SUPPLEMENTARY DATA

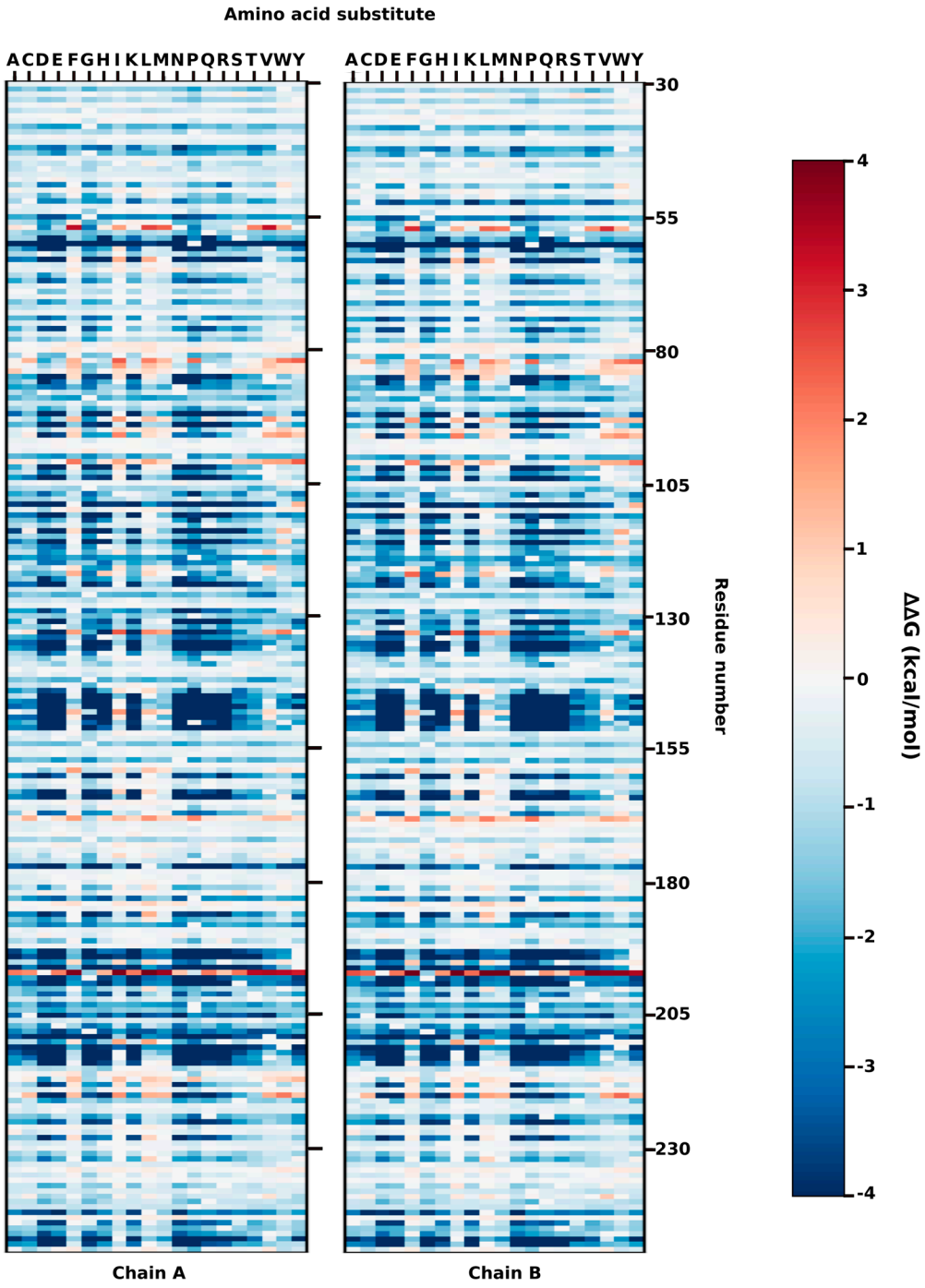
Supplementary Table S1. Protein sequence of *Nitratiruptor tergarcus* carbonic anhydrase *(*NtCA) and primers used for recombinant CA construction. In italics are the residues making up the signal peptide which were removed during cloning. Residues in bold and underline are mutations.

Gene	Protein sequence	Forward Primer (5'-3')	Reverse Primer (3'-5')
NtCA	*MKKVFVIAGILTGLLFAGVSGEGHH*AEHKHWGYSGSEGPKHWGEIDPKFRMCKFGVNQSPVDMTRFIEAKLPKLQIIYAGISQNVVNNGHTIKVTTQGKNEVVVDGIPFVLLQYHFHTPSENTINGKHFPMEAHFVHKSKDGEYLVIALMFKEGKKNRALEKVLTYLDPKVGHKQPLKEMFNPGDFFPKKLDYYRYDGSFTTPPCTEGVRWIVLKNAVEASKEQIAKMHKIMGSNNRPTQPLHARVILK	TAAGAAGGAGATATACCATGGCGGAGCATAAACATTGGGG	CAGTGGTGGTGGTGGTGGTGCTTTAATATCACGCGGGCGT
Q1 (A159F, D168P, D197L, R210L)	AEHKHWGYSGSEGPKHWGEIDPKFRMCKFGVNQSPVDMTRFIEAKLPKLQIIYAGISQNVVNNGHTIKVTTQGKNEVVVDGIPFVLLQYHFHTPSENTINGKHFPMEAHFVHKSKDGEYLVIALMFKEGKKNR**F**LEKVLTYL**P**PKVGHKQPLKEMFNPGDFFPKKLDYYRY**L**GSFTTPPCTEGV**L**WIVLKNAVEASKEQIAKMHKIMGSNNRPTQPLHARVILK	TAAGAAGGAGATATACCATGGCGGAGCATAAACATTGGGG	CAGTGGTGGTGGTGGTGGTGCTTTAATATCACGCGGGCGT

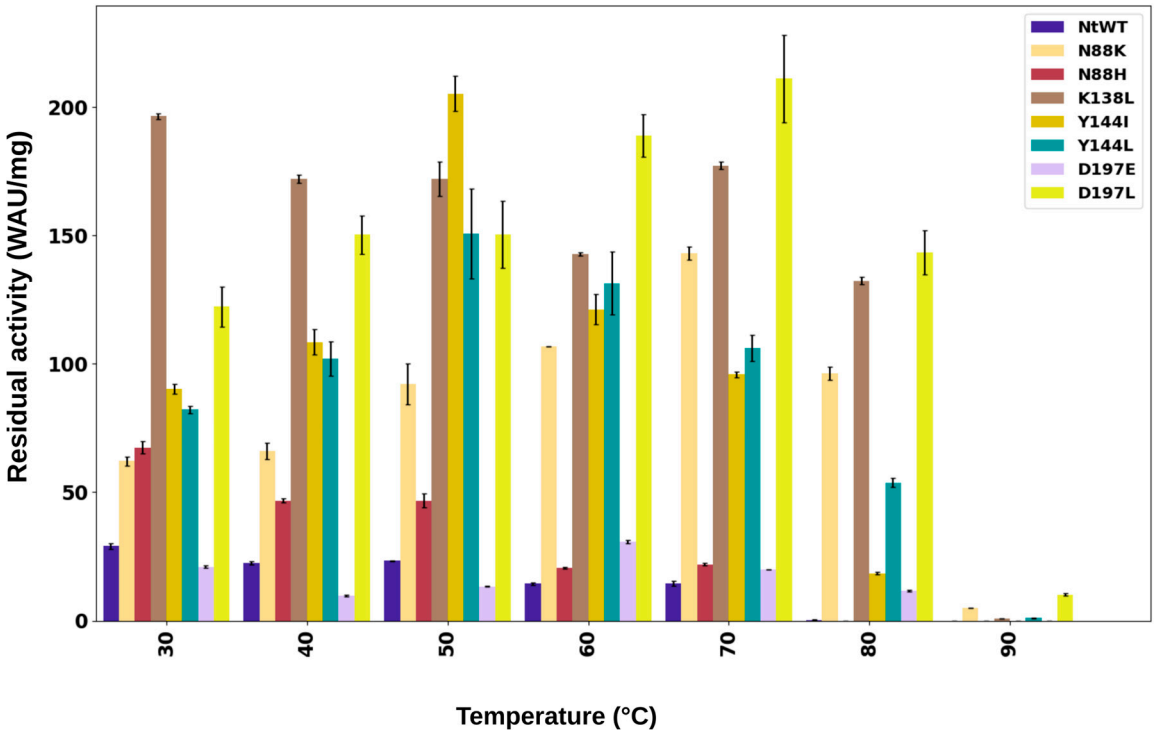
Supplementary Table S2. Sequence of primers used for NtCA site directed mutation. In bold and underlined are the codons of the mutant residue.

Mutant	Template	Forward Primer 2 (5'-3')	Reverse Primer 2 (3'-5')
N88K	NtCA	AAAATGTTGTAAAC**AAA**GGCCACACTATCAAAGTAACG	ACTTTGATAGTGTGGCC**TTT**GTTTACAACATTTTGCGATA
N88H	NtCA	AAAATGTTGTAAAC**CAT**GGCCACACTATCAAAGTAAC	TTGATAGTGTGGCC**ATG**GTTTACAACATTTTGCGAT
K138L	NtCA	TCATTTTGTTCAT**CTG**AGCAAAGACGGTGAGTATCTG	CACCGTCTTTGCT**CAG**ATGAACAAAATGAGCTTCCAT
Y144I	NtCA	GCAAAGACGGTGAG**ATT**CTGGTAATTGCCTTAATGT	AGGCAATTACCAG**AAT**CTCACCGTCTTTGCTCTTATG
Y144L	NtCA	GCAAAGACGGTGAG**CTG**CTGGTAATTGCCTTAATGTTT	AGGCAATTACCAG**CAG**CTCACCGTCTTTGCTCTTA
A159D	NtCA	GAAAGAAGAATCGC**GAC**TTAGAGAAGGTTTTAACT	AAAACCTTCTCTAA**GTC**GCGATTCTTCTTTCCTTC
A159F	NtCA	GGAAAGAAGAATCGC**TTT**TTAGAGAAGGTTTTAACTTAT	GTTAAAACCTTCTCTAA**AAA**GCGATTCTTCTTTCCTTCT

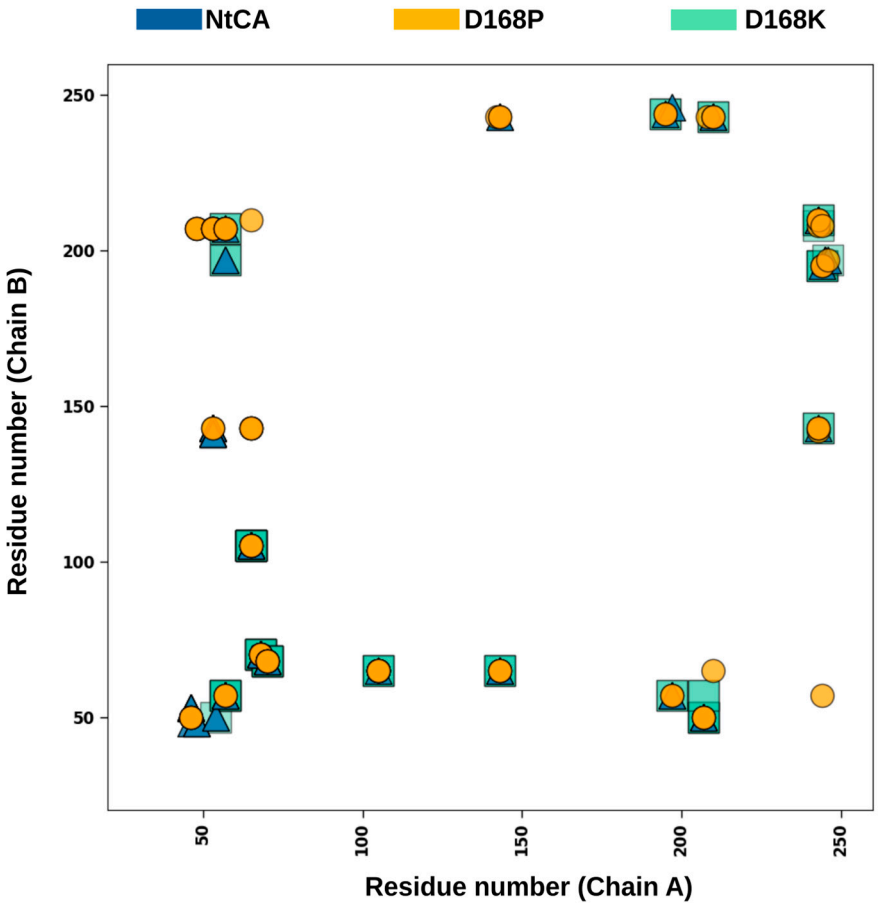
D168P	NtCA	GTTTTAACTTATCTT**CCG**CCAAA AGTAGGGCATAAGC	GCCCTACTTTTGG**CGG**AAGATAA GTTAAAACCTTCTC
D168K	NtCA	TTTAACTTATCTT**AAA**CCAAAA GTAGGGCATAAGCAA	TGCCCTACTTTTGG**TTT**AAGATAA GTTAAAACCTTC
D197L	NtCA	ATTACTACCGGTAC**CTG**GGTTC GTTCACCACCCC	TGGTGAACGAACC**CAG**GTACCGG TAGTAATCCAG
D197E	NtCA	TACTACCGGTAC**GAA**GGTTCGT TCACCACCCCCC	GGTGGTGAACGAACC**TTC**GTACC GGTAGTAATCCAG
R210L	NtCA	GCACCGAAGGGGTC**CTT**TGGA TAGTTTTAAAGAAT	TTAAAACTATCCA**AAG**GACCCCTT CGGTGCAAGGG
R210K	NtCA	CACCGAAGGGGTC**AAA**TGGAT AGTTTTAAAGAATGCTG	CTTTAAAACTATCCA**TTT**GACCCC TTCGGTGCAAGGGG
A159F, R210L	A159F	GCACCGAAGGGGTC**CTT**TGGA TAGTTTTAAAGAAT	TTAAAACTATCCA**AAG**GACCCCTT CGGTGCAAGGG
A159F, R210K	A159F	CACCGAAGGGGTC**AAA**TGGAT AGTTTTAAAGAATGCTG	CTTTAAAACTATCCA**TTT**GACCCC TTCGGTGCAAGGGG
N88K, R210L	N88K	GCACCGAAGGGGTCCTTTGGA TAGTTTTAAAGAAT	TTAAAACTATCCAAAGGACCCCTT CGGTGCAAGGG
N88K, R210K	N88K	CACCGAAGGGGTC**AAA**TGGAT AGTTTTAAAGAATGCTG	CTTTAAAACTATCCA**TTT**GACCCC TTCGGTGCAAGGGG
D168K, R210L	D168K	GCACCGAAGGGGTCCTTTGGA TAGTTTTAAAGAAT	TTAAAACTATCCAAAGGACCCCTT CGGTGCAAGGG
D168K, R210K	D168K	CACCGAAGGGGTC**AAA**TGGAT AGTTTTAAAGAATGCTG	CTTTAAAACTATCCA**TTT**GACCCC TTCGGTGCAAGGGG
Q2 (A159F, D168P, D197E, R210L)	Q1	TACTACCGGTAC**GAA**GGTTCGT TCACCACCCCCC	GGTGGTGAACGAACC**TTC**GTACC GGTAGTAATCCAG
Q3 (A159F, D168P, D197L, R210K)	Q1	CACCGAAGGGGTC**AAA**TGGAT AGTTTTAAAGAATGCTG	CTTTAAAACTATCCA**TTT**GACCCC TTCGGTGCAAGGGG
Q4 (A159F, D168P, D197E, R210K)	Q2	CACCGAAGGGGTC**AAA**TGGAT AGTTTTAAAGAATGCTG	CTTTAAAACTATCCA**TTT**GACCCC TTCGGTGCAAGGGG



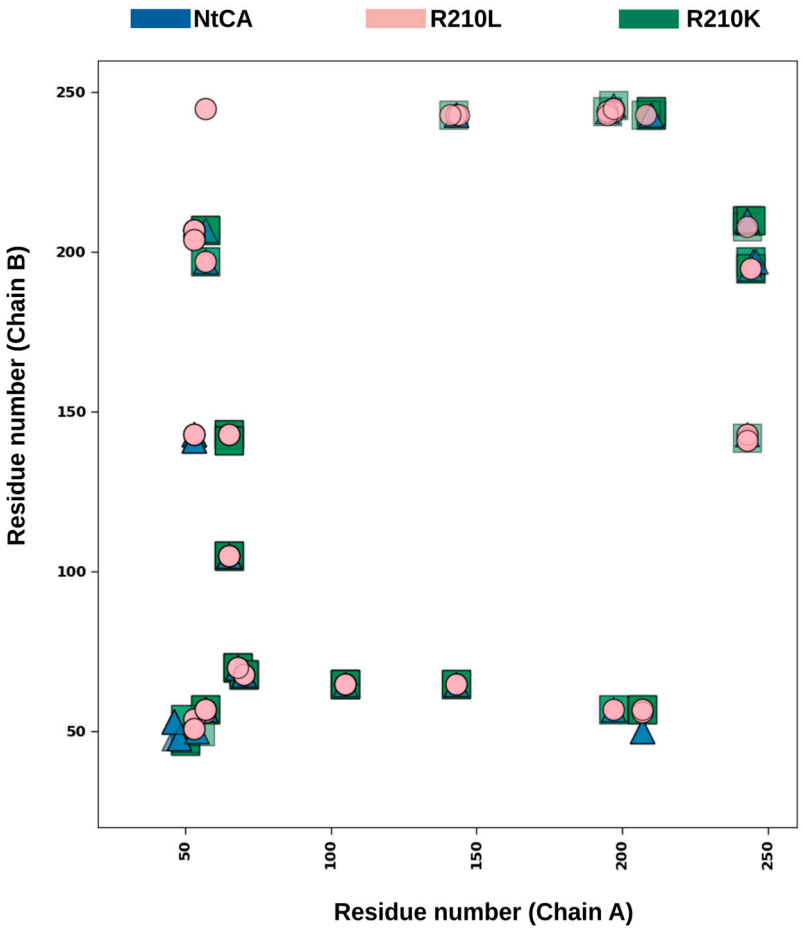
Supplementary Figure S1. Heatmap of ΔΔG values of all possible single mutations in chains A and B of NtCA. Values more than 0 suggest a more stable variant and values less than 0 suggest a less stable variant.



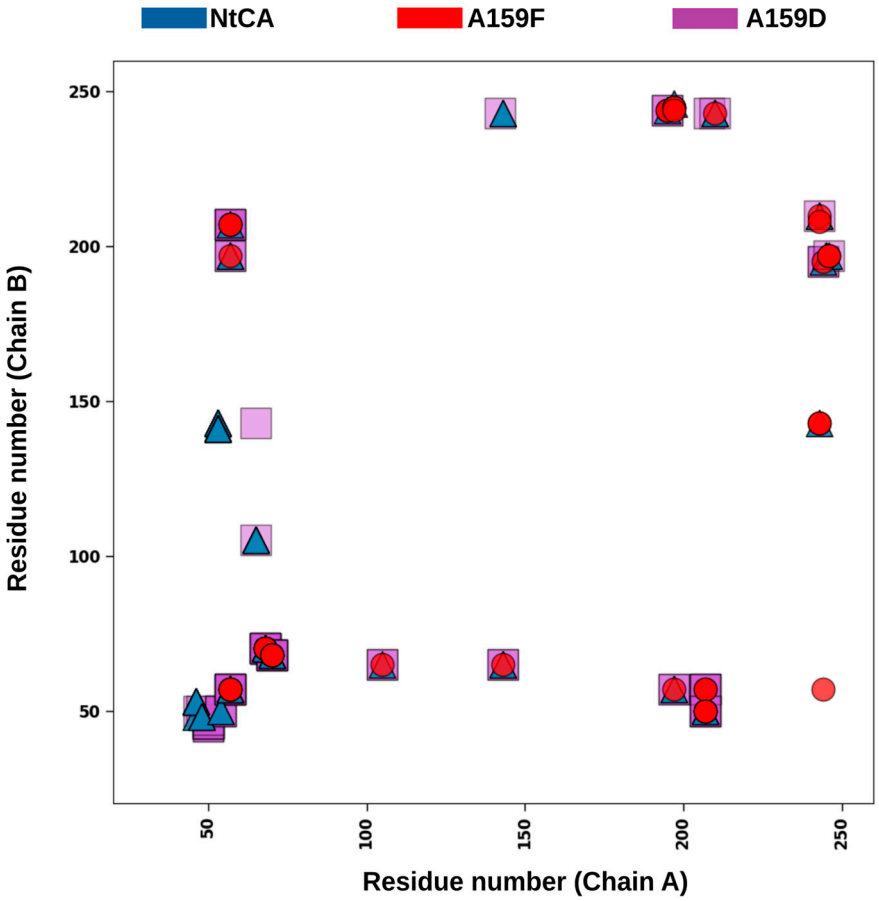
Supplementary Figure S2. Residual activity of NtCA and single mutants after incubation for 1 hr at 30 °C – 90 °C.



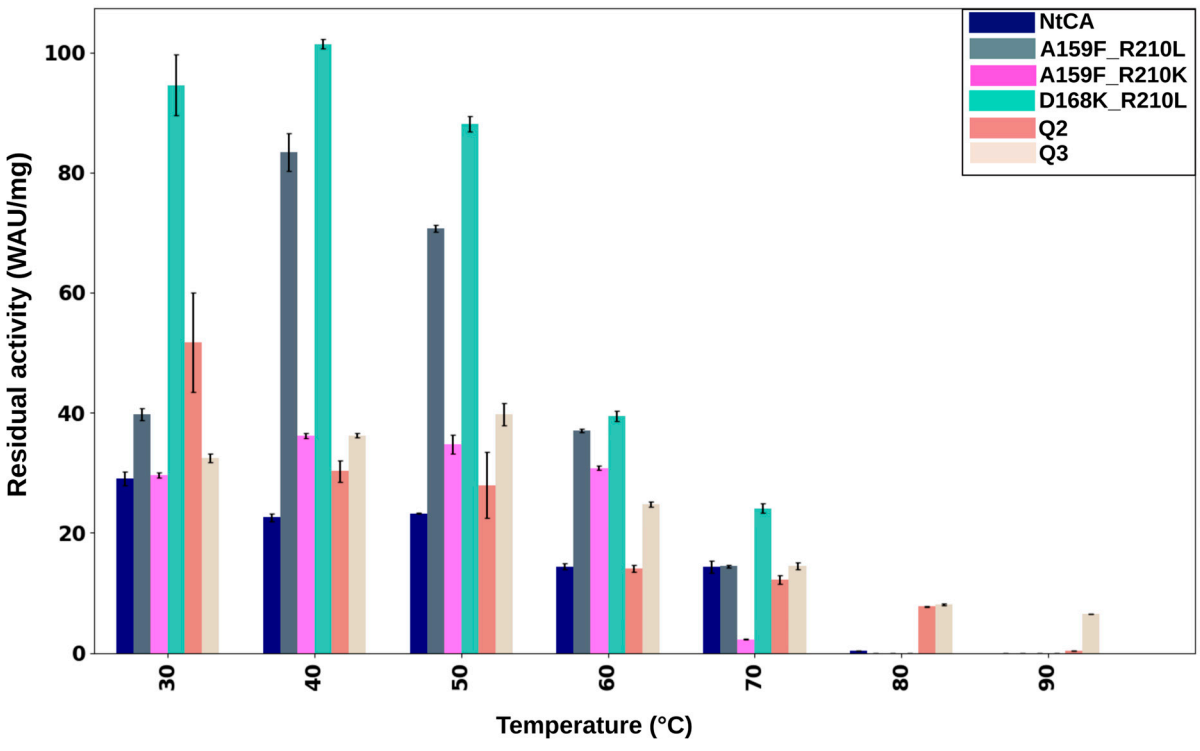
Supplementary Figure S3. Interface hydrogen bond scatter plot showing bonds formed between chain A and chain B of NtCA (blue triangles), D168P (orange circles) and D168K (light green squares).



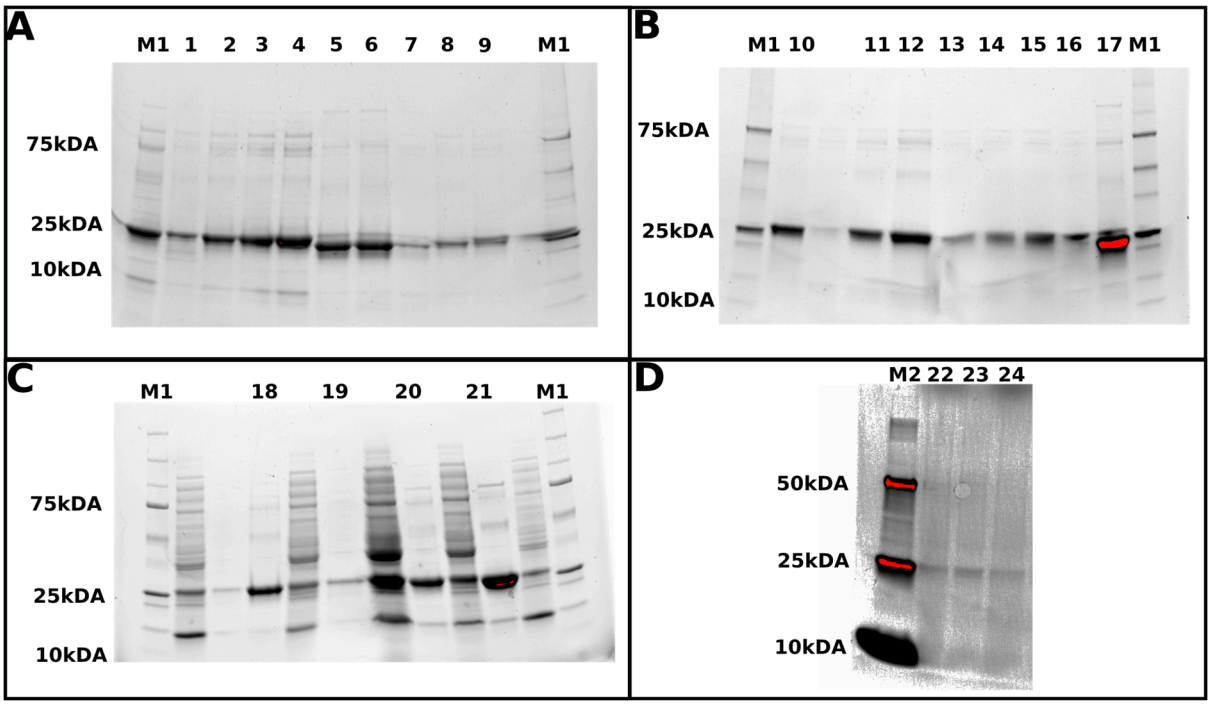
Supplementary Figure S4. Interface hydrogen bond scatter plot showing bonds formed between chain A and chain B of NtCA (blue triangles), R210L (pink circles) and R210K (green squares).



Supplementary Figure S5. Interface hydrogen bond scatter plot showing bonds formed between chain A and chain B of NtCA (blue triangles), A159F (purple circles) and A159D (purple squares).



Supplementary Figure S6. Residual activity of NtCA and combinations mutants after incubation for 1 hr at 30 – 90 °C.



Supplementary Figure S7. **SDS PAGE gels for partially purified CAs (approx. 25.4 kDA). A. Wells showing M1 - Marker proteins, 1 - NtCA, 2 – N88K, 3 - N88H, 4 - K138L, 5 - Y144I, 6 - Y144L, 7 - A159D, 8 - A159F, 9 - D168P B. Wells showing M1 – Marker proteins, 10 – D168K, 11 – D197L, 12 – D197E, 13 – R210L, 14 – R210K, 15 – A159F_R210L, 16 – A159F_R210K, 17 – N88K_R210L. C. Wells showing M1 - Marker proteins, 18 – N88K_R210K 19 – D168K_R210L, 20 - D168K, R210K, 21 – Q1. D. M2 - Marker proteins, 22 – Q2, 23 – Q3 and 24 – Q4. M1 is the Precision Plus Protein™ Unstained Protein Standards, Strep-tagged from Bio-Rad and M2 is the Unstained Protein Standard, Broad Range (10-200 kDa) from New England Biolabs, Inc. Lanes in between numbered lanes in C have crude fractions before purification.**