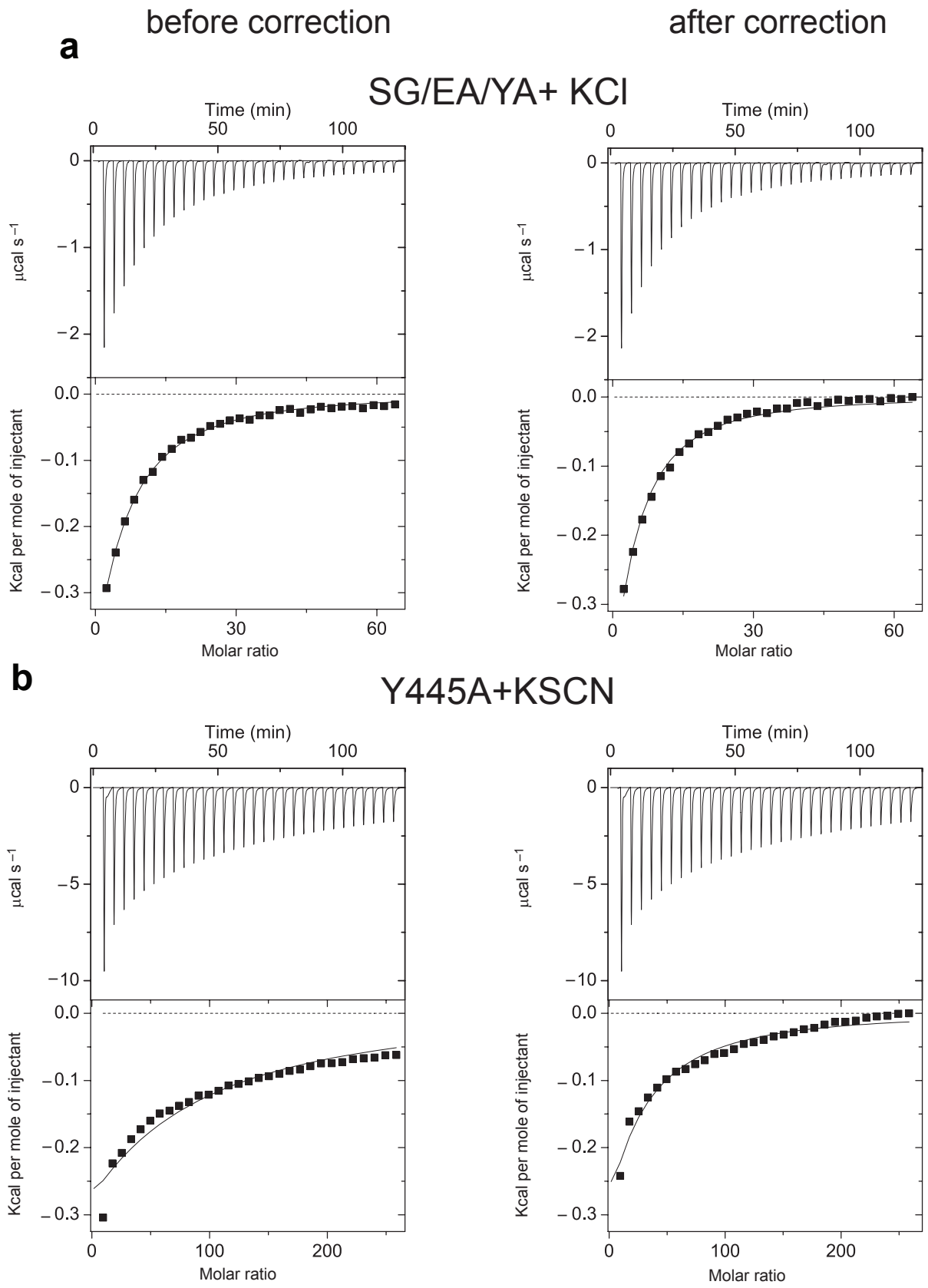
Supplementary Figure 5 Dilution heats of CLC-ec1 into buffer and of salt into buffer. Top panels: heat liberated during the injections. Bottom panels: the total heat released during each injection (squares) is calculated by integrating the area underneath each deflection. a) heat liberated when buffer is injected into the experimental chamber containing 73 μM WT CLC-ec1. b-e) Heat released when 25 mM KCl (b), 100 mM KBr (c), 100 mM KNO_3 (d) or 100 mM KSCN (e) is diluted into buffer. In all cases the injection size is 9 μl. For representation purposes the heats in b-e are plotted assuming that the protein concentration in the chamber is 100 μM.

Supplementary Figure 6



Supplementary Figure 6 Presence of an excess heat. a) Comparison of the heat resulting from dilution of 25 mM KCl into buffer (blue triangles), buffer into a 90 μM solution of the Y445A mutant (green circles), and injections of 25 mM KCl into a 94 μM solution of the Y445A protein (black squares). The sum of the separate heats of dilution (red diamonds) does not account for the total heat observed when salt is injected into protein (black squares). The colored dashed lines represent the average value of the heats in each condition. The heat released when KCl binds to WT CLC-ec1 (b) or when KNO_3 binds to the E148A mutant (c) does not plateau at 0. The dashed lines represent the excess heat, ΔQ_{excess}. (d) ΔQ_{excess} from the individual experiments for all salts and protein variants tested. Circles: KCl; Triangles: KBr; Squares: KNO_3; Diamonds: KSCN. Black: WT CLC-ec1; Red: E148A; Blue: Y445A; Magenta: S107P; Cyan: Y445L; Yellow: E148A/Y445A; Green: S107G/E148A/Y445A; Grey: E148Q. The vertical dashed line is drawn at ΔQ_{excess}=0.

Supplementary Figure 7



Supplementary Figure 7 Correction of the thermodynamic parameters due to the subtraction of ΔQ_{excess}. a) Cl^- binding to the S107G/E148A/Y445A mutant. Right panel: the total heat liberated is fit to a single site binding isotherm without subtracting ΔQ_{excess} with K_d(before)= 1170 µM, ΔH(before)=–5.33 Kcal Mol^{-1}, $T\Delta S$ (before)=–1.33 Kcal Mol^{-1}. Left panel: the total heat liberated is fit to a single site binding isotherm after subtracting ΔQ_{excess}=–15.4 cal Mol^{-1} with K_d(after)= 898 µM, ΔH(after)=–4.18 Kcal Mol^{-1}, $T\Delta S$ (after)=–0.01 Kcal Mol^{-1}. b) SCN^- binding to the Y445A mutant. Right panel: the total heat liberated is fit to a single site binding isotherm without subtracting ΔQ_{excess} with K_d(before)= 20 mM, ΔH(before)= –64 Kcal Mol^{-1}, $T\Delta S$ (before)=–62.3 Kcal Mol^{-1}. Left panel: the total heat liberated is fit to a single site binding isotherm after subtracting ΔQ_{excess}= –62 cal Mol^{-1} with K_d(after)= 6.3 mM, ΔH(after)=–20 Kcal Mol^{-1}, $T\Delta S$ (after)=–17.4 Kcal Mol^{-1}. The dashed lines are drawn at 0 Kcal Mol^{-1} of injectant.