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1 **The characterization of ancient *Methanococcales* malate dehydrogenases**
2 **reveals that strong thermal stability prevents unfolding under intense γ -**
3 **irradiation**

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15 **Abbreviations:**

16 Ancestral sequence reconstruction (ASR)

17 Bootstrap values (BV)

18 Correspondence analysis (CA)

19 Lactate dehydrogenase (LDH)

20 Malate dehydrogenase (MalDH)

21 Melting temperature ($T_{M1/2}$)

22 OGT of the 18 strains of *Methanococcales* estimated from their proteome sequences according to the
23 study by Lecocq et al. (2021) (estOGT_{CORE-PROT})

24 OGT of the 18 strains of *Methanococcales* estimated from the sequences of the MalDH according to
25 this study (estOGT_{MalDH})

26 Optimal growth temperatures (OGT)

27 Optimal temperature for activity ($A-T_{opt}$)

28 Oxaloacetate (OAA)

29 Wild type (Wt)

30

31 **Abstract**

32 Malate dehydrogenases (MalDH) (EC.1.1.1.37), which are involved in the conversion of oxaloacetate to
33 pyruvate in the tricarboxylic acid cycle, are a relevant model for the study of enzyme evolution and
34 adaptation. Likewise, a recent study showed that *Methanococcales*, a major lineage of *Archaea*, is a
35 good model to study the molecular processes of proteome thermoadaptation in prokaryotes. Here, we
36 use ancestral sequence reconstruction and paleoenzymology to characterize both ancient and extant
37 MalDHs. We observe a good correlation between inferred optimal growth temperatures (OGTs) and
38 experimental optimal temperatures for activity ($A-T_{opt}$). In particular, we show that the MalDH present in
39 the ancestor of *Methanococcales* was hyperthermostable and had an $A-T_{opt}$ of 80°C, consistent with a
40 hyperthermophilic lifestyle. This ancestor gave rise to two lineages with different thermal constraints,
41 one remaining hyperthermophilic while the other underwent several independent adaptations to colder
42 environments. Surprisingly, the enzymes of the first lineage have retained a thermoresistant behavior
43 (i.e., strong thermostability and high $A-T_{opt}$), whereas the ancestor of the second lineage shows a strong
44 thermostability, but a reduced $A-T_{opt}$. Using mutants, we mimic the adaptation trajectory towards
45 mesophily and show that it is possible to significantly reduce the $A-T_{opt}$ without altering the
46 thermostability of the enzyme by introducing a few mutations. Finally, we reveal an unexpected link
47 between thermostability and the ability to resist γ -irradiation-induced unfolding.

48

49 **Keywords**

50 **Ancestral sequence reconstruction, extremophiles, thermal adaptation, irradiation, reactive**
51 **oxygen species, COaLA evolutionary model.**

52

53 **Introduction**

54 On Earth, environmental factors such as salinity, pH, redox potential, hydrostatic pressure, temperature,
55 radiation, or desiccation, impose strong physical and chemical constraints on organisms, especially
56 microorganisms (Rothschild and Mancinelli 2001; Pikuta, et al. 2007; Merino, et al. 2019). Coping with
57 these constraints, which affect the structure, the dynamics and catalytic properties of all cellular
58 components, requires specific adaptations (Coker 2019; Ando, et al. 2021; Knop, et al. 2023). For
59 instance, it has been shown that the relative abundance of amino acids in proteins and proteomes is
60 strongly correlated with the optimal growth temperature (OGT) of prokaryotes (Zeldovich, et al. 2007;
61 Boussau, et al. 2008; Groussin and Gouy 2011; Lecocq, et al. 2021). However, other factors such as
62 the environmental salinity or the guanine and cytosine content of genomes (genomic GC content) can
63 also modulate the amino acid composition of proteomes (Dutta and Paul 2012; Vallina Estrada and
64 Oliveberg 2022; Lamolle, et al. 2023; Amangeldina, et al. 2024). Disentangling the relative contribution
65 of each factor is therefore challenging, as they may have antagonistic effects. As a result, the
66 understanding of the precise substitution processes involved in proteome thermoadaptation remains
67 elusive.

68 Paleoenzymology is a powerful tool for unravelling how amino acid substitutions have shaped enzyme
69 properties during evolution. Indeed, by coupling bioinformatic ancestral sequence reconstruction (ASR)
70 with laboratory enzyme resurrection, it is possible to experimentally characterize ancient enzymes from
71 extinct organisms (Zaucha and Heddle 2017; Selberg, et al. 2021; Schwartz, et al. 2022). This approach
72 has been used to address fundamental questions about evolution and to make inferences about
73 paleoenvironments (Gaucher, et al. 2008; Perez-Jimenez, et al. 2011; Risso, et al. 2013; Akanuma
74 2017; Garcia, et al. 2017; Nguyen, et al. 2017; Blanquart, et al. 2021). However, ASR suffers from
75 several biases (Williams, et al. 2006; Garcia and Kacar 2019). Among the many pitfalls, the most
76 common is the use of inaccurate mathematical models of evolution. In particular, most current models
77 are homogeneous, i.e., they assume that the substitution process is distributed homogeneously along
78 the phylogeny and across the sites of the sequences, an assumption that is often violated by the data
79 (Groussin, et al. 2013; Echave, et al. 2016; Naser-Khdour, et al. 2019; Del Amparo and Arenas 2022).
80 Furthermore, most ASR models treat gaps as missing/ambiguous data or ignore them, despite their key
81 role in protein evolution, and there is a real challenge to develop ASR models that efficiently account for
82 indels (see for example (Jowkar, et al. 2023; Iglhaut, et al. 2024; Tule, et al. 2024) and references

83 therein). Furthermore, tracing the evolution of enzymes from very ancient ancestors is particularly
84 difficult because the phylogenetic signal contained in the sequences is progressively eroded over time
85 (Gribaldo and Philippe 2002). Thus, the uncertainty in ancestral reconstructions increases with the age
86 of the ancestors under study. Finally, the number of genes / proteins that can be studied over large
87 evolutionary scales is very limited because the size of core gene repertoires decreases with increasing
88 phylogenetic distance (Touchon, et al. 2020).

89 For prokaryotes, the relationship between OGT and amino acid abundance in proteomes has been used
90 to build molecular thermometers (Zeldovich, et al. 2007; Boussau, et al. 2008; Groussin and Gouy 2011;
91 Smole, et al. 2011; Sauer and Wang 2019; Lecocq, et al. 2021; Kurokawa, et al. 2023). These have
92 been used to infer ancestral OGTs of key ancestors in the Tree of Life (Boussau, et al. 2008; Groussin
93 and Gouy 2011; Eme, et al. 2023), and some of these predictions have been experimentally tested in
94 the laboratory (Gaucher, et al. 2003; Gaucher, et al. 2008; Lu, et al. 2024). Surprisingly, in many cases
95 the resurrected ancestral proteins exhibit unexpectedly strong stability, in particular high thermostability
96 (Akanuma 2017; Garcia and Kacar 2019). However, whether these observations reflect the true
97 evolutionary trends or are the result of ASR artefacts is still open to debate (Trudeau, et al. 2016;
98 Wheeler, et al. 2016).

99 Methanogens have a wide range of OGTs, making their study very useful for understanding the thermal
100 adaptation process of enzymes (Prondzinsky, et al. 2023). Among these, the *Methanococcales* are
101 particularly interesting, as it has recently been shown that the OGT alone explains 70% of the variance
102 in amino acid abundance in proteomes, whereas in almost all studies the genomic GC content is the
103 dominant factor (Lecocq, et al. 2021). This is explained by the fact that the genomic GC content is very
104 stable in this archaeal order, whereas the OGTs vary widely (from 35°C for *Methanococcus vannielii* SB
105 to 90°C for *Methanocaldococcus* sp. FS406 22). Using ASR methods, Lecocq et al. (2021) showed that
106 several methanococcales species have independently adapted to moderate environments from
107 (hyper)thermophilic ancestors. They also found that amino acid substitution is the main driver of
108 thermoadaptation, sequentially shaping the amino acid composition of proteomes, with lysine playing a
109 key role in the process (Lecocq, et al. 2021).

110 In this follow-up study, we used ASR to investigate how evolution has refined the temperature-
111 dependent properties of methanococcales enzymes in response to changes in the OGT. We used the
112 NAD(P)H-dependent malate dehydrogenases (MalDHs) (EC 1.1.1.37) because they are relevant

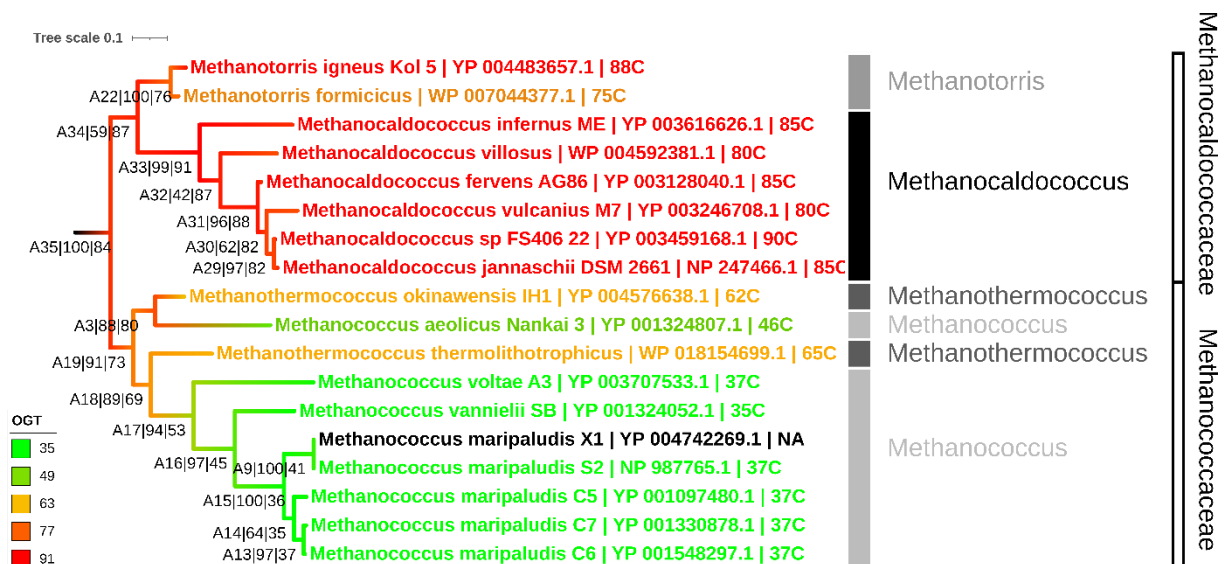
113 models to study the mechanisms of evolution and extremophilic adaptation of enzymes (Richard, et al.
114 2000; Dalhus, et al. 2002; Madern 2002; Irimia, Madern, et al. 2004; Irimia, Vellieux, et al. 2004; Tehei,
115 et al. 2005; Coquelle, et al. 2007; Kawakami, et al. 2009; Coquelle, et al. 2010; Kalimeri, et al. 2014;
116 Roche, et al. 2019; Katava, et al. 2020; Blanquart, et al. 2021; Brochier-Armanet and Madern 2021;
117 lorio, et al. 2021). MalDHs belong to the large super-family of Lactate/Malate Dehydrogenases
118 (LDH/MalDH) (Birktoft, et al. 1982; Boucher, et al. 2014; Brochier-Armanet and Madern 2021; Robin, et
119 al. 2023). They are involved in the tricarboxylic acid cycle by converting oxidized oxaloacetate (OAA) to
120 malate, using either NADH or NADPH as a coenzyme, whereas LDH are involved in the anaerobic
121 metabolism of glucose by catalysing the conversion of pyruvate to lactate. Regarding the oligomeric
122 state, MalDHs are found as homodimers or homotetramers (Talon, et al. 2014; Roche, et al. 2019;
123 McCue and Finzel 2022). We found that the ancestral OGTs of *Methanococcales* inferred from MalDH
124 sequences are consistent with those obtained from core protein families (Lecocq, et al. 2021). As a first
125 step, we inferred the phylogeny of present-day *Methanococcales* MalDH and used it to predict the
126 ancestral MalDH sequences. We then analyzed the effect of temperature on the conformational stability
127 and catalytic activity of MalDH at key positions of the phylogeny. We showed that the ancestral MalDHs
128 display strong conformational stability, as do the present-day MalDHs from hyperthermophiles, but their
129 enzymatic activity profiles differ. Analysis of the substitution trajectories across the MalDH sequences
130 provided interesting clues as to how the mesophilic properties might have evolved from
131 hyperthermophilic ancestors. This was tested experimentally by using the *Methanocaldococcus*
132 *jannaschii* MalDH (*M. jann* MalDH) as a scaffold. Finally, as a recent work on LDH has shown a link
133 between the thermal stability of LDHs and their ability to resist unfolding induced by γ -rays irradiation
134 (Halgand, et al. 2020), we tested the response of some ancestral and modern MalDHs when exposed
135 to γ irradiation. We found that these enzymes remained folded even when they accumulated numerous
136 oxidative damages due to high doses of irradiation. Taken together, our data support a strong link
137 between resistance to thermal and oxidative stress. They also show that such a peculiar phenotype is
138 an ancient trait.

139

140 **Results**

141 **1) Evolution of MalDH from Methanococcales**

142 We assembled a dataset encompassing the MalDH sequences from the 18 strains of *Methanococcales*
 143 from the study by Lecocq et al. (2021) and seven strains of *Methanomada* used as outgroup. Although
 144 it is based on the analysis of a single protein and therefore on a limited number of amino acid positions,
 145 the MalDH phylogeny is overall well supported (most Bootstrap Values (BV) > 90%) and consistent with
 146 the current taxonomy (Fig. 1). In particular, the monophyly of the families *Methanocaldococcaceae* and
 147 *Methanococcaceae*, and genera *Methanocaldococcus* and *Methanotorris* is recovered, while, as in most
 148 studies, *Methanothermococcus* and *Methanococcus* strains are mixed in the tree (see for instance
 149 (Takai, et al. 2002; Kendall, et al. 2006; Sakai, et al. 2019; Lecocq, et al. 2021)). This recurring
 150 observation urges the need to redefine the contours of these two genera. The first speciation event in
 151 the MalDH phylogeny separates *Methanocaldococcaceae* (i.e., *Methanocaldococcus* and
 152 *Methanotorris*, BV = 59%) from *Methanococcaceae* (*Methanothermococcus* and *Methanococcus*,
 153 BV = 91%). With respect to OGTs, the described *Methanocaldococcus* are all hyperthermophilic (OGT
 154 $\geq 80^\circ\text{C}$), *Methanotorris* are either thermophilic (i.e., *Methanotorris formicicus*, OGT = 75°C) or
 155 hyperthermophilic (i.e., *Methanotorris igneus*, OGT = 88°C), the two *Methanothermococcus* are
 156 thermophilic ($45^\circ\text{C} < \text{OGT} < 80^\circ\text{C}$) and *Methanococcus* are either slightly thermophilic (i.e.,
 157 *Methanococcus aeolicus*, OGT = 46°C) or mesophilic (all other *Methanococcus*, OGT $\leq 45^\circ\text{C}$) (Fig.1).
 158



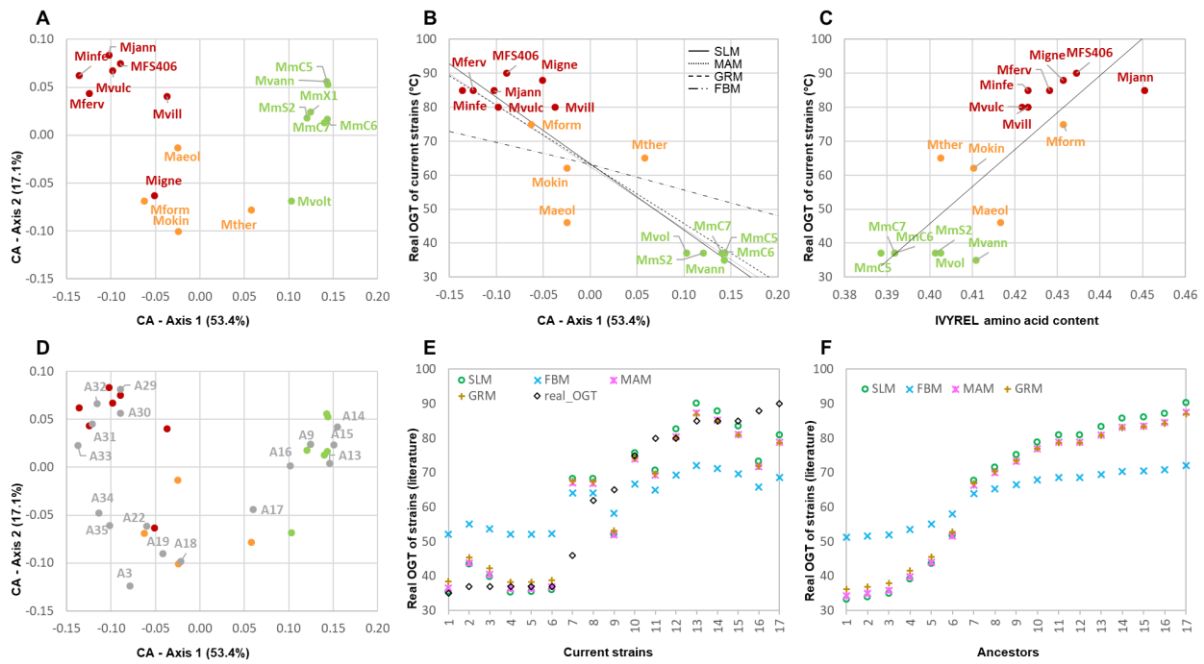
159
 160
 161 **Fig. 1. Rooted maximum likelihood phylogeny of MalDH from *Methanococcales*.** For clarity the
 162 outgroup is not shown. The full rooted tree (including the seven strained used as outgroup) is provided
 163 as Supplementary Fig. S1. Taxonomic information is indicated with vertical bars: grey bars correspond

164 to the genera (i.e., *Methanococcus*, *Methanocaldococcus*, *Methanotorris*, *Methanothermococcus*), white
165 bars to the families (i.e., *Methanocaldococcaceae* and *Methanococcaceae*). Numbers at nodes
166 correspond to ancestor names | bootstrap_values | estimated_OGT_with_the_standard_linear_model
167 (see below). The scale bar corresponds to the average number of substitutions per site. Colours of
168 strains correspond to OGT from the literature (Huber, et al. 1982; Jones, et al. 1983; Burggraf, et al.
169 1990; Jeanthon, et al. 1998; Takai, et al. 2002; Takai, et al. 2004; Kendall, et al. 2006; Mehta and Baross
170 2006; Bellack, et al. 2011; Whitman, et al. 2015) (see also Supplementary Table S1). Branch colours
171 correspond to inferred ancestral OGTs along the phylogeny.

172

173 To test whether there is a correlation between the amino acid content of MalDH sequences and the
174 OGT of strains, we performed a correspondence analysis (CA). We found that the first two axes
175 explained 70.5% of the observed variance (first axis: 53.4% and second axis: 17.1%, Fig. 2A), whereas
176 the third axis explained only 8.2% of the variance (not shown). We also observed that OGT strongly
177 correlated with the MalDH sequence scores on the first axis of the CA (Fig. 2B, $r = -0.928$,
178 $pvalue = 1.3 \times 10^{-07}$), but not with the second axis ($r = 0.158$, $pvalue = 0.5451$) nor the third axis ($r = -$
179 0.048 , $pvalue = 0.8534$). These results are consistent with those obtained on the complete proteomes
180 and core proteins of *Methanococcales* (Lecocq, et al. 2021), which means that in these archaea, the
181 signal associated with thermoadaptation is strong enough to be observed at the level of a single protein.
182 We also observed a significant but slightly weaker correlation between OGT and the amount of I, V, Y,
183 R, E, L amino acids in MalDH sequences ($r = 0.826$, $pvalue = 4.4 \times 10^{-05}$, Fig. 2C), which has been
184 proposed as a good predictor of OGT (Zeldovich, et al. 2007). Regarding MalDH gene GC content, we
185 did not find any significant correlation with any of the first three axes of the CA (all $pvalues > 0.05$, not
186 shown) nor with the OGT ($r = -0.312$, $pvalue = 0.2234$, not shown).

187



188

189

190 **Fig. 2. Analysis of the signal contained in MalDH sequences.** The dots on panels A-D represent

191 MalDH sequences from the studied *Methanococcales* strains and their ancestors, while the marks on

192 panels E and F represent estOGT_{MalDH} with different linear models. Present-day hyperthermophiles are

193 shown in red (Migne = *Methanoterris igneus* Kol_5, Mvulc = *Methanocaldococcus vulcanius* M7,

194 Mvill = *Methanocaldococcus villosus*, MFS406 = *Methanocaldococcus* sp. FS406-22,

195 Mjann = *Methanocaldococcus jannaschii* DSM2661, Minfe = *Methanocaldococcus infernus* ME,

196 Mferv = *Methanocaldococcus fervens* AG86), thermophiles in orange (Mform = *Methanoterris*

197 *formicicus*, Mther = *Methanothermococcus thermolithotrophicus*, Mokin = *Methanothermococcus*

198 *okinawensis* IH1, Maeol = *Methanococcus aeolicus* Nankai-3), and mesophiles in green

199 (Mvann = *Methanococcus vanniellii* SB, MmS2 = *Methanococcus maripaludis* S2,

200 MmC7 = *Methanococcus maripaludis* C7, MmC6 = *Methanococcus maripaludis* C6,

201 MmC5 = *Methanococcus maripaludis* C5, Mvolt = *Methanococcus voltae* A3), while ancestors are in

202 gray. **(A)** Representation of the first two axes of the correspondence analysis (CA), which explained

203 70.5% of the observed variance in amino acid content. The first axis explains 53.4% and the second

204 17.1%. **(B)** Correlation between OGT of the strains and scores of their MalDH sequences

205 on the first axis of the correspondence analysis CA ($r = -0.928$, $pvalue = 1.3 \times 10^{-07}$) according to the

206 standard linear model (SLM). Other lines correspond to the models by Martins and Hansen (MAM), by

207 Grafen (GRM), and by Felsenstein (FBM). **(C)** Correlation between the OGT of the strains and the I, V,

208 Y, R, E, L amino acid content of their MalDH sequences according to a SLM ($r = 0.826$, $p\text{value} = 4.4 \times 10^{-05}$). **(D)** Representation of the first two axes of the (CA) including both present-day methanococcales
209 strains and their ancestors. **(E)** Estimated OGT ($\text{estOGT}_{\text{MalDH}}$) of present-day strains according to the
210 scores of their MalDH on axis 1 of the CA using standard linear model (SLM), and models by Martins
211 and Hansen (MAM), by Grafen (GRM), and by Felsenstein (FBM). Real OGTs according to the literature
212 are indicated by empty black diamonds. Numbers on the X-axis correspond to strains: 1=Mvann, 2=Mvolt, 3=MmS2, 4=MmC5, 5=MmC6, 6=MmC7, 7=Maeol, 8=Mokin, 9=Mther, 10=Mform, 11=Mvill,
213 12=Mvulc, 13=Minfe, 14=Mferv, 15=Mjann, 16=Migne, and 17=MFS406. **(F)** $\text{EstOGT}_{\text{MalDH}}$ of ancestors
214 according to the scores of their MalDH on axis 1 of the CA using the standard linear model (SLM), and
215 models by Martins and Hansen (MAM), by Grafen (GRM), and by Felsenstein (FBM). Numbers on the
216 X-axis correspond to ancestors: 1=A14, 2=A15, 3=A13, 4=A9, 5=A16, 6=A17, 7=A18,
217 8=*Methanococcaceae*/A19, 9=A22, 10=A3, 11=A29, 12=A30, 13=LMCA/A35,
218 14=*Methanocaldococcaceae*/A34, 15=A32, 16=A31, and 17=A33. Confidence intervals and numeric
219 values of $\text{EstOGT}_{\text{MalDH}}$ are provided as Supplementary Fig. S2 and Table S2, respectively.
220
221

222

223 We then used the correlation between the OGT of present-day methanococcales strains and the scores
224 of MalDH sequences on the first axis of the CA to construct molecular thermometers. We used four
225 models, the standard linear model and three regression models that account for the phylogenetic
226 relationships between MalDH sequences (i.e., the Grafen model, the Martins and Hansen model, and
227 the Felsenstein Brownian model, see Methods). The first two provided a regression similar to the
228 standard model, while the slope of the Felsenstein Brownian model was weaker (Fig. 2B). To test the
229 reliability of the thermometer, we used the regression lines to predict the OGT of current
230 methanococcales strains (referred to as $\text{estOGT}_{\text{MalDH}}$). Estimations performed with the linear, the models
231 by Grafen and by Martins and Hansen were close to the real OGTs, excepted for four strains (i.e., Maeol,
232 Migne, Mther, and MFS406, Fig. 2E) Interestingly, the OGTs of three of these strains were difficult to
233 predict, even when all core proteins were considered (Lecocq, et al. 2021). In contrast, the Felsenstein
234 model tended to underestimate high OGTs and overestimate low OGTs (Fig. 2E and Supplementary
235 Fig. S2 and Table S2). Taken together, these results suggest that although the standard model does
236 not account for phylogenetic relationships between sequences, it is not biased by them. Determining
237 the reasons why the Brownian model produced different results is beyond the scope of this study.

238 Then, we applied these models to the ancestral MalDH sequences to predict the OGT of the
239 corresponding ancestors. Again, the standard linear model, the model by Grafen, and the model by
240 Martins and Hansen provided very close OGT estimates (Figs. 2D, 2F, and Supplementary Fig. S2 and
241 Table S2). Since the standard linear model and two of the three models accounting for phylogenetic
242 relationships between strains gave very similar results, for the rest of the analysis $estOGT_{MalDH}$ will refer
243 to the OGTs estimated from the MalDH sequences using the standard linear model. In line with these
244 inferences, the ancestor of the *Methanococcales* was predicted to be slightly hyperthermophilic
245 (LMCA/A35, $estOGT_{MalDH} = 84^{\circ}C$, Fig. 1), as was the ancestor of the *Methanocaldococcaceae* (A34,
246 $estOGT_{MalDH} = 87^{\circ}C$, Fig. 1). Subsequently, a decrease in OGT was observed at the base of the genus
247 *Methanotrorris* (A22, $estOGT_{MalDH} = 76^{\circ}C$), followed by a significant increase in *Methanotrorris igneus*
248 (OGT = $88^{\circ}C$), and a more moderate increase in *Methanotrorris formicicus* (OGT = $75^{\circ}C$) (Fig. 1). In the
249 ancestor of the genus *Methanocaldococcus*, an increase in OGTs was observed (A33,
250 $estOGT_{MalDH} = 91^{\circ}C$), whereas in the *Methanococcaceae*, very different trends were observed (Fig. 1).
251 From a thermophilic ancestor (A19, $estOGT_{MalDH} = 73^{\circ}C$), two independent adaptations toward cooler
252 temperatures occurred in the lineage leading to *Methanococcus aeolicus* (OGT = $46^{\circ}C$) and in the
253 lineage leading to *Methanococcus voltae*, *Methanococcus vannielii*, and *Methanococcus maripaludis*
254 (OGTs = $35-37^{\circ}C$), while OGTs remained almost unchanged in the two *Methanothermococcus* lineages
255 (OGTs = 62 and $65^{\circ}C$, Fig. 1).

256 **2) Success and failure in methanococcales MalDHs purification**

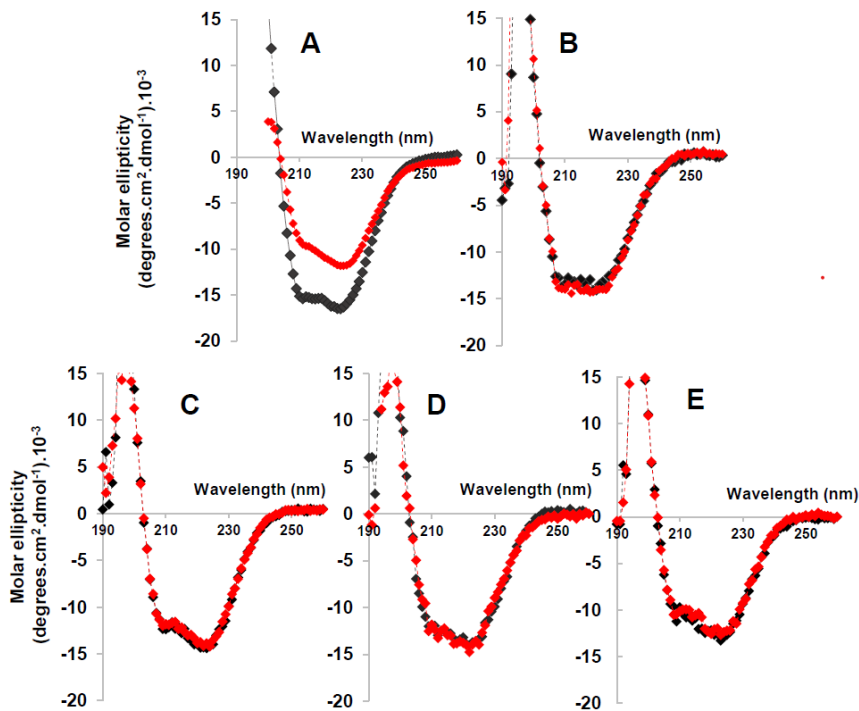
257 Over the years, we have developed overexpression and purification procedures that are routinely used
258 to obtain pure recombinant MalDH from various organisms, including mesophiles (Richard, et al. 2000;
259 Dalhus, et al. 2002; Madern 2002; Irimia, Madern, et al. 2004; Irimia, Vellieux, et al. 2004; Tehei, et al.
260 2005; Coquelle, et al. 2010; Kalimeri, et al. 2014; Roche, et al. 2019; Katava, et al. 2020; Blanquart, et
261 al. 2021; Brochier-Armanet and Madern 2021; Iorio, et al. 2021). Here, we successfully purified the
262 MalDH from *Methanococcus infernus*, *Methanocaldococcus jannaschii*, *Methanotrorris formicicus*,
263 *Methanopyrus kandleri* (outgroup), and *Methanothermobacter thermautotrophicus* (outgroup). However,
264 we encountered major difficulties in purifying MalDH from both present-day and ancestral mesophilic
265 methanococcales (see below), which prevented their experimental characterization. We tried several
266 overexpression protocols, overexpression plasmids, and different *E. coli* strains. Unfortunately, none of
267 the tests yielded sufficient amounts of soluble enzyme. We also tried refolding procedures, but to no

268 avail. This is particularly intriguing as we have had no difficulty in producing MalDH from mesophiles
269 belonging to other taxonomic groups. This suggests that in mesophilic methanococcales, MalDH may
270 follow a specific chaperone-assisted folding pathway, which cannot be mimicked within the *E. coli*
271 cytoplasm. Fortunately, when expressed in *E. coli*, the MalDH sequences inferred in the three deepest
272 methanococcales ancestors (LMCA/A35, *Methanocaldococcaceae*/A34, and *Methanococcaceae*/A19)
273 and two intermediate ancestors A30 and A33 were correctly folded and soluble, allowing the appropriate
274 purification procedure to be set up as indicated in the Materials and Methods section. At the end of the
275 purification, the tetrameric state was assessed using either a calibrated size exclusion chromatography
276 column or analytical centrifugation (Supplementary Fig. S3-S5). With the LMCA/A35 MalDH, we found
277 that in addition to the tetramer, a fraction of the enzyme exists as an octamer.

278

279 **3) Thermal-dependent conformational stability of the ancestral methanococcales MalDHs.**

280 In the first part of this work, we addressed the question concerning the thermal properties of enzymes
281 at the root of *Methanococcales*. To achieve this goal, we monitored the circular dichroism (CD) spectra
282 of the three deepest ancestral MalDHs, i.e., the ancestors of all *Methanococcales* (LMCA/A35),
283 *Methanocaldococcaceae* (A34), and *Methanococcaceae* (A19), before and after incubation at 90°C to
284 test their conformational thermostability. In parallel, we used the LDH of the bacterium *Selenomonas*
285 *ruminantium* (Bertrand, et al. 2023) and the MalDH of *M. kand* as control proteins representative of
286 mesophiles and hyperthermophiles, respectively (Fig. 3A-B). The CD spectra of the three ancestral
287 enzymes, *M. kand* MalDH and *S. rumi* LDH recorded at 25°C are typical of folded proteins, with a strong
288 negative molar ellipticity below 200 nm and a peak with positive values centered at 195 nm (Fig. 3B-E,
289 black diamonds). After incubation at 90°C, MalDH CD spectra remained unchanged, demonstrating that
290 the three ancestral MalDHs exhibit behavior typical of modern hyperthermophilic MalDHs (Fig. 3B-E,
291 red diamonds). Remarkably, the spectrum of the *S. rumi* LDH observed after incubation at 60°C is
292 already modified, indicating that the thermal treatment has strongly altered the folding state of the
293 enzyme (Fig. 3A, black and red diamonds).

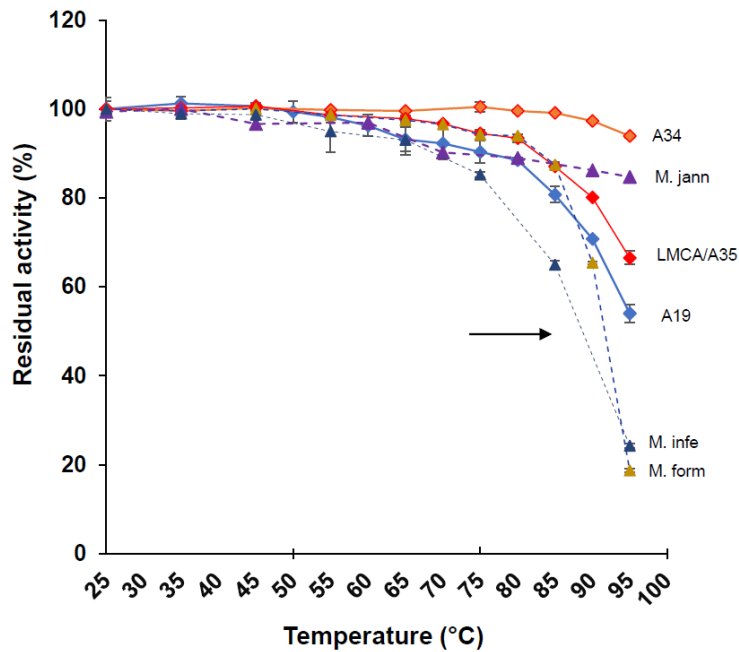


295 **Fig. 3. Effect of temperature on the circular dichroism (CD) spectra on the three deepest**
 296 ***Methanococcales* ancestral MalDHs and controls.** Curves correspond to CD spectra taken at 25°C
 297 before the thermal treatment (TT) (black diamonds) and after the thermal treatment (red diamonds). **(A)**
 298 *S. rumi* LDH, TT = 60°C (OGT = 37°C). **(B)** *M. kand* MalDH, TT = 90°C (OGT = 98°C). **(C)** Ancestor of
 299 *Methanococcales* MalDH (LMCA/A35, estOGT_{MalDH} = 84°C), TT = 90°C. **(D)** Ancestor of
 300 *Methanocaldococcaceae* MalDH (A34, estOGT_{MalDH} = 87°C), TT = 90°C. **(E)** Ancestor of
 301 *Methanococcaceae* MalDH (A19, estOGT_{MalDH} = 73°C), TT = 90°C.

302

303 To test whether the hyperthermophilic modern and ancestral enzymes could be unfolded, we added
 304 10% v/v of pure HCl to the 300 μ L of sample in the cuvette used for CD and incubated for an additional
 305 15 min at 70°C. The resulting spectra showed a strong loss of the negative molar ellipticity, indicating
 306 that the three ancestral MalDHs tested unfold after application of such a stringent treatment
 307 (Supplementary Fig. S6). The CD spectrum of *M. kand* MalDH was less affected, suggesting that the
 308 enzyme is more resistant to the treatment (Supplementary Fig. S6A). Altogether, this suggests that the
 309 three deepest *Methanococcales* ancestral MalDHs are hyper thermostable and require drastic treatment
 310 to unfold.

311 We also recorded the stability of these enzymes using residual activity measurements (see Methods).
 312 This method is commonly used to evaluate the effect of a chemical-physical treatment on the
 313 functionality of MalDH and LDH enzymes (Coquelle, et al. 2007; Coquelle, et al. 2010; Blanquart, et al.
 314 2021; Robin, et al. 2023). The method allows to extract the apparent melting temperature ($T_{M1/2}$), i.e.,
 315 the temperature at which 50% of the enzyme is still active.



316 **Fig. 4. MalDH thermostability**

317 **probed by residual activity measurement.** Present day and ancestral MalDHs are indicated by
 318 triangles and diamonds, respectively. MalDH of *M. jann* (OGT = 85°C) is in dark purple, *M. form*
 319 (OGT = 75°C) in brown, *M. infe* (OGT = 85°C) in dark blue, while the MalDH of the ancestor of
 320 *Methanococcales* (LMCA/A35, estOGT_{MalDH} = 84°C) is in red, *Methanocaldococcaceae* (A34,
 321 estOGT_{MalDH} = 87°C) in orange, and *Methanococcaceae* (A19, estOGT_{MalDH} = 73°C) in cyan. The arrow
 322 designates $T_{M1/2}$.

323 Here, we tested the ancestral MalDHs at the root of the *Methanococcales* (LMCA/A35,
 324 estOGT_{MalDH} = 84°C), *Methanocaldococcaceae* (A34, estOGT_{MalDH} = 87°C), and *Methanococcaceae*
 325 (A19, estOGT_{MalDH} = 73°C), as well as those from three present-day species: *M. form* (OGT = 75°C), *M.*
 326 *jann* (OGT = 85°C), and *M. infe* (OGT = 85°C). We found that these six MalDHs had $T_{M1/2}$ values above
 327 90°C (Fig. 4), demonstrating that they behave as hyper thermostable enzymes. Note that the *M. infe*
 328 MalDH profile suggested a slightly lower resistance to high temperature, probably due to differences in

329 the kinetic processes leading to deactivation and unfolding. However, the full characterization of these
330 processes is beyond the scope of this study.

331 We concluded that at the root of the *Methanococcales*, the LMCA/A35 MalDH had a strong
332 conformational stability that allowed it to resist to unfolding induced by the high environmental
333 temperature. This correlates well with estimated ancestral OGTs, which suggests that ancestor
334 LMCA/A35 was hyperthermophilic (estOGT_{MalDH} = 84°C, Fig.1). This ancestral phenotype was
335 conserved in the ancestor of *Methanocaldococcaceae* (A34), whose present-day descendants live in
336 hot ecological niches (Fig. 1). This suggests that the amino acid substitutions that occurred in MalDH
337 sequences in this lineage were quite neutral with respect to thermostability. More surprisingly, this
338 characteristic was also conserved in *M. form*, which has been characterized as thermophilic (Takai, et
339 al. 2004), and in the ancestor of the *Methanococcaceae*, which we predicted to be thermophilic (A19,
340 estOGT_{MalDH} = 73°C).

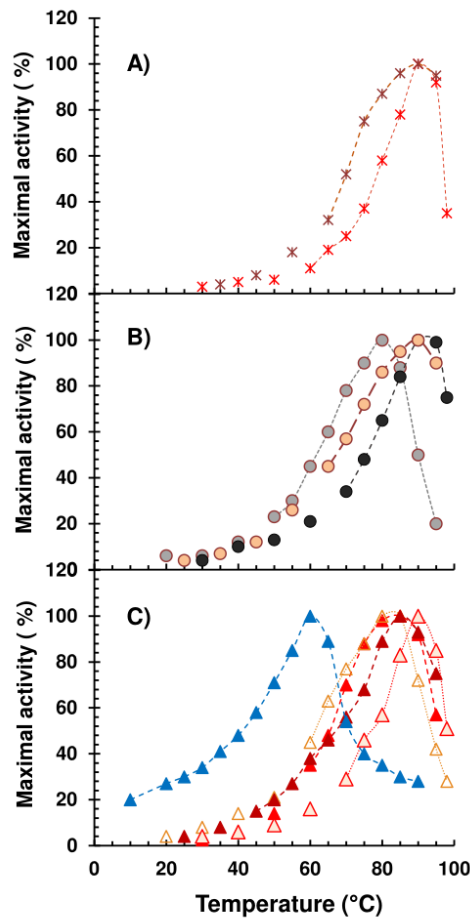
341 With regard to adaptation to lower environmental temperatures, the inability to purify mesophilic MalDH
342 prevents us from drawing definitive conclusions about the changes in conformational stability that may
343 have accompanied these changes in OGT. However, two scenarios can be suggested. First, one could
344 hypothesize that both the conformational stability and the activity of MalDHs were reduced during the
345 OGT shifts. Alternatively, it is possible that the high conformational thermal stability was maintained
346 while the activity profile was shifted to lower temperatures. According to the first hypothesis, the
347 substitutions that occurred at the basis of the mesophilic lineages must have had an effect on both the
348 conformational stability and the activity of MalDH. In the second case, these substitutions must only
349 have affected the activity and not the conformational stability of MalDH.

350

351 **4) Temperature dependence of enzymatic activity.**

352 Amino acid substitutions can affect not only conformational stability, but also the dynamic properties of
353 an enzyme and, by extension, its catalytic properties (Yu and Dalby 2018; Bigman and Levy 2020).
354 Therefore, we recorded the enzymatic activity of several present-day and ancestral MalDHs at different
355 temperatures: the MalDH of the three deepest ancestors (LMCA/A35, *Methanocaldococcaceae*/A34 and
356 *Methanococcaceae*/A19), two intermediate ancestors along the hyperthermophilic *Methanocaldococcus*
357 lineage (A33 and A30), and three present-day *Methanocaldococcaceae* species (*M. jann*, *M. infe*, and

358 *M. form*) (Fig. 5). The profiles of two MalDHs from *M. kand* and *M. therm*, both belonging to the outgroup,
 359 were also recorded.



360
 361 **Fig 5. Plot of the relative activity as a function of temperature.** The averaged values are expressed
 362 as % of activity relative to the maximal value recorded for each enzyme. For a given MalDH, optimal
 363 temperature for activity ($A-T_{opt}$) corresponds to the peak of the curve, i.e., the maximal value recorded.
 364 **(A)** Enzymes from the outgroup: MalDH from *M. kand* (OGT = 98°C, red crosses, $A-T_{opt}$ = 90°C) and *M.*
 365 *ther* (OGT = 65°C, brown crosses, $A-T_{opt}$ = 90°C). **(B)** Present-days enzymes: MalDH from *M. jann*
 366 (OGT = 85°C, black circles, $A-T_{opt}$ = 93°C), *M. form* (OGT = 75°C, orange circles, $A-T_{opt}$ = 90°C), *M. infe*
 367 (OGT = 85°C, grey circles, $A-T_{opt}$ = 80°C). **(C)** Ancestral enzymes: MalDH from the *Methanococcales*
 368 ancestor (LMCA/A35, $estOGT_{MalDH}$ = 84°C, closed red triangles, $A-T_{opt}$ = 80°C), the
 369 *Methanocaldococcaceae* ancestor (A34, $estOGT_{MalDH}$ = 87°C, dark brown triangles, $A-T_{opt}$ = 81°C), two
 370 intermediate ancestors within the *Methanocaldococcus* lineage (A33, $estOGT_{MalDH}$ = 91°C, open red
 371 triangles, $A-T_{opt}$ = 91°C and A30, $estOGT_{MalDH}$ = 82°C, open orange triangles, $A-T_{opt}$ = 82°C,

372 respectively), and the *Methanococcaceae* ancestor (A19, $\text{estOGT}_{\text{MalDH}} = 73^{\circ}\text{C}$, blue triangles, A-
373 $T_{\text{opt}} = 67^{\circ}\text{C}$).

374

375 The profiles showed that all enzymes from modern (hyper)thermophilic organisms, i.e., *M. jann*, *M. infe*,
376 *M. form*, *M. ther*, and *M. kand* had the expected typical profile with an optimal temperature for activity
377 ($A-T_{\text{opt}}$) in the range $80-95^{\circ}\text{C}$, and a relative activity decreasing to 20% in the range $50-65^{\circ}\text{C}$ (Fig.5).
378 The enzymatic activity for the LMCA/A35 and *Methanocaldococcaceae*/A34 MalDHs showed equivalent
379 profiles, demonstrating that they were fully functional at high temperature. In contrast, the enzymatic
380 profile of the MalDH inferred in the *Methanococcaceae* ancestor (A19) was strongly shifted towards
381 lower temperatures, with an $A-T_{\text{opt}}$ of 60°C . In this case, the threshold temperature for maintaining 20%
382 of the activity was 15°C . This suggested that in the ancestor of the *Methanococcales* (LMCA/A35,
383 $\text{estOGT}_{\text{MalDH}} = 84^{\circ}\text{C}$), MalDH exhibited both strong conformational stability and enzymatic activity at
384 high temperature, two characteristics that were conserved in the *Methanocaldococcaceae* lineage (A34,
385 A33, A30, *M. jann*, *M. infe*, and *M. form*, all $\text{estOGT}_{\text{MalDH/OGT}} \geq 75^{\circ}\text{C}$), independently of amino acid
386 substitutions that may have occurred in the MalDH sequences. In contrast, the MalDH inferred in the
387 ancestor of *Methanococcaceae* (A19, $\text{estOGT}_{\text{MalDH}} = 73^{\circ}\text{C}$) showed a decoupling between its
388 conformational stability and its enzymatic activity. This suggested that the 13 amino acid substitutions
389 that occurred in the stem of *Methanococcaceae*, i.e., along the branch linking ancestors A35 and A19,
390 resulted in an enzyme that was still hyperthermostable, but with optimal activity at more moderate
391 temperatures.

392 For many years the activity/stability trade-off was the dominant concept to explain thermal adaptation of
393 enzymes (Goldstein 2011). It implies that mesophilic and psychophilic enzymes must sacrifice their
394 stability to achieve an increase in conformational flexibility crucial for activity. This point of view has been
395 challenged by two studies based on ASRs of the adenylate kinase and the 3-isopropylmalate
396 dehydrogenase (Nguyen, et al. 2017; Furukawa, et al. 2020). In particular, it was shown that their
397 ancestral states were both thermostable and catalytically efficient at low temperature. Our data based
398 on MalDH confirmed that a catalytic activity at moderate temperature can be observed on a thermally
399 stable scaffold. Crystallographic investigations using the ancestral MalDHs from *Methanococcales* are
400 currently under way and will help to analyze this phenomenon.

401

402 **5) Does OGT and A-T_{opt} correlate?**

403 One of the aims of the study was to investigate the extent to which the ancestral estOGT_{MalDH} and the
 404 A-T_{opt} correlated. The measurements of A-T_{opt} for all the enzymes studied here, were done at a
 405 concentration of 0.8 mM OAA. Such a value was a compromise (i) to ensure a non-limiting effect of OAA
 406 concentration at the beginning of the reaction and (ii) to avoid inhibition by high OAA concentration.
 407 Determination of the optimal OAA value for each enzyme and at different temperatures in order to gain
 408 in A-T_{opt} accuracy was beyond the scope of this study. Furthermore, the experimental determination of
 409 A-T_{opt} is difficult, particularly at high temperatures, and requires the use of temperature-controlled
 410 apparatus, so we assumed that all values are within a confidence limit of +/- 5°C. The results are shown
 411 in Table 1. The OGT and MalDH A-T_{opt} values of *M. kand* (outgroup), *M. infe*, and *M. jann* were close to
 412 each other. For the thermophilic *M. ther* (outgroup) and *M. form*, OGT values were higher than the A-
 413 T_{opt} of their MalDHs. We cannot exclude that the experimental OGT was underestimated for these two
 414 species. This could indeed be the case for the *M. form*, where the OGT could actually be close to 80°C.
 415 (Philippe Oger and Priscilla Gillet, personal communication) For the resurrected ancestral MalDHs, we
 416 observed almost identical values for the inferred OGT and A-T_{opt}. This is particularly noticeable with the
 417 A-T_{opt} of the MalDH inferred at the root of the *Methanococcaceae* (A19) lineage (A-T_{opt} = 60°C), which
 418 is consistent with the strong shift towards low OGT predicted using molecular thermometers applied to
 419 MalDHs (estOGT_{MalDH} = 73°C, this study) and proteomes (estOGT_{MalDH} = 64.5/65°C) (Lecocq, et al.
 420 2021).

421
 422 **Table 1.** Table showing OGTs according to data from the literature, estimated OGTs from proteome
 423 sequences (estOGT_{CORE-PROT}) (Lecocq, et al. 2021), estimated OGT from MalDH protein sequences
 424 (estOGT_{MalDH}, this study), and experimentally determined A-T_{opt} (this study), for two outgroup species:
 425 *M. kand* (*Methanopyrales*) and *M. ther* (*Methanobacteriales*), three present-day species of
 426 *Methanococcales*: *M. jann*, *M. infe*, *M. form*, and five methanococcales ancestors: LMCA/A35,
 427 *Methanocaldococcaceae*/A34, *Methanococcaceae*/A33, and two intermediate ancestors along the
 428 *Methanocaldococcaceae*: A30, and A19.

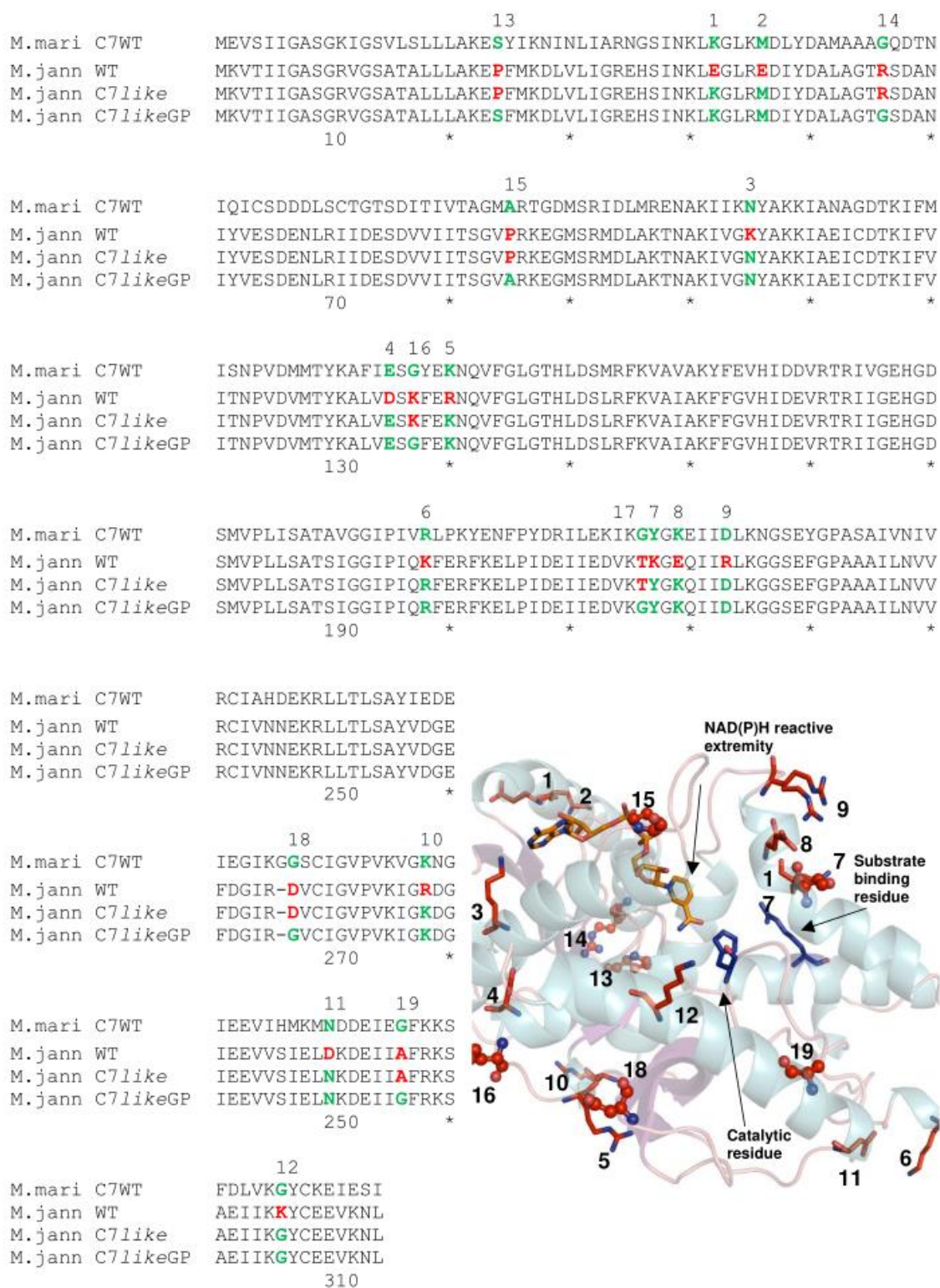
Taxa	Experimental OGT (°C)	estOGT _{CORE-PROT} (°C)	estOGT _{MalDH} (°C)	Experimental A-T _{opt} (°C)
Outgroup				
<i>M. kand</i>	98 (Kurr, et al. 1991)	-	-	90
<i>M. ther</i>	65 (Zeikus and Wolfe 1972)	-	-	90
Methanococcales				
<i>M. jann</i>	85 (Jones, et al. 1983)	85-90	-	90
<i>M. infe</i>	85 (Jeanthon, et al. 1998)	90-93	-	80

<i>M. form</i>	75 (Takai, et al. 2004)	75-86	-	90
LMCA, A35	-	81-83	84	85
A34	-	NA	87	85
A33	-	84-86	91	90
A30	-	NA	82	80
A19	-	64.5-65	73	60

429

430 **6) Mimicking mesophilic adaptation.**

431 To overcome the inability to purify MalDH from present-day mesophilic Methanococcales, we applied a
432 mimicry strategy based on a sequence comparison between MalDH sequences from the
433 hyperthermophilic *M. jann*, for which a structure exists (Protein data bank, PDB accession
434 number,1HYG), and the mesophilic *M. mari* C7. In fact, it is well known that present day
435 hyperthermophilic proteins have an increased number of salt-bridges compared to their mesophilic and
436 psychrophilic counterparts (see (Coquelle, et al. 2007; Pinney, et al. 2021) and references therein). For
437 example, in proteins from hyperthermophiles, E and R amino acids are preferentially used over D and
438 K, because they have longer side chains that can form ionic interactions with more partners and complex
439 networks of salt bridges that greatly increase the thermal stability of the proteins (Donald, et al. 2011;
440 Sammond, et al. 2016). This is also the case for MalDH, where the *M. mari* enzyme contains 21 E and
441 11 R, whereas the *M. jann* protein contains 27 E and 18 R (Supplementary Table S2). Other studies
442 suggested that G and P amino acids play a role in the thermal adaptation of enzymes to low
443 temperatures (Feller 2010; Michetti, et al. 2017). This is due to their opposite influence on conformational
444 dynamics: G, which lacks a β carbon, has the highest degree of conformational entropy, whereas P can
445 sample only a few states and has the lowest degree of conformational entropy (Prajapati, et al. 2007;
446 Feng, et al. 2020). The ability to increase the thermostability of enzymes by adding P residues to their
447 sequences has been successfully exploited (Gojberg, et al. 2007; Tian, et al. 2010; Zhou, et al. 2010).
448 To test the impact of these residues, we designed two mutants using the hyperthermophilic Wild-type
449 (Wt) *M. jann* MalDH as a template.



450

451 **Fig. 6. Multiple alignment of Wt *M. jann*, Wt *M. mari* C7, and *M. jann* C7 like and C7GP like mutants.**

452 The multiple alignment shows the 19 amino acids of the Wt *M. mari* C7 (green, first sequence) that have
 453 been introduced into the Wt *M. jann* MalDH sequence at equivalent positions (red, second sequence).

454 The *M. jann* C7 like mutant corresponds to the Wt *M. jann* MalDH with 12 of the 19 targeted amino acids

455 present in *M. mari* C7 added (1 to 12 in green, third sequence). The *M. jann* C7 like GP mutant contained
456 the full set of targeted amino acids present in Wt *M. mari* C7 (1 to 19 in green, fourth sequence). The
457 linear numbering of amino acids is shown below the alignment.

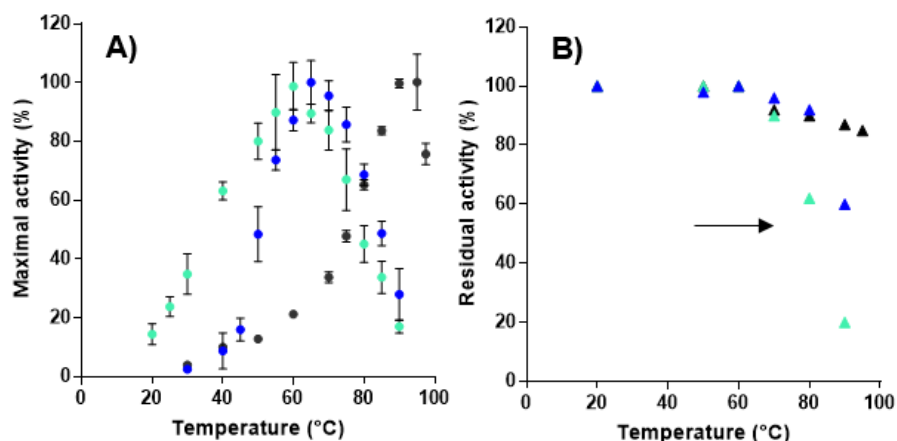
458 The small inset shows the location of mutations on the *M. jann* MalDH structure of the monomer (PDB
459 accession number, 1HYG). α -helices, β -strands and loops are in blue, purple and pale orange,
460 respectively. The first set of mutated amino acids is represented in red stick, the second is in small red
461 balls. The numbering refers to their location indicated on the sequence alignment. The catalytic histidine
462 (H178) and the substrate binding residue (R154) are shown in blue sticks. The coenzyme reactive
463 extremity (orange stick) is indicated.

464

465 We carefully inspected the *M. jann* MalDH structure as a guide to discriminate between substitutions
466 that preferentially target intra-monomeric salt bridges rather than those located at interfaces that
467 contribute to tetramerization. The first mutant, *M. jann* C7 MalDH, contained 12 substitutions that target
468 the salt bridges, while the second mutant, *M. jann* C7GP, contained seven additional substitutions (i. e.,
469 five glycine were added and two proline residues removed) located in loops, which connect β -strands
470 and α -helices. In both mutants, the targeted residues were substituted by the amino acids found in *M.*
471 *mari* C7 at the same position (Fig. 6). The position of the substitutions is shown on the structure of the
472 Wt *M. jann* MalDH monomer (Fig. 6). Recall that none of these amino acids participate to substrate-
473 binding or catalytic reaction.

474 The resulting *M. jann* C7 and *M. jann* C7GP MalDHs, were purified and their temperature-dependent
475 activity profiles were recorded. As previously noted, the Wt *M. jann* MalDH has the typical profile of a
476 hyperthermophilic enzyme with an $A-T_{opt}$ close to 90°C. The activity profile of *M. jann* C7 MalDH is
477 strongly shifted towards lower temperatures with an $A-T_{opt}$ value of 65°C (Fig. 7A). Our data showed
478 that disruption of intra-monomer ionic interactions could be one of the mechanisms that drove adaptation
479 toward mesophily in *Methanococcales*.

480



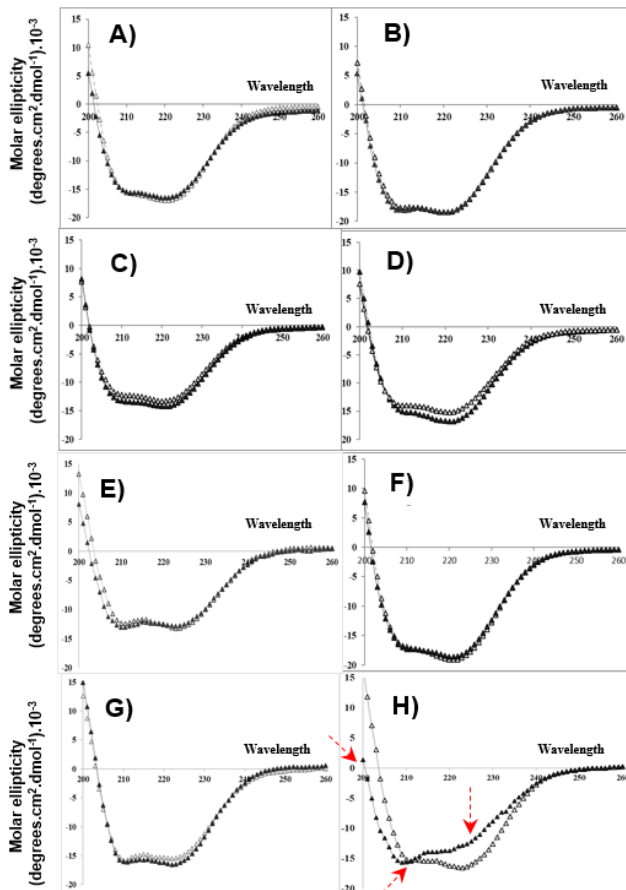
481 **Fig 7. Effects of mesophilic-like mutations on *M. jann* MalDH. (A)** Temperature-dependent activity
 482 profile of Wt *M. jann* MalDH, black circles. *M. jann* C7 MalDH mutant, dark blue circles. *M. jann* C7GP
 483 MalDH mutant, cyan circles. The averaged values are expressed as % of activity relative to the maximal
 484 value recorded for each enzyme. For a given MalDH, optimal temperature for activity ($A-T_{opt}$)
 485 corresponds to the peak of the curve, i.e., the maximal value recorded. Error bars are shown. **(B)**
 486 Thermostability probed by residual activity measurement. Wt *M. jann* MalDH is in black triangles, *M.*
 487 *jann* C7 MalDH in dark blue triangles and *M. jann* C7GP MalDH in cyan triangles. The arrow indicates
 488 $T_{M1/2}$ values.

489
 490 For the *M. jann* C7GP MalDH, the resulting behavior was different. The $A-T_{opt}$ has a value of 60°C,
 491 indicating that modifying the G and P content did not have a major effect on this parameter compared
 492 to *M. jann* C7. However, the profile of *M. jann* C7GP MalDH showed that the relative maximum activity
 493 below $A-T_{opt}$ (20-60°C range) was significantly increased compared to the *M. jann* C7 enzyme (Fig. 7A).
 494 Without data based on the Wt *M. mari* MalDH, a comparison with its activity profiles was not possible.
 495 We also recorded the apparent conformational stability using residual activity measurements. The data
 496 showed that the two sets of mutations affected the thermal stability by shifting the $T_{M1/2}$ towards lower
 497 temperature compared to the Wt *M. jann* MalDH. However, $T_{M1/2}$ of 83°C and 90°C indicated that the
 498 mutants still behave as hyper thermostable scaffolds (Fig. 7B). We noted that the characterization of the
 499 adenylate kinase from *M. mari* reports an excellent activity over a moderate temperature range and a
 500 high thermal conformational stability (Davlieva and Shamoo 2010).

501

502 **7) Conformational stability of ancestral and present-day *Methanococcales* MalDHs under γ -**
503 **irradiation oxidation by $\bullet\text{OH}$ and Cl_2^- free radicals.**

504 To test whether high conformational thermal stability favours the capacity to resist unfolding under strong
505 γ -irradiation-induced oxidation, we monitored the circular dichroism (CD) spectrum at 25°C of a set of
506 present-day *Methanococcales* MalDHs (*M.infe* and *M. jann* (both OGTs = 85°C), and *M. form*
507 (OGT = 75°C)), and their deepest ancestors (LMCA/A35 (estOGT_{MalDH} = 84°C),
508 *Methanocaldococceae/A34* (estOGT_{MalDH} = 87°C) and *Methanococceae/A19*
509 (estOGT_{MalDH} = 73°C)) before and after exposure to a ⁶⁰Cobalt source in oxidizing conditions (Fig. 8A).
510 The LDH from *S. rumi* (OGT = 37°C) and the MalDH from *M. kand* (OGT = 98°C) were also tested (Fig.
511 8B). In the irradiation conditions, the medium is strongly oxidising as the reducing free radicals are
512 scavenged. For the set of *Methanococcales*, the comparison showed no significant differences between
513 the irradiated and non-irradiated enzymes (Fig. 8A). This was also the case for MalDH from the
514 hyperthermophilic archaea *M. kand* (Fig. 8B). On the other hand, for the *S. rumi* enzyme, the CD
515 spectrum obtained after irradiation showed strong modifications: the value of the molar ellipticity at 222
516 nm increased, the lowest intensity peak was shifted to 208 nm, the zero value of the molar ellipticity at
517 200 nm was shifted, and the positive signal disappeared, showing a transition towards a less structured
518 and less compact state (see arrows in Fig. 8B).



519

520 **Fig. 8. Effect of γ -irradiation on the circular dichroism spectra of ancestral and present-day**

521 ***Methanococcales* MalDHs.** Open and closed triangles correspond to CD spectra taken before and

522 after irradiation. MalDH from (A) *M. infe* (OGT = 85°C), (B) *M. jann* (OGT = 85°C), (C) *M. form*

523 (OGT = 75°C), (D) LMCA/A35 (estOGT_{MalDH} = 84°C), (E) *Methanocaldococcaceae*/A34

524 (estOGT_{MalDH} = 87°C), (F) *Methanococcaceae*/A19 (estOGT_{MalDH} = 73°C), (G) *M. kand* (OGT = 98°C,

525 outgroup), and (H) the LDH from *S. rumi* (OGT = 37°C). To the exception of the *S. rumi* enzyme, for

526 which the applied dose was 1,500 Gy, the other enzymes received (in average) a dose of 4,000 Gy.

527

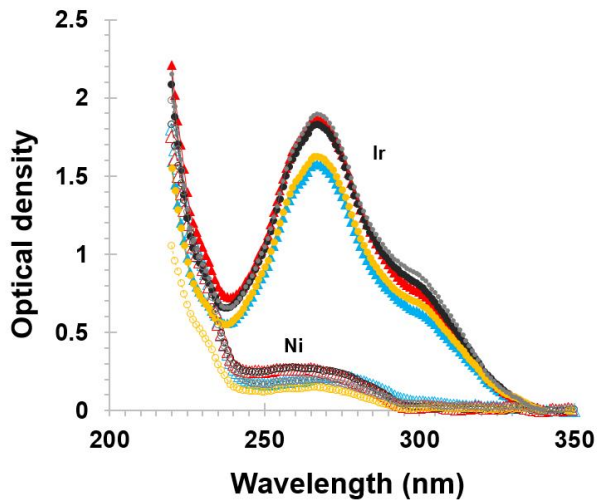
528 The modification of UV-visible spectra of a protein after γ -irradiation is a strong indicator of protein

529 residue oxidized (Houee-Levin and Bobrowski 2013). Accordingly, we checked whether

530 methanococcales MalDHs were subjected to attack by reactive species by recording their UV-visible

531 spectra before and after irradiation. A set of two ancestral (LMCA/A35 and *Methanococcaceae*/A19)

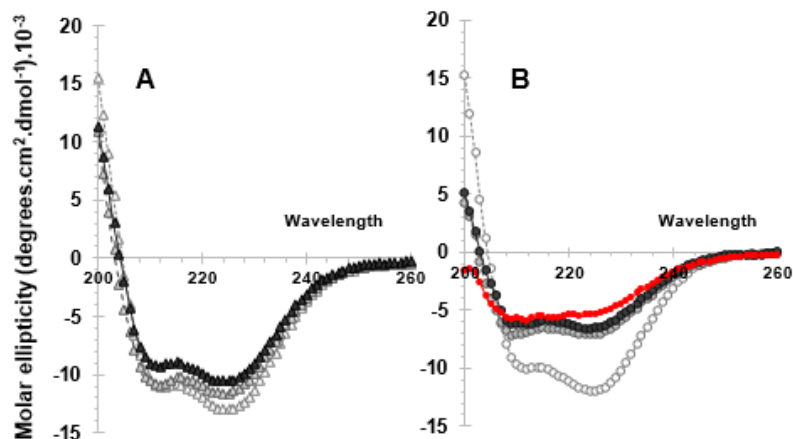
532 and two present-day *Methanococcales* MalDHs (*M. jann* and *M. form*) were tested (Fig. 9).



534 **Fig. 9. Effect of γ -irradiation on the UV-visible spectra of some ancestral and present-day**
 535 **methanococcales MalDHs.** Ni: non-irradiated, open symbols, Ir : irradiated, closed symbols. Ancestral
 536 MalDHs : LMCA/A35 (estOGT_{MalDH} = 84°C), red triangles; *Methanococcaceae*/A19
 537 (estOGT_{MalDH} = 73°C), blue triangles. Present-day MalDHs: *M. form* (OGT = 75°C), orange circles; *M.*
 538 *jann* (OGT = 85°C), black circles; *M. infe* (OGT = 85°C), grey circles.

539

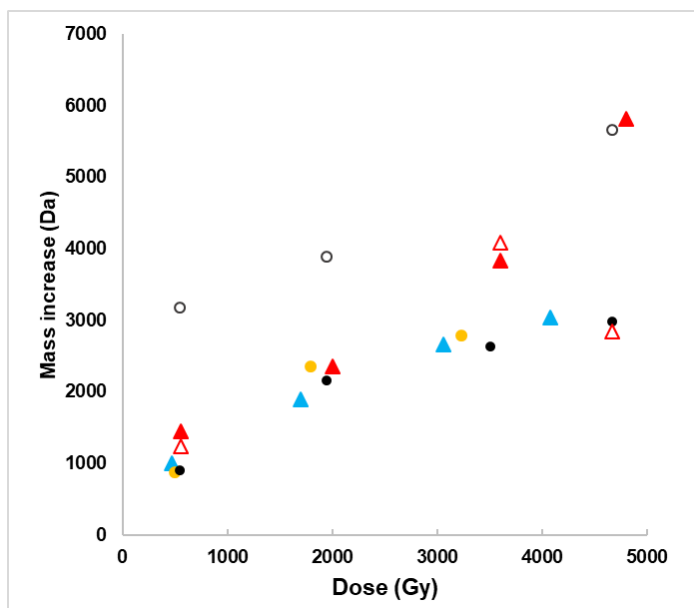
540 The data clearly showed that chemical modifications occurred after irradiation. We concluded that even
 541 if OH-Cl₂⁻ radicals have oxidized numerous amino acids this was not sufficient to unfold the
 542 hyperthermostable MalDHs, suggesting that strong stabilizing interactions are still efficient in the
 543 tetrameric scaffold. Due to the limited amount of N₂O in the samples to be irradiated, further
 544 investigations at higher doses were difficult to perform. However, we tried to test the conformational
 545 stability of the most ancestral enzyme at doses of 7,200 and 10,500 Gy by recording the CD spectra.
 546 The data showed that these doses have a small effect on the CD spectrum of the LMCA/A35 suggesting
 547 that only a small fraction of the molecules begin to unfold (Fig. 10A). As previously, further drastic
 548 treatments are necessary to induce unfolding, even if the enzyme has been subjected to oxidation (Fig.
 549 10B). The thermal treatment applied to the irradiated enzyme produces an intermediate state that is
 550 different from the typical unfolded state obtained after acidification of the sample.



551
 552
 553 **Fig. 10. Effect of high irradiation doses on LMCA/A35 MalDH. (A)** CD spectra taken before (Open
 554 triangles) and after irradiation at 7,200 Gy (Gray triangles) and 10,500 Gy (Dark triangles). **(B)** CD
 555 spectra after adding thermal treatment and acidification to samples used in A. 0 Gy (Open circles), 7,200
 556 Gy (Gray circles), 10,500 Gy (Dark circles) and 10,500 Gy+ HCl (red circles).

557
 558 **8) Quantification of γ -irradiation oxidative damages.**

559 Native mass spectrometry was used to characterize the extent of protein damage after irradiation. This
 560 method allows the observation of the oligomeric state (homotetramer) of the protein under study and its
 561 molecular mass to be monitored simultaneously. Some of the mass spectrometry spectra are shown as
 562 Supplementary Fig. S7. They showed that the enzymes remained tetrameric after irradiation. We
 563 observed also that, after irradiation, chemical modifications by reactive species induced an increase in
 564 the molecular mass of each MalDH at the dose administered (Fig. 11, Supplementary Table S3). We
 565 observed that LMCA/A35, and *M. kand* MalDHs accumulated a higher number of modifications than the
 566 other enzymes. At this stage, it was not possible to determine the reasons for this, even it can be
 567 assumed that differences in the amino acid sequence may lead to different reactivity with oxidative
 568 species. As the SEC was not efficient enough to remove salts, knowing that the KCl concentration was
 569 of 0.4 M, the peaks of the tetramers are large and the calculated molecular weight is overestimated. For
 570 this reason, we also performed reversed phase chromatography to obtain more reliable mass increases.
 571 Under these conditions, the mass increases were lower than under native conditions. However, at very
 572 high irradiation doses, the chemical modifications were so numerous that it was sometimes impossible
 573 to measure a molecular weight.



574

575

576 **Fig. 11. Effect of irradiation doses on molecular mass of some ancestral and present-day**

577 **MalDHs.** The molecular mass changes detected by mass spectrometry are expressed in Dalton for each

578 enzyme investigated. Ancestral MalDHs: LMCA/A35 (estOGT_{MalDH} = 84°C), red triangles,

579 *Methanococcaceae/A19* (estOGT_{MalDH} = 73°C), blue triangles. Modern MalDHs: *M. form* (OGT = 75°C),

580 orange circles, *M. jann* (OGT = 85°C), black circles, *M. kand* (OGT = 98°C, outgroup), open circles.

581

582 9) Effect of γ -irradiation induced oxidation on MalDH enzymatic activity.

583 We then investigated the effect of irradiation on the enzymatic capacity of some ancestral and extant

584 MalDHs from *Methanococcales* (Fig. 12). After irradiation, all samples were stored at 25°C prior to

585 enzymatic assay. All assays were performed at 70°C, with the exception of *Methanococcaceae/A19* and

586 *S. rumi* LDH for which the temperature was 60°C. With *S. rumi* LDH, taken as a mesophilic example,

587 the data showed it was strongly deactivated with a maximum activity value of 10% after irradiation at

588 420 Gy. The ancestral and modern methanococcales MalDHs shared a common tendency to deactivate

589 as the irradiation dose increased. The MalDH *Methanococcaceae/A19* completely lost its activity at

590 4,080 Gy, suggesting that it was the most sensitive ancestral MalDH in *Methanococcales*. The data did

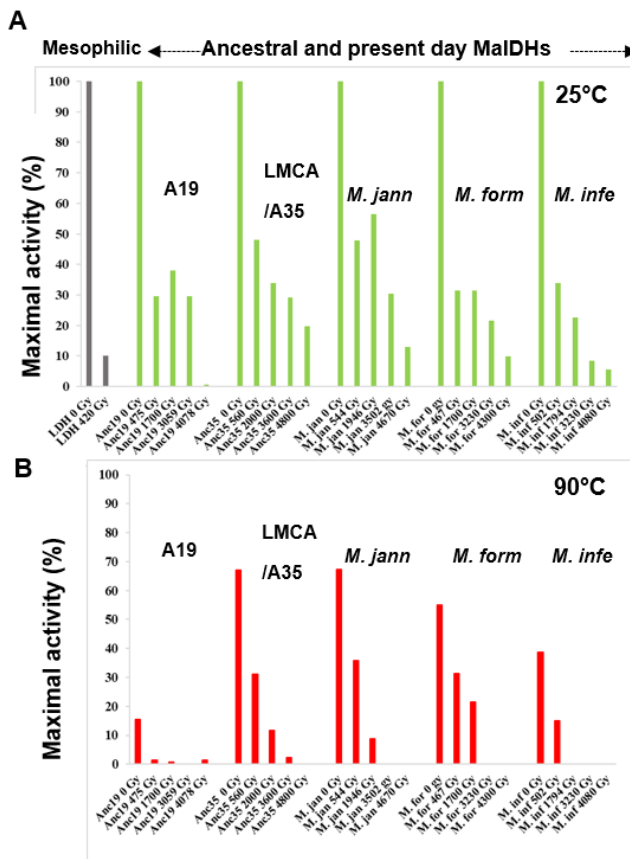
591 not allow us to distinguish between two possible explanations for the deactivation. In the first one, only

592 a fraction of the enzymes was inactivated, the rest being able to maintain full activity. In the second, the

593 activities of all the enzyme molecules are modified (without loss of structure, as previously monitored by

594 CD), so that their enzymatic parameters (maximum rate and/or affinities for substrate and coenzyme)

595 were altered in a way that reduces their efficiency. The more oxidized the residues, the less effective
 596 the enzymes. Since the reactive species are produced homogeneously in solution during irradiation, the
 597 second scenario was the most likely. We decided to incubate the irradiated and non-irradiated enzymes
 598 at 90°C for 15 min and monitor the activity again to explore the effect of an additional physicochemical
 599 stress (Fig. 12B). The data showed that the ancestor of *Methanococcaceae*/A19 was completely
 600 inactivated, whereas the other MalDHs retained a low enzymatic capacity. It is therefore reasonable to
 601 assume that incubation at high temperature selected for a population of conformers with reduced
 602 enzymatic capacity.



603
 604 **Fig.12. Effect of γ -irradiation dose on enzymatic activity.** LMCA/A35 (estOGT_{MalDH} = 84°C),
 605 *Methanococcaceae*/A19 (estOGT_{MalDH} = 73°C), *M. form* (OGT = 75°C), *M. jann* (OGT = 85°C), *M. infe*
 606 (OGT = 85°C). **(A)** Changes in enzymatic activity (using OAA as substrate) of different methanococcales
 607 MalDHs after exposure to different doses of γ -irradiation. *S. rumi* LDH (using pyruvate as substrate) was
 608 used to illustrate the behavior of an enzyme from a mesophile. The results are expressed as % of the
 609 enzymatic activity recorded before irradiation (0 Gy) for each enzyme. **(B)** Effect of an additional
 610 incubation at 90°C on irradiated and non-irradiated samples.

611

612 In the case of the MalDH from the ancestor of *Methanococcaceae/A19* (OGT = 67°C), which has an A-
613 T_{opt} of 60°C, the amplitude of the effect was maximal, whereas for the other enzymes, which have an A-
614 T_{opt} greater than 80°C, the effect was reduced. When the enzymes were modified by irradiation, the
615 effect of thermal treatment on the population of the conformers with lower activity was enhanced.
616 Consequently, at high doses, all enzymes were inactive after thermal treatment. Our protocol confirmed
617 that the ancestor of *Methanococcaceae/A19* behaves differently from enzymes with hyperthermophilic
618 properties.

619 In a previous study using LDHs from organisms with different OGTs, it was observed that none of the
620 amino acids involved in catalysis were damaged by oxidation after irradiation, although the enzymes
621 were inactive (Halgand, et al. 2020). It was proposed that this phenomenon is based on the ability to
622 maintain LDHs in a conformational state unproductive for catalysis (Halgand, et al. 2020). We tried to
623 apply the same experimental strategy to the different MalDHs analyzed here. Unfortunately, we were
624 unable to find efficient conditions to induce limited proteolysis on the irradiated sample, which prevented
625 the use of mass spectrometry on the various fragments obtained by this method and thus the
626 identification of the amino acids affected by reactive species.

627

628 **10) Thermal and irradiation stresses alter the substrate-binding affinity of MalDHs.**

629 Several works have shown that the loss of enzymatic activity during thermal or chemical unfolding
630 precedes large conformational changes (Yao, et al. 1984; Xie and Tsou 1987; Zhang, et al. 1993). Later,
631 using a set of eight enzymes, the mechanism by which they lose activity as temperature rises was
632 analyzed, leading to the conceptualization of the equilibrium model (reviewed in (Daniel and Danson
633 2013)). The model states that the transition from the folded to the unfolded state, proceeds through an
634 intermediate folded state, which can sample two (active or inactive) catalytic site conformational sub
635 states. Due to their intrinsic dynamical properties, temperature-driven activity shifts of enzymes could
636 be therefore the consequence of (i) catalytic site reorganizations that affect the pKa of amino acids
637 involved in catalysis, (ii) the maximal velocity rate or (ii) modifications in the substrate-binding affinity.
638 Our findings are consistent with this model. Indeed, we have shown that the loss of MalDH activity was
639 not necessarily associated with unfolding. This was clearly the case for the MalDH of the
640 *Methanococcaceae/A19* ancestor, which loses activity at temperatures above 60°C, while remaining

641 folded. This was also the case for all MalDHs after irradiation. In order to understand the mechanisms
 642 of inactivation, we recorded the kinetic parameters of three hyperthermophilic MalDHs (*M.jann*, *M.infe*,
 643 and *M. form*) at 75°C and the *Methanococcaceae*/A19 ancestor at 60°C (Table 2).

644

645 **Table 2.** Kinetic parameters of MalDH from present-day *Methanococcales* (*M. jann*, *M. infe*, and
 646 *M.form*), the ancestors of *Methanococcales* (LMCA/A35), *Methanocaldococcaceae* (A34) and
 647 *Methanococcaceae* (A19).

Taxa	Experimental temperatures	“Stress”	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ .s ⁻¹)
<i>M. infe</i>	75°C		0.9	1,150	1.3 10 ⁶
<i>M. jann</i>	75°C		1.1	1,031	0.9 10 ⁶
<i>M. form</i>	75°C		0.8	1,189	1.4 10 ⁶
<i>Methanococcaceae</i> (A19) ancestor	60°C		0.6	1,143	1.9 10 ⁶
	75°C	Higher than A-T _{opt}	7.7	686	8.9 10 ⁴
<i>Methanococcales</i> ancestor (A35)	75°C		0.3	960	3.2 10 ⁶
	75°C	Irradiation at 2,000 Gy	2.9	732	2.5 10 ⁵
<i>Methanocaldococcaceae</i> (A34) ancestor	75°C		1.0	915	0.9 10 ⁶

648

649 Under these conditions, k_{cat} values between 915 and 1189 s⁻¹ were quite similar for all enzymes and
 650 close to typical values for MalDHs (Shimozawa, et al. 2022). Phylogenetic analyses have shown that
 651 methanococcales MalDHs belong to an intermediate group of enzymes between the clade of tetrameric
 652 MalDH type 3 and LDH (Brochier-Armanet and Madern 2021). Present day and ancestral
 653 methanococcales MalDHs have K_m values for OAA in the range of 0.3 to 1.1 mM, indicating a lower
 654 affinity than that of MalDH type 3 (Brochier-Armanet and Madern 2021), an observation that was
 655 previously reported for another hyperthermophilic MalDH from the intermediate group (Roche, et al.
 656 2019). A detailed description of this phenomenon required the acquisition of structural information on
 657 the respective enzymes used in this study, work that is currently underway.

658 We then recorded parameters using *Methanococcaceae*/A19 MalDH at 75°C, a temperature higher than
 659 its A-T_{opt}. We also analyzed the effects of an irradiation dose of 2,000 Gy, using LMCA/A35 MalDH as
 660 the target enzyme. The data showed that these two enzymes became less efficient, as indicated by their
 661 k_{cat}/K_m values, which fall well below the values recorded at A-T_{opt}. This was mainly because their K_m
 662 values for OAA increased strongly (Table 2). This might indicate that their respective substrate binding

663 site was in an unfavorable configuration to accommodate OAA. A previous work on an hyperthermophilic
664 LDH showed that irradiation-induced inactivation maintains the enzyme in a low-affinity pyruvate-binding
665 state, preventing the enzyme from accessing its competent state for catalysis (Halgand, et al. 2020).

666

667 **Discussion**

668 One of the aims of this study was to experimentally investigate the process of enzyme thermoadaptation
669 using *Methanococcales* MalDH as model. The inference of OGT along the *Methanococcales* phylogeny
670 using present-day MalDH sequences is consistent with the seminal work performed on complete
671 proteomes by Lecocq et al. (2021), indicating that the thermoadaptation was so strong that it is still
672 detectable today at the level of a single protein. Specifically, we showed that (i) the LMCA lived in a hot
673 environment and (ii) some of its descendants evolved to colonize cooler environmental niches. We then
674 sought to determine whether there was a good correlation between the thermal properties of the MalDH
675 model enzyme and the OGT of ancestral and extant *Methanococcales*. We found that the ancestral
676 MalDH in *Methanococcales* was both a hyperthermostable and thermoactive enzyme, confirming the
677 hot origin of this archaeal order. We also observed that although numerous amino acid substitutions
678 have accumulated in the sequences of present-day MalDH since the LMCA, their effects have been
679 buffered to maintain the original phenotype. We observed that the first step in the evolutionary process
680 leading to the mesophilic lineages was different. In fact, the MalDH inferred in the
681 *Methanococcaceae/A19* ancestor showed a maximal activity at moderate temperature without
682 compromising its high thermal stability. Furthermore, the $A-T_{opt}$ value of this enzyme (60°C) is close to
683 the $estOGT_{MalDH}$ of the *Methanococcaceae/A19* ancestor (73°C). This correlation shows that monitoring
684 the $A-T_{opt}$ of enzymes may be more reliable than monitoring their conformational stability. The behavior
685 of the *Methanococcaceae/A19* ancestor MalDH is consistent with the microstate ensemble model of
686 protein structure. This model proposes that temperature samples a set of conformational sub-states that
687 are structurally close to the native state, but for which the catalytic efficiency and substrate binding
688 capacity are different (Wrabl, et al. 2011). Molecular dynamics simulations have shown that the onset
689 of the activity drop, which can occur well below the melting temperature, is determined by the activation
690 energy between the productive and unproductive conformers (Maffucci, et al. 2020). Consequently, any
691 shifts in catalytic activation energy induced by mutations will alter the balance between these states,

692 allowing thermal adaptation to proceed (Dong, et al. 2018; Fusco, et al. 2022; Koenekoop and Aqvist
693 2023).

694 Here, the substitutions selected in the ancestral MalDH of *Methanococcaceae*/A19 have most likely
695 lowered the energy barriers to access catalytically competent conformers without the need for high
696 thermal energy, allowing for a rapid and efficient adaptive response. The characterization of this MalDH
697 also allowed us to propose that incompetent conformers sampled at high temperature exhibit local
698 perturbations within their catalytic site, which reduce substrate binding efficiency. In the context of these
699 observations, the introduction of mesophilic-like mutations, using a MalDH from a modern
700 hyperthermophile as a scaffold, shifts the $A-T_{opt}$ towards low temperatures with a limited effect on
701 thermal stability. Our work illustrates that the thermal adaptation of enzymes is much more complex than
702 a uniform shift in the melting and optimum activity temperatures. Structural and dynamic studies are
703 being carried out on several of the enzymes used in this work to go further in the understanding of
704 thermal adaptation process. We also investigated the relationship between high thermal stability and a
705 putative ability to resist γ -irradiation induced unfolding. We found that the MalDH inferred in the
706 LMCA/A35 ancestor is a polyextremophilic enzyme capable of withstanding both strong thermal and
707 radiative stress. In a previous study, we proposed that resistance to γ -irradiation might be an indirect
708 consequence of the overall stability of the proteins rather than a specific adaptation (Halgand, et al.
709 2020). Consistent with the present work, it is reasonable to propose that adaptation to elevated
710 temperature is mediated by increased stability of the protein, which may also favor resistance to γ -
711 irradiation. Further studies are needed to gain insight into this phenomenon. The ability to resist to
712 γ -irradiation induced unfolding has been retained (but hidden) in modern hyperthermophilic enzymes,
713 mainly because of the strong selective pressure continuously exerted by environmental temperature.

714

715 **Materials and Methods**

716 **Phylogenetic analyses and ancestral MalDH sequence reconstruction**

717 MalDH protein sequences were retrieved from the *nr* protein sequence database at the NCBI. The
718 dataset encompassed MalDH sequences from ten *Methanococcaceae*: *Methanococcus maripaludis* C5
719 (YP_001097480), *Methanococcus maripaludis* C6 (YP_001548297.1), *Methanococcus maripaludis* C7
720 (YP_001330878.1), *Methanococcus maripaludis* S2 (NP_987765.1), *Methanococcus maripaludis* X1
721 (YP_004742269.1), *Methanococcus vannielii* SB (YP_001324052.1), *Methanococcus voltae* A3

722 (YP_003707533.1), *Methanothermococcus thermolithotrophicus* (WP_018154699.1), *Methanococcus*
723 *aeolicus* Nankai-3 (YP_001324807.1), and *Methanothermococcus okinawensis* IH1 (YP_004576638.1),
724 and eight *Methanocaldococcaceae*: *Methanotorris formicicus* (WP_007044377.1), *Methanotorris igneus*
725 Kol5 (YP_004483657.1), *Methanocaldococcus fervens* AG86 (YP_003128040.1), *Methanocaldococcus*
726 *jannaschii* DSM 2661 (NP_247466.1), *Methanocaldococcus* sp. FS406-22 (YP_003459168.1),
727 *Methanocaldococcus vulcanius* M7 (YP_003246708.1), *Methanocaldococcus villosus*
728 (WP_004592381.1), and *Methanocaldococcus infernus* ME (YP_003616626.1), and seven sequences
729 from other *Methanomada* lineages: six *Methanobacteriales* (*Methanobacterium* sp. AL-21
730 (YP_004289802.1), *Methanobacterium* sp. SWAN-1 (YP_004520598.1), *Methanothermobacter*
731 *marburgensis* str. Marburg (YP_003849548.1), *Methanothermobacter thermautotrophicus* str. Delta H
732 (NP_275331.1), *Methanobrevibacter ruminantium* M1 (YP_003423997.1), *Methanosphaera*
733 *stadtmanae* DSM 3091 (YP_447713.1)), and one member of *Methanopyrales* (*Methanopyrus kandleri*
734 AV19 (NP_614352.1)). Sequences were aligned using MAFFT v7.505 with the accurate option L-INSI
735 (Kato and Standley 2013). A maximum likelihood phylogeny was inferred using PhyML with the LG
736 model, a gamma distribution to consider across site rate variations (four discrete categories), as
737 suggested by ModelFinder implemented in IQ-TREE (version 2.3.4) according to the Bayesian
738 Information Criterion (Nguyen, et al. 2015; Kalyaanamoorthy, et al. 2017), and the NNI+SPR option to
739 optimize the tree topology. The length of the branches of the resulting tree was optimized using BPPML
740 with the branch heterogeneous model COALA, assuming the non-stationarity of the evolutionary
741 process, and with a gamma distribution to model the heterogeneity of evolutionary rates among sites
742 (Dutheil and Boussau 2008; Groussin, et al. 2013). At each node of the *Methanococcales* phylogeny,
743 ancestral sequences were reconstructed using BPPANCESTOR (Dutheil and Boussau 2008), with the
744 evolutionary parameters estimated with BPPML and the optimized ML tree as input. For details
745 regarding the parameters used for BPPML and BPPancestor (see Supplementary Table S4). As most
746 ASR methods, BPPANCESTOR ignores gaps. However, MalDH sequences of *Methanococcales*
747 contains only two one aa-gaps, at positions 262 and 267. The first one corresponds to a deletion found
748 only in *Methanocaldococcus villosus* and *Methanocaldococcus infernus*, while the second is shared by
749 *Methanococcus maripaludis*, *Methanococcus vannielli*, *Methanococcus voltae*, and
750 *Methanothermococcus thermolithotrophicus*. Since the number of gaps in the sequences is small and
751 their positioning in the alignment is clear, we expect that they should not have a major impact ASR. The

752 multiple alignment of present-day MalDH, the ML tree file, and ancestral MalDH sequences are provided
753 as online supplementary material.

754

755 **Estimation of present-day and ancestral OGT**

756 We performed a correspondence analysis (CA) of the amino acid content of the MalDH sequences using
757 the R libraries ADE4, APE and NLME (Dray and Dufour 2007; Paradis and Schliep 2019; Pinheiro and
758 Bates 2024). Note that we did not consider tryptophan (W) because almost all MalDH protein sequences
759 lack this amino acid, with the exception of MalDH from *M. okin* IH1, *M. volt* A3, and *M. aeol* Nankai-3,
760 which contain a single tryptophan. Pearson's product-moment correlation were computed with ADE4.

761 To build the molecular thermometer, we used four linear models: the standard linear model (SLM), and
762 models by Martins and Hansen (MAM) (Martins and Hansen 1997), by Grafen (GRM) (Grafen 1989),
763 and by Felsenstein (FBM) (Felsenstein 1985), the three latter accounting for phylogenetic relationships
764 (Paradis and Schliep 2019). The models were used to correlate the scores of the MalDH protein
765 sequences on the first axis of the CA with the OGT of the present-day Methanococcales strains. For
766 MAM, FBM, and GRM models, we used the ML tree of the MalDH as input.

767 The intercept and slope coefficients of the regression were: 63.45098 and -195.4594 (SLM); 62.48197
768 and -182.24615 (MAM); 63.17399 and -173.70077 (GRM); 62.3114 and -71.0827 (FBM). The reliability
769 of the models was tested by predicting OGT of the 17 methanococcales strains for which OGT are
770 known. They were then used to predict ancestral OGT at each node of the *Methanococcales* phylogeny
771 using the inferred ancestral MalDH protein sequences. OGTs were also predicted applying the standard
772 linear model on the content of amino acids I, V, Y, R, E and L highlighted by Zeldovich et al (2007).

773

774 **Gene synthesis and protein expression and purification.**

775 All the ancestral and present day MalDH gene sequences studied in this work were subjected to codon
776 optimization for over expression in *Escherichia coli*. The genes have been synthesized by the Genecust
777 company. In the case of the two *M. jann* MalDH mutants, an extension of six histidine (His tag) was
778 fused to their C-terminus in order to facilitate the purification using affinity chromatography. The other
779 genes were synthesized without any extension.

780 All the genes were sub-cloned into the pet 20a vector between the *NdeI* and *BamHI* restriction sites.

781 The constructs were transformed into BL21(DE3) plysS cells and selected on LB-agar plate containing

782 100 µg ml⁻¹ ampicilin. A single colony was cultured overnight at 37°C in 100 ml LB medium containing
783 100 µg ml⁻¹ ampicilin. 25 ml of this culture were transferred into 1L of LB medium containing 100 µg ml⁻¹
784 1 ampicilin. The cells were grown at 37°C until an OD 600 of 0.6 was reached. The temperature was
785 quickly lowered to 4°C and the cells were kept on ice for three hours, then IPTG was added at a final
786 concentration of 0.2 mM to induce expression, which was continued overnight at 20°C. The bacterial
787 cells were then harvested by centrifugation. The pellets were suspended in 50 mM Tris-HCl pH 7, 50
788 mM NaCl (Buffer A) and frozen at -20.

789 After thawing of the cells, DNase at 5 µg ml⁻¹ was added to the suspension. The cell suspension was
790 sonicated with a Branson sonicator at 25% intensity during three cycles of 1 min. The extract was
791 centrifuged at 18,000 g for 30 min at 4°C. The supernatant was filtered on a 0.45 µm (Amicon). With the
792 two His-tagged mutants, the extract was passed through a 5mL HisTrap Q HP column (GE Healthcare
793 Life Science). The column was washed with 20 mL of 50 mM Tris-HCl pH7, 200 mM NaCl and by 25
794 mL of 20 mM imidazole pH 7, 200 mM NaCl. Then, the two *M. jann* MalDH mutants were eluted with
795 300 mM imidazole pH 7, 50 mM NaCl. The resulting enzyme preparation was concentrated and dialyzed
796 in Buffer A using Amicon Ultra-30 concentrators. The enzyme was then loaded on an anion exchange
797 Uno Q (BioRad) and eluted with a linear gradient from 0 to 1M NaCl buffered by 50 mM Tris-HCl pH7.
798 The active fractions were pooled, concentrated and loaded on a size exclusion chromatography column
799 (SEC) equilibrated with buffer A. An Enrich 650 (Biorad) column was used.

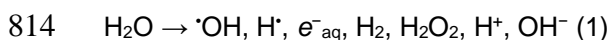
800 With all the other enzymes without His-tag extension, the crude extract obtained after sonication was
801 incubated 15 min at 70°C and centrifugated at 18,000 g for 30 min at 4°C. The supernatant was filtered
802 on a 0.45 µm (Amicon). The clear supernatant was loaded on a Q sepharose (2x5cm) and eluted using
803 a linear gradient of gradient from 0 to 1M NaCl in buffer A. The active fractions were pooled,
804 concentrated and loaded on the SEC equilibrated with buffer A. Fractions containing the enzymes under
805 study were concentrated and stored at -80°C in buffer A with 20% glycerol. In the case of *M. kand* MalDH
806 the SEC was equilibrated with 0.4 M KCl, Tris-HCl 50 mM pH7 (Brochier-Armanet and Madern 2021).

807 **Protein irradiation**

808 The proteins were dissolved to a final concentration of 0.3 mg/mL (2.4 µM) in 50 mM Tris-HCl pH 7+KCl
809 0.4M. They were irradiated in N₂O atmosphere in the panoramic ⁶⁰Co γ-source IL60PL (Cis-Bio
810 International (France)) at the University Paris-Saclay (Orsay, France). The absorbed dose was

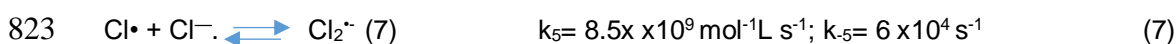
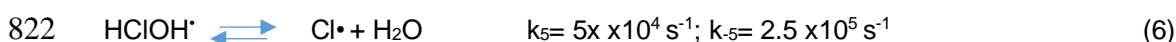
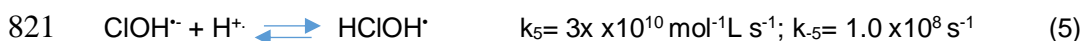
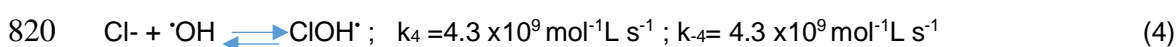
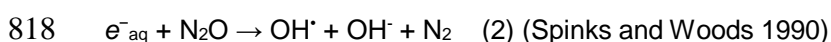
811 determined by Fricke dosimetry (Spinks and Woods 1990). The doses were comprised between 500
812 and 10,000 Gy, delivered with a dose rate up to 31.8 Gy/min.

813 Interaction of ionizing radiations with water leads to the formation of free radicals following equation 1:



815 In N_2O atmosphere, e^-_{aq} is scavenged leading to the formation of OH radicals according to equation
816 (2)).

817 in the presence of Tris buffer and KCl and in an atmosphere of N_2O , the following reactions take place:



824 Thanks to reaction (2) the yield of $\cdot\text{OH}$ radicals is doubled: $0.54 \mu\text{mol J}^{-1}$. The total amount of $\cdot\text{OH}$ radicals
825 is proportional to the dose, i.e., the total $[\cdot\text{OH}]$ delivered to the solution was between 0.27 mM and 5.4
826 mM. The H_2O_2 yield is lower ($0.07 \mu\text{mol J}^{-1}$). In the presence of Tris buffer and KCl, $\cdot\text{OH}$ radicals can
827 react either with the protein or with the buffer or with Cl^- ions. Reaction (3) produces carbon-centered
828 radicals that are reductants and usually poorly reactive toward proteins.

829 At pH around neutrality equilibria (4) to (7) are involved. As a consequence, a mixture of $\text{OH}^\cdot - \text{Cl}_2^{\cdot-}$ is
830 created in the solution. Both are oxidizing free radicals, $\text{Cl}_2^{\cdot-}$ being less reactive than OH radicals (Valiev,
831 et al. 2009).

832

833 **Circular dichroism spectroscopy**

834 CD spectra were recorded at 25°C , on a MOS 500 circular dichroism spectropolarimeter (BioLogic)
835 using a quartz cuvette with a path-length of 0.1 cm. The spectra were recorded at 50 nm / min with a
836 bandwidth of 2.0 nm and an integration time of 6 sec, over the wavelength range 190-260 nm.

837

838 **Enzymatic assays**

839 The standard assay was performed at 60°C when using Anc 19 MalDH and at 70°C with all the other
840 enzymes used in this study, respectively. The standard assay mixture of 0.5 ml contained 0.2 mM NADH,

841 the various enzymes under study and 50 mM Tris-HCl pH 7. A 3 minutes pre –incubation period was
842 applied to the mixture. Then, the reaction was started by the addition of the oxaloacetate solution (0.8
843 mM final concentration) and the dA/dt was immediately followed during 120 sec by recording the
844 decrease of NADH absorbance (A) at 340 nm in a thermostated Jasco spectrophotometer.

845 To record the temperature-dependent enzymatic activity profile, the reaction mixture for each enzyme
846 was incubated at various temperature ranging from 25° to 95°C. The measurements were done in
847 triplicates. The data were expressed as a percentage of the maximal activity recorded for a given
848 enzyme.

849 To record the effect of irradiation on enzymatic activity, an aliquot of each enzyme was taken from the
850 various irradiated solutions. The data were expressed in % with respect to the activity recorded using
851 non-irradiated samples.

852 Our protocol allows comparing directly the effects of different treatments on activity.

853

854 **Size-exclusion chromatography (SEC) coupled to mass spectrometry (MS).**

855 SEC-MS coupling was set up using a TSK 3000 SW gel filtration column (Interchim, 1 x 300 mm). Elution
856 buffer was 100 mM ammonium acetate, pH 7, infused at 0.02 ml/min flow rate and directly injected in
857 the electrospray ionization source. Ten μ l of standard and irradiated protein solution initially at 90 μ M
858 concentration were loaded on the SEC column. Mass spectrometry experiments were performed on a
859 QToF instrument (Synapt G2-Si, Waters Company, Manchester, UK). Calibration was performed using
860 sodium trifluoroacetate. The typical standard error was below 5 ppm. The native conditions used to
861 detect the intact tetramer require soft ionization parameters. Reliable and accurate detection of native
862 proteins were obtained by using an optimization procedure (Hall and Robinson 2012; Van der Rest and
863 Halgand 2017). In brief, for analysis under non-denaturing conditions the following instrumental
864 parameters were used: capillary voltage = 4.5 kV, sampling cone = 150 V, source offset, 150 V,
865 nebulization gas pressure = 5 bar, source temperature, 40 °C, desolvation temperature, 75 °C. Data
866 were processed with MassLynx.

867

868 **Reverse phase chromatography (RP) coupled to mass spectrometry (MS).**

869 Reverse phase chromatography was performed on an Acquity UPLC CSH C18 with a 2.0x100 mm.
870 Acetonitrile gradient was a linear increase of this last between 5 to 90 % in 35 min with a flow rate of 0.2

871 ml/min flow rate. Data were acquired on a Synapt instrument (Waters, Manchester, UK) equipped with
872 an electrospray ionization source. In brief, instrumental parameters were the following: capillary voltage
873 = 3.5 kV; source voltage = 50 V; source offset = 50 V; desolvation gas = 5 bar and desolvation
874 temperature was 650 °C. Calibration was performed using sodium trifluoroacetate. The typical standard
875 error was below 5 ppm.

876 **Author contributions**

877 DM and CBA conceived the project. CBA and ABD conducted evolutionary analyses. DM, SAA and SC,
878 CB performed samples preparation and biochemical characterization. DM and SMS analyzed thermal
879 properties. CHL performed irradiation experiments. FH performed mass spectrometry measurements.
880 DM and CBA wrote the first draft manuscript. All the authors analyzed the data and wrote the final
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896 **Conflict of interest**

897 The authors declare that they have no conflicts of interest with the contents of this article. The data are
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899 **Data Availability**

900 The data underlying this article are available in the online supplementary material.

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