Supplemental Material to:

Stepwise adaptations to low temperatures as revealed by multiple mutants of a psychrophilic α-amylase from an Antarctic bacterium

Alexandre Cipolla[‡], Salvino D'Amico[‡], Roya Barumandzadeh[§],

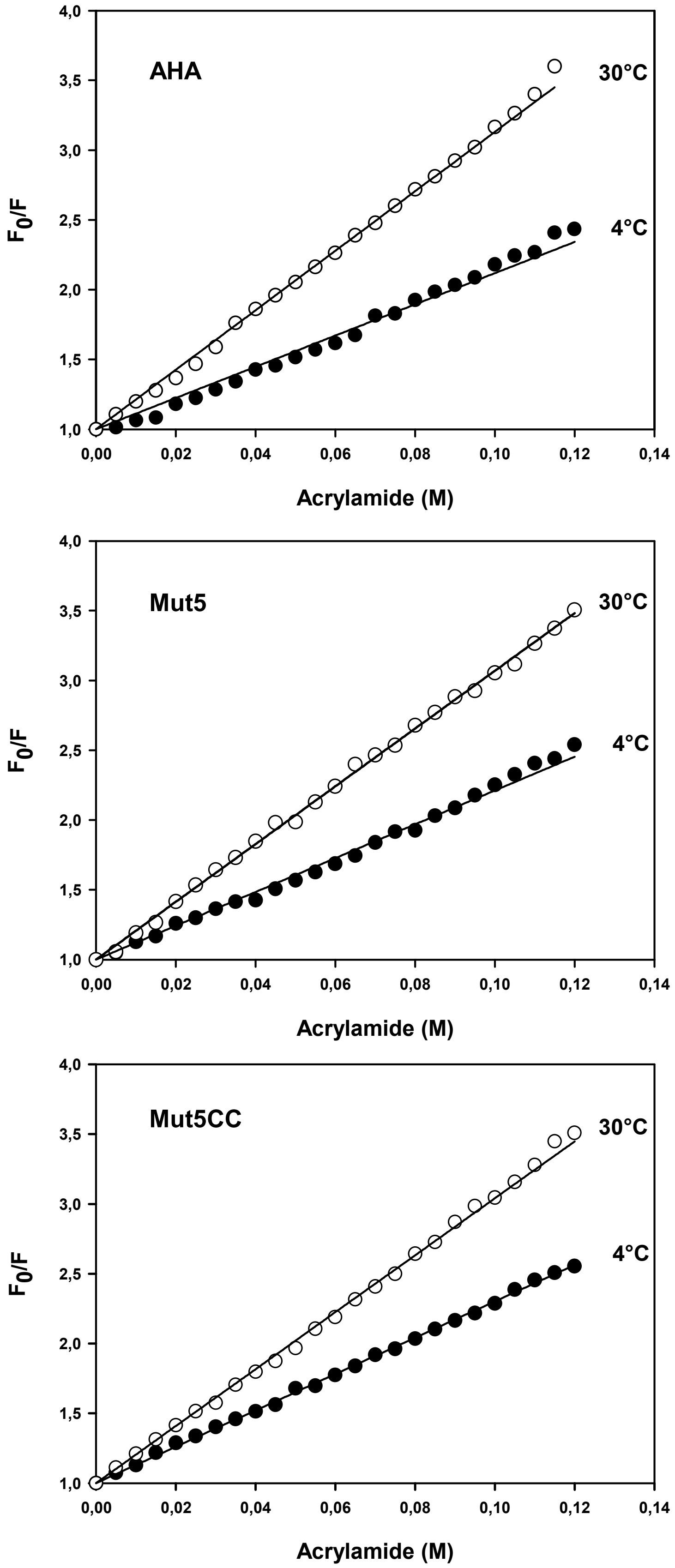
André Matagne[§] and Georges Feller[‡1]

From the [‡]Laboratory of Biochemistry and the [§]Laboratory of Enzymology and Protein Folding, Centre for Protein Engineering, University of Liège, B-4000 Liège-Sart Tilman, Belgium

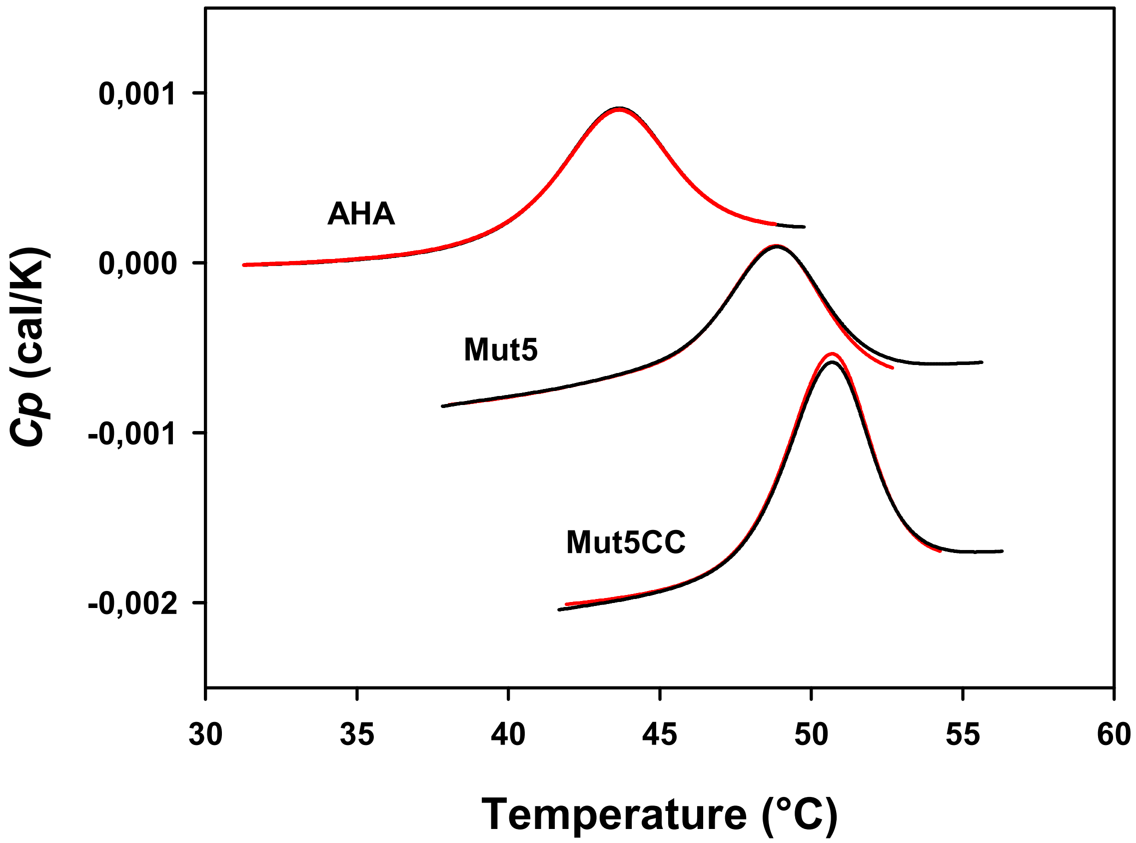
Contents

```
AHA             --------TPTTFVHLFEWNWQDVAQECEQYLGPKGYAAVQVSPPNEHITGS----QWWT  48
Mut5            --------TPTTFVHLFEWNWQDVAQECEQYLGPKGYAAVQVSPPNEHITGS----QWWT  48
Mut5CC          --------TPTTFVHLFEWNWQDVAQECEQYLGPKGYAAVQVSPPNEHITGS----QWWT  48
PPA             QYAPQTQSGRTSIVHLFEWRWVDIALECERYLGPKGFGGVQVSPPNENIVVTNPSRPWWE  60

AHA             RYQPVSYELQSRGGNRAQFIDMVNRCSAAGVDIYVDTLINHMA---AGSGTGT-AGNSFG 104
Mut5            RYQPVSYELQSRGGNRAQFIDMVNRCSAAGVDIYVDTLINHMA---AGSGTGT-AGNSFG 104
Mut5CC          RYQPVSYELCSRGGNRAQFIDMVNRCSAAGVDIYVDTLINHMA---AGSGTGT-CGNSFG 104
PPA             RYQPVSYKLCTRSGNENEFRDMVTRCNNVGVRIYVDAVINHMCGSGAAAGTGTTCG-SYC 119

AHA             N---KSFP--IYSPQDFHES-CTINNS--DYGNDRYRVQNCELVGLADLDTASNYVQNTI 156
Mut5            N---KSFP--IYSPQDFHES-CTINNS--DYGNDRYRVQNCELVGLADLDTASDYVQNTI 156
Mut5CC          N---KSFP--IYSPQDFHES-CTINNS--DYGNDRYRVQNCELVGLADLDTASDYVQNTI 156
PPA             NPGNREFPAVPYSAWDFNDGKCKTASGGIESYNDPYQVRDCQLVGLLDLALEKDYVRSMI 179

AHA             AAYINDLQAIGVKGFRFDASKHVAASDIQSLMAKVN---------GS-PVVFQEVIDQGG 206
Mut5            AAYINDLIAIGVKGFRFDASKHVAASDIQSLMAKVN---------GS-PFVFQEVIDQGG 206
Mut5CC          AAYINDLIAIGVKGFRFDASKHVAASDIQSLMAKVN---------GS-PFVFQEVIDQGG 206
PPA             ADYLNKLIDIGVAGFRIDASKHMWPGDIKAVLDKLHNLNTNWFPAGSRPFIFQEVIDLGG 239

AHA             EAVGASEYLSTGLVTEFKYSTELGNTFRNGS---LAWLSNFGEGWGFMPSSSAVVFVDNH 263
Mut5            EAVGASEYLSTGLVTEFKYSTELGNVFRNGS---LAWLSNFGEGWGFMPSSSAVVFVDNH 263
Mut5CC          EAVGASEYLSTGLVTEFKYSTELGNVFRNGS---LAWLSNFGEGWGFMPSSSAVVFVDNH 263
PPA             EAIQSSEYFGNGRVTEFKYGAKLGTVVRKWSGEKMSYLKNWGEGWGFMPSDRALVFVDNH 299

AHA             DNQRGHGGAGN-VI-TFEDGRLYDLANVFMLAYPYGYPKVMSSYDF----HGDTDA---- 313
Mut5            DNQRGHGGAGN-VI-TFEDGRLYDLANVFMLAYPYGYPRVMSSYDF----HGDTDA---- 313
Mut5CC          DNQRGHGGAGN-VI-TFEDGRLYDLANVFMLAYPYGYPRVMSSYDF----HGDTDA---- 313
PPA             DNQRGHG-AGGASILTFWDARLYKVAVGFMLAHPYGFTRVMSSYRWARNFVNGQDVNDWI 358

AHA             GGPNVPVHN-----NGNLECFASNWKCEHRWSYIAGGVDFRNNTADNWAVTNWWDNTNNQ 368
Mut5            GGPNVPVHN-----NGNLECFASNWKCEHRWSYIAGGVDFRNNTADNWAVTNWWDNTNNQ 368
Mut5CC          GGPNVPVHN-----NGNLECFASNWKCEHRWSYIAGGVDFRNNTADNWAVTNWWDNTNNQ 368
PPA             GPPNNNGVIKEVTINADTTC-GNDWVCEHRWRQIRNMVWFRN-VVDGQPFANWWANGSNQ 416

AHA             ISFGRGSSGHMAINKEDSTLTATVQTDMASGQYCNVLKGELSADAKSCSGEVITVNSDGT 428
Mut5            ISFGRGSSGHMAINKEDSTLTATVQTDMASGQYCNVLKGELSADAKSCSGEVITVNSDGT 428
Mut5CC          ISFGRGSSGHMAINKEDSTLTATVQTDMASGQYCNVLKGELSADAKSCSGEVITVNSDGT 428
PPA             VAFGRGNRGFIVFNNDDWQLSSTLQTGLPGGTYCDVISGDKVG--NSCTGIKVYVSSDGT 474

AHA             INLNIG--AWD-AMAIHKNAKLNTSSAS 453
Mut5            INLNIG--AWD-AMAIHKNAKLNTSSAS 453
Mut5CC          INLNIG--AWD-AMAIHKNAKLNTSSAS 453
PPA             AQFSISNSAEDPFIAIHAESKL------ 496
```

Supplemental Figure S1: Sequence alignment of the psychrophilic AHA, its multiple mutants Mut5 and Mut5CC, and of the mesophilic PPA. The mutations engineered in Mut5 and in Mut5CC are shown in red. The 24 conserved residues forming the active site cleft are shown in blue.



Supplemental Figure S2: Stern-Volmer plots of fluorescence quenching by acrylamide. The quenching constant K_{SV} values corresponding to the plot slope are 11.7, 12.1 and 13.0 M^{-1} at 4°C and 21.3, 20.7 and 20.4 M^{-1} at 30°C for AHA, Mut5 and Mut5CC, respectively.



Supplemental Figure S3: Unfolding reversibility of the mutants in the presence of a nondetergent sulfobetaine. Thermograms were recorded in 30 mM Mops, 50 mM NaCl, 1 mM Ca Cl_2, 1 M 3-(1-pyridinio)-1-propanesulfonate, pH 7.2 at a scan-rate of 60 K h^{-1}. Red traces: first up-scans interrupted after completion of the unfolding transition. Black traces: second up-scans performed after sample cooling. Raw data have been displaced along the Y axis for clarity.

Supplemental TABLE S1

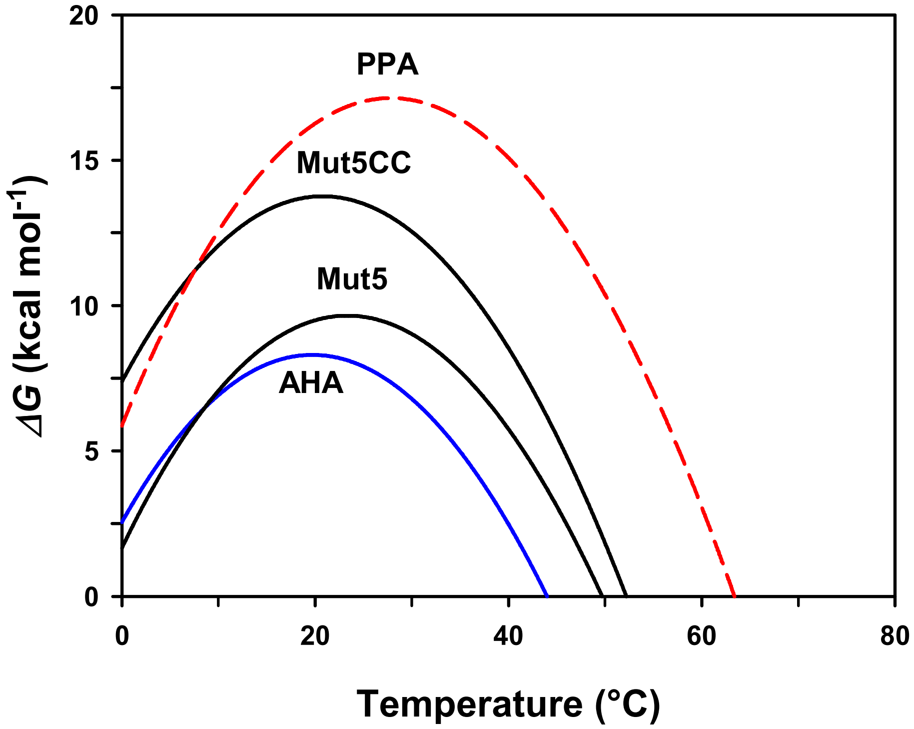
Thermodynamic parameters of unfolding derived from reversible DSC endotherms

	T_m °C	ΔH_{cal} kcal mol^{-1}	ΔH_{eff} kcal mol^{-1}	ΔH_{cal} / ΔH_{eff}	Reversibility %
AHA	44.0	214	203	1.05	>99
Mut5	49.7	233	204	1.14[1]	>98
Mut5CC	52.2	280	270	1.04	>98
PPA[2]	65.6	319	--[3]	--[3]	none

[1] despite a ratio close to 1, unfolding deviates from a two-state model as a result of an asymmetric transition

[2] data from (44)

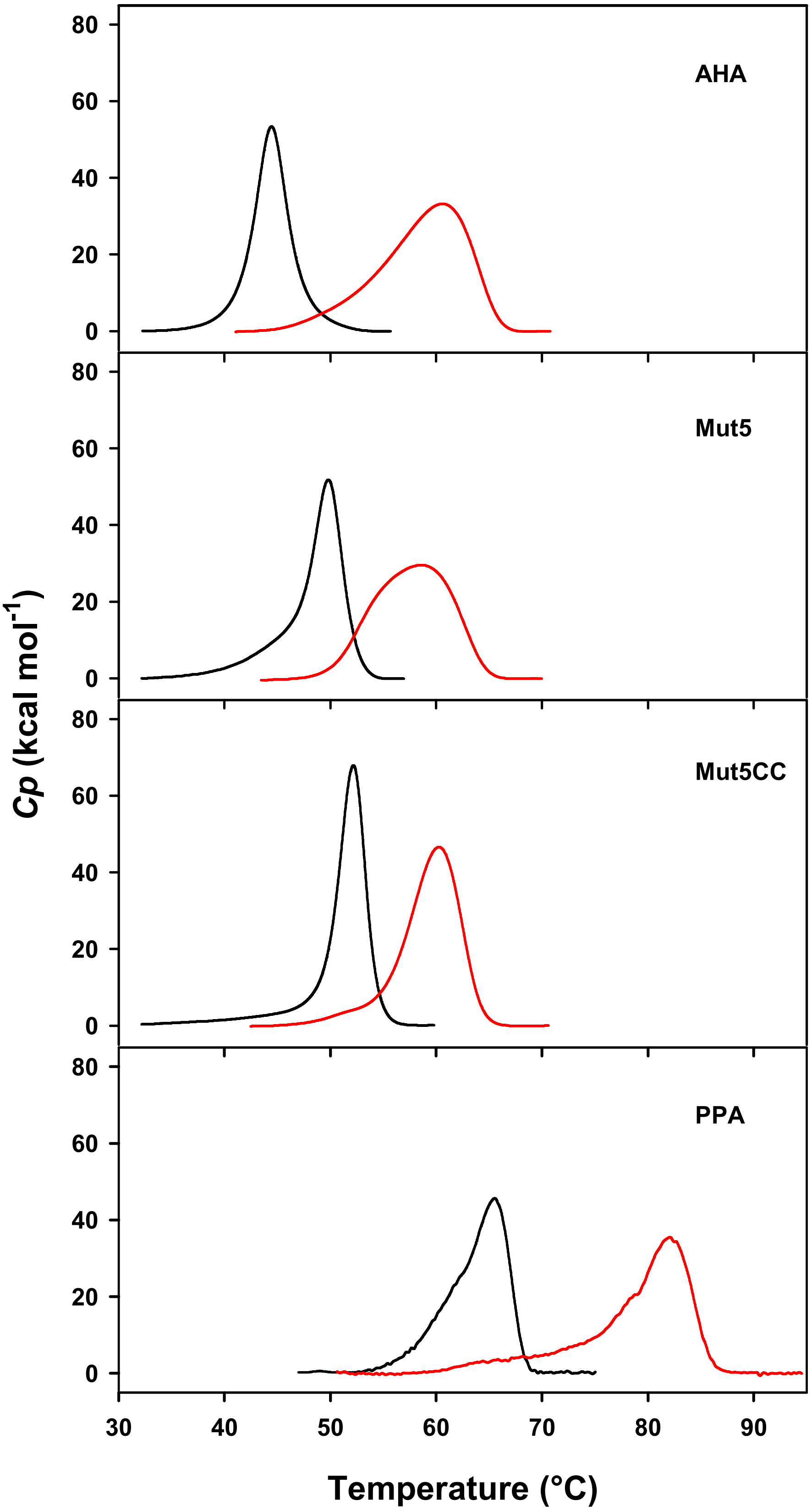
[3] not applicable, biphasic transition



Supplemental Figure S4: Stability curves calculated from DSC data. The Gibbs free energy of unfolding was calculated as described (22) by the relation:

$$\Delta G(T) = \Delta H_{cal}(1-T/T_m) + \Delta Cp(T-T_m) - T\Delta Cp \ln (T/T_m)$$

using ΔCp = 8.47 kcal mol^{-1} K^{-1} as determined experimentally for AHA (44).
Dashed, estimation of PPA stability from its irreversible unfolding parameters.



Supplemental Figure S5: Stability of α-amylase-acarbose complexes. DSC endotherms of α-amylases in the free state (black lines) and in complex with the transition state analog acarbose (red lines). T_{max} corresponds to the top of the transition and ΔH_{cal} to the surface below the transition (Table S2). Baseline subtracted data have been normalized for protein concentration.

Supplemental TABLE S2

Microcalorimetric parameters of thermal unfolding for α-amylases in the free state and in complex with the pseudosaccharide inhibitor acarbose.

	Free enzyme		Enzyme-acarbose complex			
	T_{max} °C	ΔH_{cal} kcal mol^{-1}	T_{max} °C	ΔH_{cal} kcal mol^{-1}	ΔT_{max} °C	$\Delta\Delta H_{cal}$ kcal mol^{-1}
AHA	44.0	214	60.6	329	16.6	115
Mut5	49.4	229	58.4	282	9.0	53
Mut5CC	51.8	277	60.1	293	8.3	16
PPA[a]	65.6	295	81.9	306	16.3	11

[a] data from (22)