Supplementary information

Table S1. Primers used in this study.

Name	Sequence (5´ → 3´)
KHY-F	ttccatatgatggaggcgacccttcc
KHY-R	ttcgaattcctagcgccagaggaccac
Y194H-F	gctacccctcccaccgcctgccc
Y194H-R	gggcaggcggtgggaggggtagc
K166R-F	tgaccccgagagggtgagggcgatcct
K166R-R	aggatcgccctcaccctctcggggtca
H174R-F	ggcttccttcccgcgccgccatccc
H174R-R	gggatggcggcgcgggaaggaagcc

Restrictions sites are shown underlined.

Table S2. Plamids used in this study.

Plasmid	Description	Reference
pET22b(+)	Amp^r, lacl. Expression of genes in *E. coli*, dependent of T7 phague RNA polymerase	Novagen
pET22b-KHY	Amp^r. NOX overexpression without C-terminal His-tag (NdeI/EcoRI). DNA fragment (TTC0057 gene from *T. thermophilus* HB27) amplified with KHY-F and KHY-R primers	[1]
pET22b-KHH	Amp^r. KHH variant overexpression without C-terminal His-tag (NdeI/EcoRI). The mutation was introduced with Y194H-F and Y194H-R primers, replacing Y/H in Nox postion 194 and using pET22b-KHY as a template	This study
pET22b-RHY	Amp^r. RHY variant overexpression without C-terminal His-tag (NdeI/EcoRI). The mutation was introduced with K166R-F and K166R-R primers, replacing K/R in NOX position 166 and using pET22b as a template	This study
pET22b-KRY	Amp^r. KRY variant overexpression without C-terminal His-tag (NdeI/EcoRI). The mutation was introduced with H174R-F and H174R-R primers, replacing H/R in Nox position 174 and using pET22b-KHY as a template	This study
pET22b-RHH	Amp^r. RHH variant overexpression without C-terminal His-tag (NdeI/EcoRI). The mutation was introduced with K166R-F and K166R-R primers, replacing K/R in NOX position 166 and Y/H in NOX position 194 and using pET22b-KHH as a template	This study
pET22b-KRH	Amp^r. RRH variant overexpression without C-terminal His-tag (NdeI/EcoRI). The mutation was introduced with H174R-F and H174R-R primers and using pET22b-KHY as a template.	This study
pET22b-RRH	Amp^r. RRH variant overexpression without C-terminal His-tag (NdeI/EcoRI). The mutation was introduced with K166R-F and K166R-R primers and using pET22b-KRH as a template.	This study

Table S3. Quantification of flavin cofactor content.

Tt-NOX variant	**Cofactor**	**HPLC method (µM)**	**Spectrophotometric method (µM)**	**Enzyme molecules containg flavin cofactor (%)**
K166/R174/Y194	FAD	traces	ND	-
	FMN	1.15	1.1	5.5
K166/H174/Y194	FAD	traces	ND	-
	FMN	5.6	5.84	29

Experiments were performed with 20 µM of purified protein. Flavin content was determined by HPLC and spectrophotometric method as described in methods section. ND: non-detectable.

Table S4. Structural alignment of Tt27-NOX using Dali Server. Best 50 results.

PDB id.	UNIPROT id.	Chain	Resolution (Å)	Name	Organism	Z-value	RMSd (Å)	id (%)
1nox	Q60049	A	1.59	NADH oxidase		33.6	0.0	100
3gbh	A0A0H2VHN8	A	2.00	Putative NAD(P)H:FMN Oxidoreductase	*Staphylococcus epidermidis ATCC 12228*	23.9	1.8	23
3bem	P96692	A	1.65	Putative NAD(P)H Nitroreductase YDFN	*Bacillus subtilis*	23.5	1.7	24
3ge6	B1YG32	A	1.70	Nitroreductase	*Exiguobacterium sibiricum*	23.3	2.0	28
3of4	Q5R179	B	1.90	FMN/FAD- and NAD(P)H-dependent Nitroreductase	*Idiomarina loihiensis L2TR*	23.2	1.9	21
3gag	Q8DVW4	B	1.70	Nitroreductase-like protein (smu.346)	*Streptococcus mutans*	22.6	2.1	25
2b67	A0A0H2UP38	B	1.90	Nitroreductase	*Streptococcus pneumoniae TIGR4*	22.3	2.3	27
4qly	U6C5W9	D		Enone reductase CLA-ER	*Lactobacillus plantarum*	22.1	2.4	23
1nec 1kqb 1kqc 1kqd 5j8d 5j8g	Q01234	A	1.95	Nitroreductase	*Eneterobacter cloacae*	20.2	2.6	25
1icu 3x22 3x21 1yki 1ylr 1ylu 1idt 1oo5 1oo6 1oon 1ooq 1icr 1icv 1ds7 1idt	P38489	C	1.80	Nitroreductase	*Escherichia coli BL21*	20.2	2.6	24
2hay	Q9A120	B	2.11	Putative NAD(P)H-Flavin Oxidoreductase	*Streptococcus pyogenes*	20.0	2.3	24

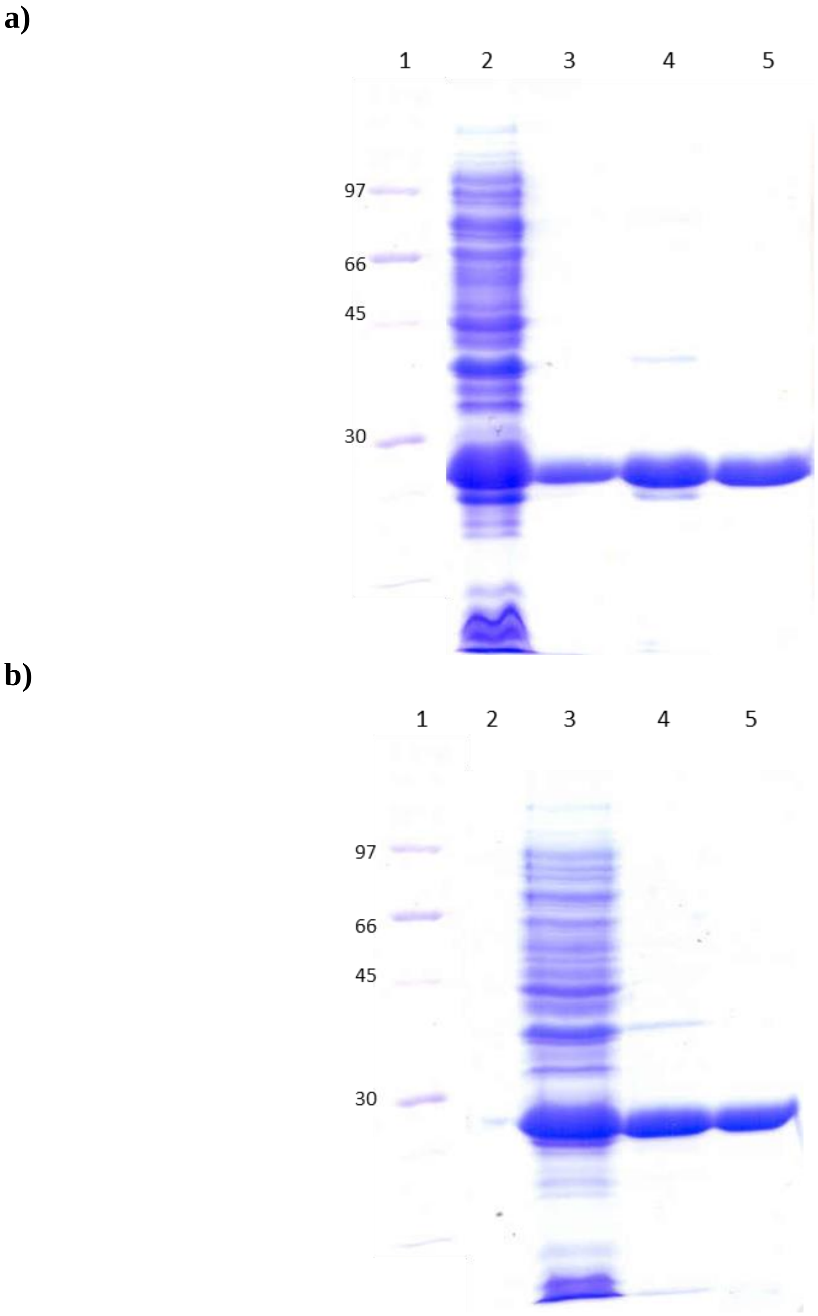
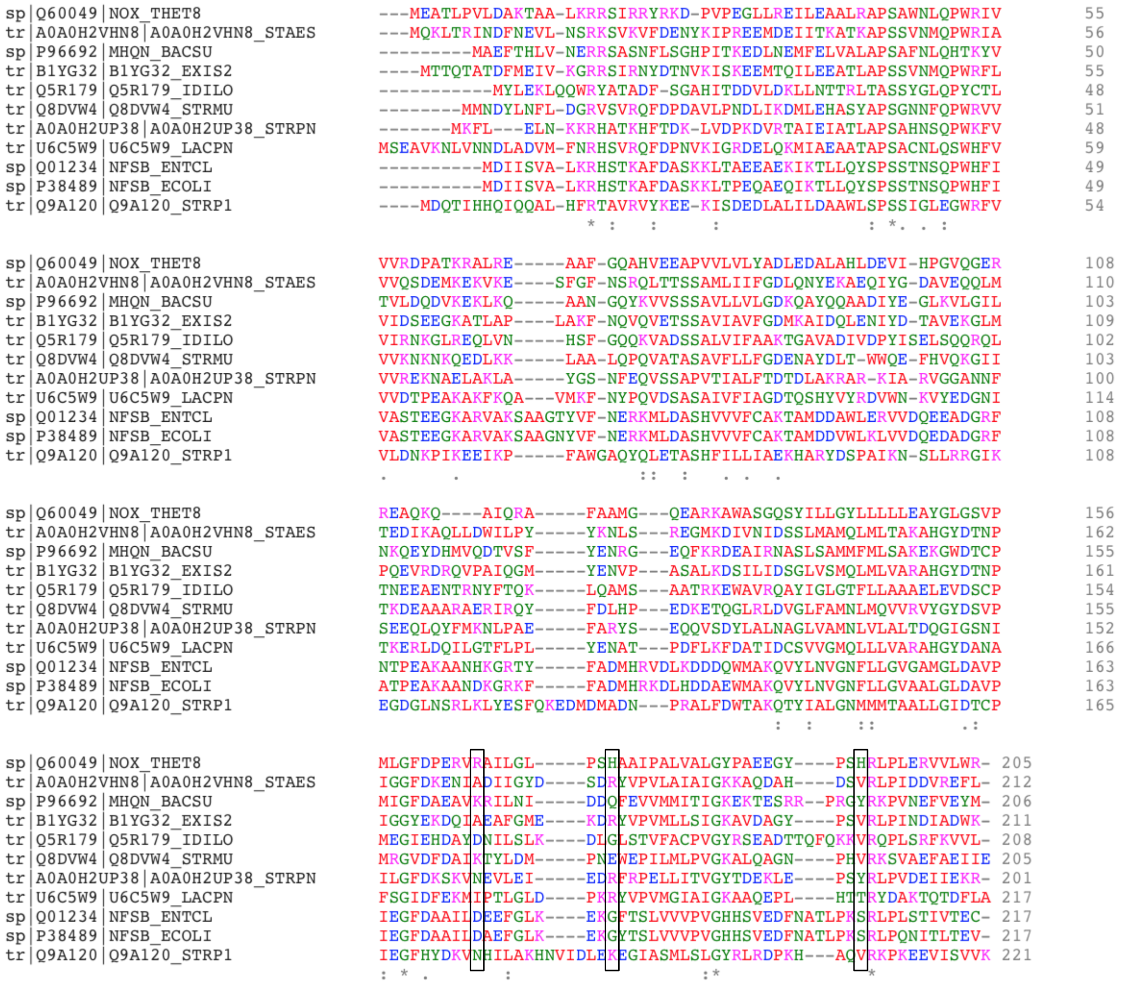


Figure S1. SDS-PAGE analysis of the purification process of the **a)** K166/H174/H194 and **b)** K166/R174/Y194 variants. **a)** Lanes: 1) Molecular weight marker (kDa); 2) Crude protein extract; 3) Supernatant after heat treatment at 80 ℃ for 45 minutes; 4) Supernatant after incubation in presence of PEI-Ag for 45 minutes; 5) Supernatant after incubation in the presence of DS-Ag for 45 minutes. **b)** Lanes: 1) Molecular weight marker (kDa); 2) Empty lane; 3) Crude protein extract; 4) Supernatant after heat treatment at 70 ℃ for 60 minutes; 5) Supernatant after incubation in presence of PEI-Ag for 45 minutes.



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sp|Q60049|NOX_THET8                 ---MEATLPVLDAKTAA-LKRRSIRRYRKD-PVPEGLLREILEAALRAPSAWNLQPWRIV        55
tr|A0A0H2VHN8|A0A0H2VHN8_STAES      ---MQKLTRINDFNEVL-NSRKSVKVFDENYKIPREEMDEIITKATKAPSSVNMQPWRIA        56
sp|P96692|MHQN_BACSU                ---------MAEFTHLV-NERRSASNFLSGHPITKEDLNEMFELVALAPSAFNLQHTKYV        50
tr|B1YG32|B1YG32_EXIS2              ----MTTQTATDFMEIV-KGRRSIRNYDTNVKISKEEMTQILEEATLAPSSVNMQPWRFL        55
tr|Q5R179|Q5R179_IDILO              -----------MYLEKLQQWRYATADF-SGAHITDDVLDKLLNTTRLTASSYGLQPYCTL        48
tr|Q8DVW4|Q8DVW4_STRMU              --------MMNDYLNFL-DGRVSVRQFDPDAVLPNDLIKDMLEHASYAPSGNNFQPWRVV        51
tr|A0A0H2UP38|A0A0H2UP38_STRPN      -------MKFL---ELN-KKRHATKHFTDK-LVDPKDVRTAIEIATLAPSAHNSQPWKFV        48
tr|U6C5W9|U6C5W9_LACPN              MSEAVKNLVNNDLADVM-FNRHSVRQFDPNVKIGRDELQKMIAEAATAPSACNLQSWHFV        59
sp|Q01234|NFSB_ENTCL                ----------MDIISVA-LKRHSTKAFDASKKLTAEEAEKIKTLLQYSPSSTNSQPWHFI        49
sp|P38489|NFSB_ECOLI                ----------MDIISVA-LKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFI        49
tr|Q9A120|Q9A120_STRP1              ----MDQTIHHQIQQAL-HFRTAVRVYKEE-KISDEDLALILDAAWLSPSSIGLEGWRFV        54
                                                        * :   :     :              : *. . :

sp|Q60049|NOX_THET8                 VVRDPATKRALRE-----AAF-GQAHVEEAPVVLVLYADLEDALAHLDEVI-HPGVQGER        108
tr|A0A0H2VHN8|A0A0H2VHN8_STAES      VVQSDEMKEKVKE----SFGF-NSRQLTTSSAMLIIFGDLQNYEKAEQIYG-DAVEQQLM        110
sp|P96692|MHQN_BACSU                TVLDQDVKEKLKQ-----AAN-GQYKVVSSSAVLLVLGDKQAYQQAADIYE-GLKVLGIL        103
tr|B1YG32|B1YG32_EXIS2              VIDSEEGKATLAP----LAKF-NQVQVETSSAVIAVFGDMKAIDQLENIYD-TAVEKGLM        109
tr|Q5R179|Q5R179_IDILO              VIRNKGLREQLVN-----HSF-GQQKVADSSALVIFAAKTGAVADIVDPYISELSQQRQL        102
tr|Q8DVW4|Q8DVW4_STRMU              VVKNKNKQEDLKK-----LAA-LQPQVATASAVFLLFGDENAYDLT-WWQE-FHVQKGII        103
tr|A0A0H2UP38|A0A0H2UP38_STRPN      VVREKNAELAKLA-----YGS-NFEQVSSAPVTIALFTDTDLAKRAR-KIA-RVGGANNF        100
tr|U6C5W9|U6C5W9_LACPN              VVDTPEAKAKFKQA---VMKF-NYPQVDSASAIVFIAGDTQSHYVYRDVWN-KVYEDGNI        114
sp|Q01234|NFSB_ENTCL                VASTEEGKARVAKSAAGTYVF-NERKMLDASHVVVFCAKTAMDDAWLERVVDQEEADGRF        108
sp|P38489|NFSB_ECOLI                VASTEEGKARVAKSAAGNYVF-NERKMLDASHVVVFCAKTAMDDVWLKLVVDQEDADGRF        108
tr|Q9A120|Q9A120_STRP1              VLDNKPIKEEIKP-----FAWGAQYQLETASHFILLIAEKHARYDSPAIKN-SLLRRGIK        108
                                    .      .                 ::  :   . .  .

sp|Q60049|NOX_THET8                 REAQKQ----AIQRA-----FAAMG---QEARKAWASGQSYILLGYLLLLLEAYGLGSVP        156
tr|A0A0H2VHN8|A0A0H2VHN8_STAES      TEDIKAQLLDWILPY-----YKNLS---REGMKDIVNIDSSLMAMQLMLTAKAHGYDTNP        162
sp|P96692|MHQN_BACSU                NKQEYDHMVQDTVSF-----YENRG---EQFKRDEAIRNASLSAMMFMLSAKEKGWDTCP        155
tr|B1YG32|B1YG32_EXIS2              PQEVRDRQVPAIQGM-----YENVP---ASALKDSILIDSGLVSMQLMLVARAHGYDTNP        161
tr|Q5R179|Q5R179_IDILO              TNEEAENTRNYFTQK-----LQAMS---AATRKEWAVRQAYIGLGTFLLAAAELEVDSCP        154
tr|Q8DVW4|Q8DVW4_STRMU              TKDEAAARAERIRQY-----FDLHP---EDKETQGLRLDVGLFAMNLMQVVRVYGYDSVP        155
tr|A0A0H2UP38|A0A0H2UP38_STRPN      SEEQLQYFMKNLPAE-----FARYS---EQQVSDYLALNAGLVAMNLVLALTDQGIGSNI        152
tr|U6C5W9|U6C5W9_LACPN              TKERLDQILGTFLPL-----YENAT---PDFLKFDATIDCSVVGMQLLLVARAHGYDANA        166
sp|Q01234|NFSB_ENTCL                NTPEAKAANHKGRTY-----FADMHRVDLKDDDQWMAKQVYLNVGNFLLGVGAMGLDAVP        163
sp|P38489|NFSB_ECOLI                ATPEAKAANDKGRKF-----FADMHRKDLHDDAEWMAKQVYLNVGNFLLGVAALGLDAVP        163
tr|Q9A120|Q9A120_STRP1              EGDGLNSRLKLYESFQKEDMDMADN---PRALFDWTAKQTYIALGNMMMTAALLGIDTCP        165
                                                                          :  :    ::        .:

sp|Q60049|NOX_THET8                 MLGFDPERVRAILGL-----PSHAAIPALVALGYPAEEGY----PSHRLPLERVVLWR- 205
tr|A0A0H2VHN8|A0A0H2VHN8_STAES      IGGFDKENIADIIGYD----SDRYVPVLAIAIGKKAQDAH----DSVRLPIDDVREFL- 212
sp|P96692|MHQN_BACSU                MIGFDAEAVKRILNI-----DDQFEVVMMITIGKEKTESRR--PRGYRKPVNEFVEYM- 206
tr|B1YG32|B1YG32_EXIS2              IGGYEKDQIAEAFGME----KDRYVPVMLLSIGKAVDAGY----PSVRLPINDIADWK- 211
tr|Q5R179|Q5R179_IDILO              MEGIEHDAYDNILSLK----DLGLSTVFACPVGYRSEADTTQFQKKVRQPLSRFKVVL- 208
tr|Q8DVW4|Q8DVW4_STRMU              MRGVDFDAIKTYLDM-----PNEWEPILMLPVGKALQAGN----PHVRKSVAEFAEIIE 205
tr|A0A0H2UP38|A0A0H2UP38_STRPN      ILGFDKSKVNEVLEI-----EDRFRPELLITVGYTDEKLE----PSYRLPVDEIIEKR- 201
tr|U6C5W9|U6C5W9_LACPN              FSGIDFEKMIPTLGLD----PKRYVPVMGIAIGKAAQEPL----HTTRYDAKTQTDFLA 217
sp|Q01234|NFSB_ENTCL                IEGFDAAILDEEFGLK----EKGFTSLVVVPVGHHSVEDFNATLPKSRLPLSTIVTEC- 217
sp|P38489|NFSB_ECOLI                IEGFDAAILDAEFGLK----EKGYTSLVVVPVGHHSVEDFNATLPKSRLPQNITLTEV- 217
tr|Q9A120|Q9A120_STRP1              IEGFHYDKVNHILAKHNVIDLEKEGIASMLSLGYRLRDPKH---AQVRKPKEEVISVVK 221
                                    : * .       :                  :*              *
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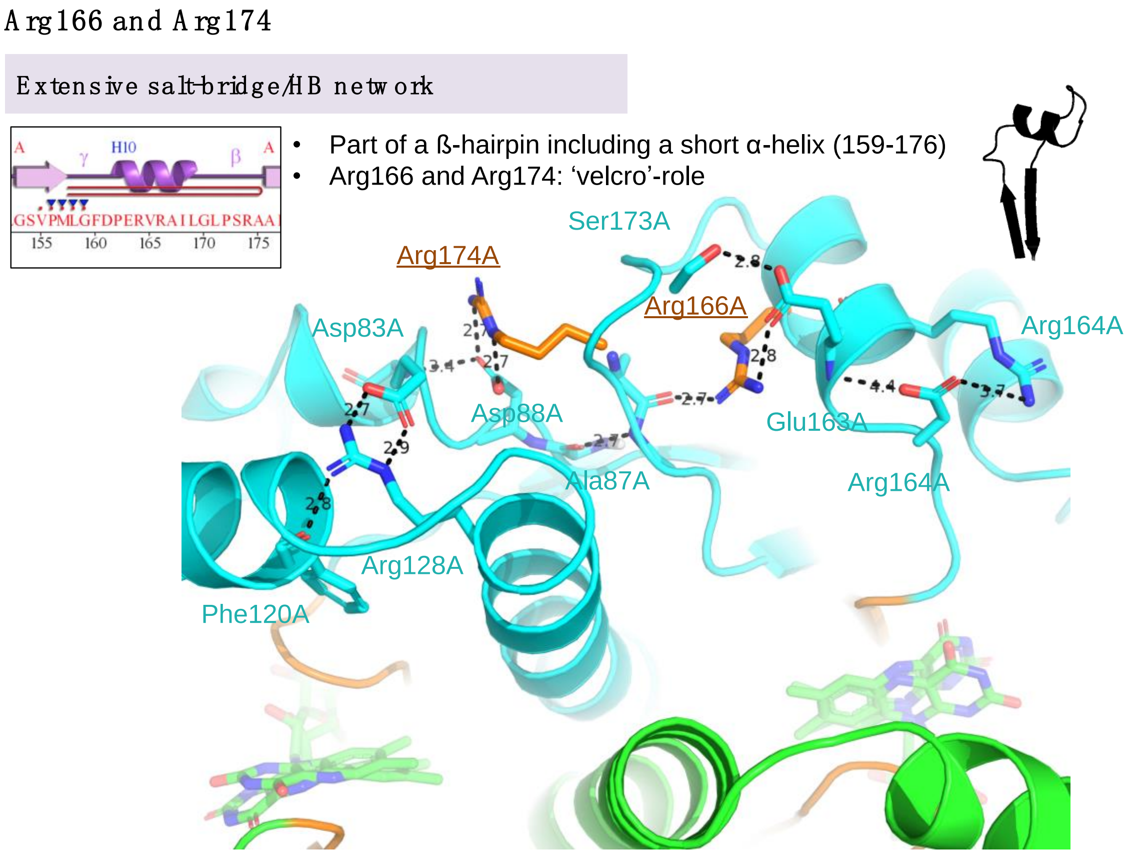
Figure S2. Sequence alignment of enzymes included in Table S1.



Arg166 and Arg174

Extensive salt-bridge/HB network

- Part of a ß-hairpin including a short α-helix (159-176)
- Arg166 and Arg174: ‘velcro’-role

Figure S3. Hydrogen bond network of Arg174 and Arg166 of Tt27-NOX (PDB id. 1NOX). The two monomers of the enzyme are colored in green and in blue, respectively. FMN is shown in sticks.

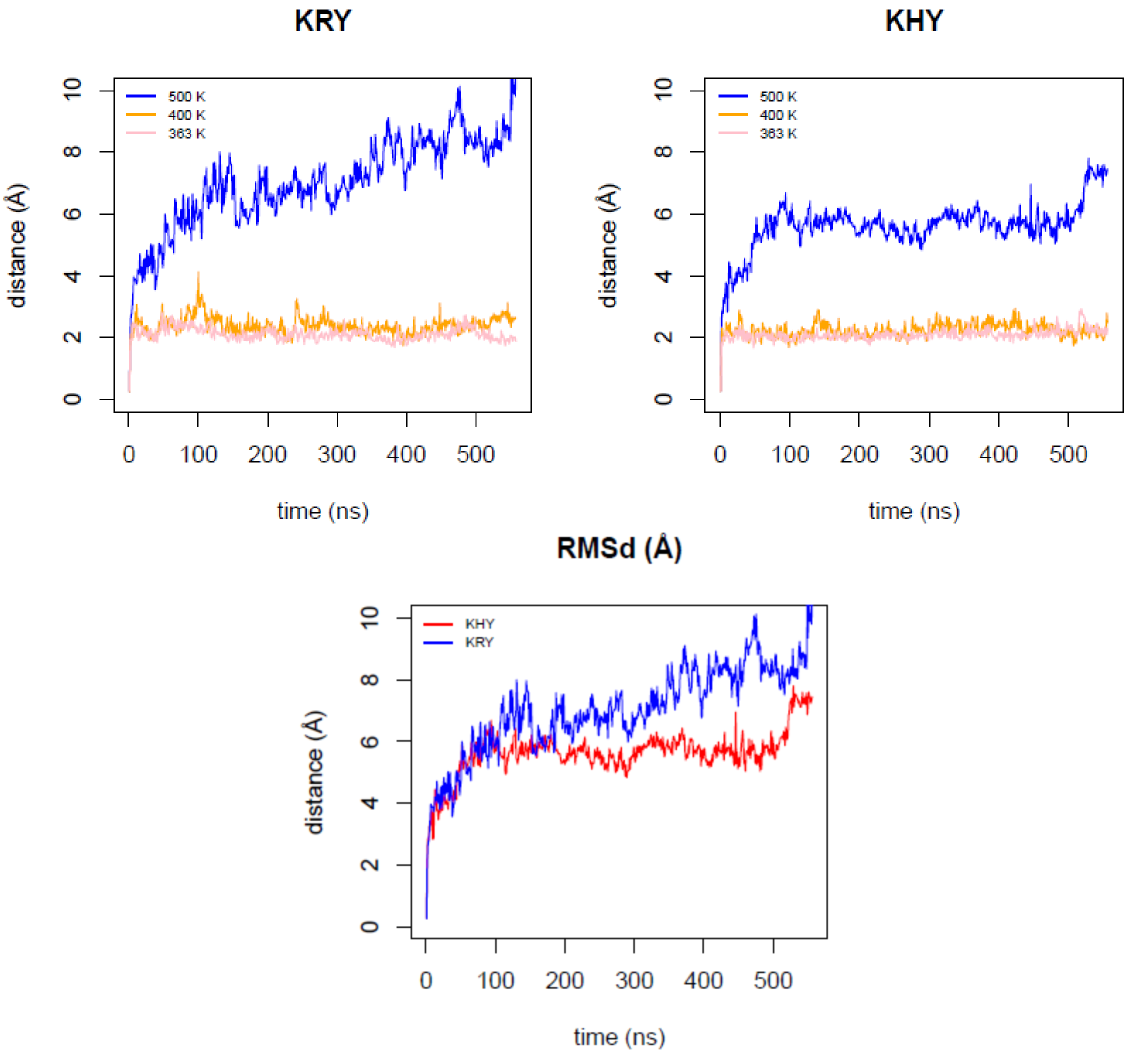


Figure S4. RMSd (Å) evolution of the two variants K166/H174/Y194 and K166/R174/Y194 along 0.5 μs of unbiased MD simulation.

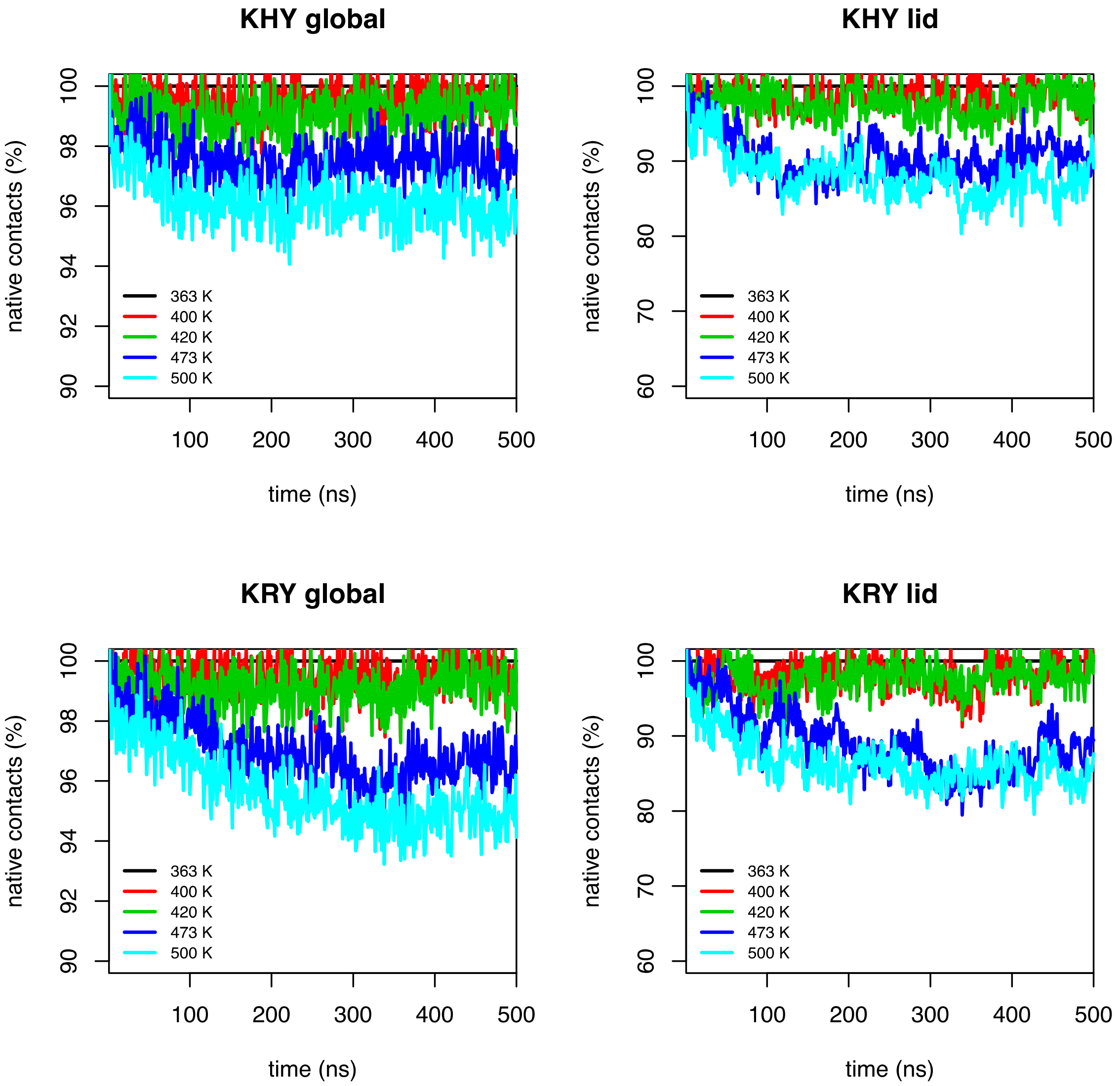


Figure S5. Native contacts (%) evolution of the two variants K166/H174/Y194 and K166/R174/Y194 along 0.5 μs of unbiased MD simulations at different temperatures. Together with the global number of native contacts, the evolution of the residues of the lid is also shown.