

Supplementary Information

A mechanism to prevent production of reactive oxygen species by *Escherichia coli* respiratory complex I

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Supplementary Note on binding of NAD⁺ and NADH to *A. aeolicus* NuoEF.

a)

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A.ae  -----MFKTEFEFPEELKTKLQEHINYFPK--KRQAILLCLHEIQNYGYIPPESLKPL 52
T.th  -----MGFFDDKQDFLEETFAYKYPPEGRRAAIMPLLRVQQEEGWIRPERIEEI 49
E.co  MHENQQPQTEAFELSAAREAEIHEMHYED--PRAASIEALKIVQKQRGWVPDGAIHAI 58
      : :      : :. : :      * * : * : *: * : : .. :

A.ae  ADMLELPLNHVEGVVAFYDMFDREDKAKYRIRVCVSVICHLMGTNKLKALENILGIKPG 112
T.th  ARLVGTTPTVEVMGVASFYSYYQFVPTGKYHLQVCATLSCKLAGAEELWDYLTETLGIGPG 109
E.co  ADVLGIPASDVEGVATFYSQIFRQPVGRHVIRYCDVSVCHINGYQGIQAALCKKLNKPG 118
      * : : . . . * * * . : : . : : : * : : * : : * : : * : * *

A.ae  EVTPDGKFKIVPVQCLGACSEAPVMVNDDEY-KFESEVQLNEILSRYT----- 160
T.th  EVTPDGLFSVQKVECLGSCHTAPVIQVNDPEYVECVTRARLEALLAGLRAGKRLLEEIELP 169
E.co  QTTFDGRFTLLPTCCLGNCDKGNMMDIEDTH-AHLTPEAIPPELLERYK----- 166
      : . * * * . : . * * * * . * : : : : : : : : : *

T.th  GKCGHHVHEVEV 181
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b)

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A.ae  MRSYPAIPRIYAETTLNMLLKRAKKPRVHSIDEYLDGGYQALEKALN-MSPEEIIDWVD 59
T.th  -MTGPILSGLDPRFER-TLYAHVGKEGSWTLDYLRHGGYETAKRVLKEKTPDEVIEEVK 58
E.co  --MKNIIR--TPETHPLTWRLRDDKQPVW-LDEYRSKNGYEGARKALTGLSPDEIVNQVK 55
      : ..      : *      : * * ..* : ..* . : * : : * .

A.ae  KSTLRGRGGAGFPTGKKWKFAVQNG--PRYFICNADESEPGTFKDRIIIRDPHLLIEG 117
T.th  RSGLRGRGGAGFPTGLKWSFMPKDDGK-QHYLICNADESEPGSFKDRYILEDVPHLLIEG 117
E.co  DAGLKGRGGAGFSTGLKWSLMPKDESMNIRYLLCNADEMEPGTYKDRLLMEQLPHLLVEG 115
      : * : * * * * * . * * : : . : * : * * * * * * * : * * * * * *

A.ae  IISSYAIGANEAYIYIRGEYPAGYYILRDAIEEAKKKGFLGKNILGSGFDLEIYVARGA 177
T.th  MILAGYAIRATVGYIYVRGEYRRAADRLEQAIKEARARGYLGNLFGTDFSDLHVHARGA 177
E.co  MLISAFALKAYRGYIFLRGEYIEAAVNLRRRAIAEATEAGLLGKNIMGTGDFELFVHTGA 175
      : : : : : * . * : : * * . * . * * * * * * : : . * : : * *

A.ae  GAYICGEETALIESLEGKRGHPRLKPPYPVQKGLWGKPTVVNNVETIANVPFIISMGWEE 237
T.th  GAYICGEETALMNSLEGLRANPRLKPPFPAQSGLWGKPTTINNVTLASVVPIMERGADW 237
E.co  GRYICGEETALINSLEGRRANPRSKPPFPATSGAWGKPTCVNNVETLCNVPAILANGVEW 235
      * * * * * * * : * * * * * . * * * * * : * * * * * . * : * :

A.ae  YRYIGPSDYAGPKLFPVSGKVKKPGVYELPMNTTLREVIFKYAGGTGNGKKVAVFSGAL 297
T.th  FAQMGTESKGMKLYQISGPVKRPGVYELPMGTTFRELIYEWAGGPLEP--IQAIIPGGS 295
E.co  YQNISKSKDAGTKLMGFSGRVKNPGLWELPFGTTAREILEDYAGGMRDGLKFKAWQPGGA 295
      : : . . . * * * . * * * * : * * * * * * * : : * * .

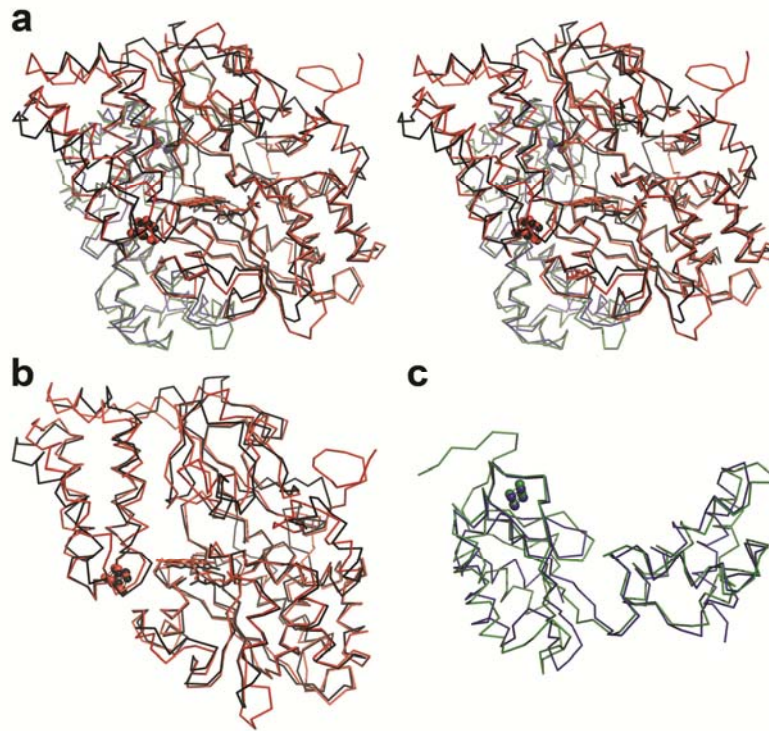
A.ae  DC---FSSEELDIPMDYSPLGFG---GTGTIVLVEEDDIVEAALKIAEFYEHETCGQ 349
T.th  STPPLPFTEEVLDTPMSYHLQAKGSMLGTGGVILIPERVSMVDAMWNLTRFYAHESCGK 355
E.co  GTD--FLTEAHLDPMEFESIGKAGSRLGTALAMAVDHEINMVSLVRNLEEFFARESCGW 353
      . : : . * * * * : : * * . : : . . : * . : : * : * *

A.ae  CTPCRVGCY-EQANLLEKIYKGEATEQDWEGFDFVNRNIQP-TSICGLGAVAGRILRQTL 407
T.th  CTPCREGVAGFMVNLFAKIGTGQGEKDVENLEALLPLIEG-RSFCPLADAAVWPVKGSL 414
E.co  CTPCRDGLP-WSVKILRALERGEQPGDIETLEQLCRFLGPGKTFCAHAPGAVEPLQSAI 412
      * * * * * . : : : * : . * * : : : : : : * . * : : :

A.ae  EKFPPEWEKYRKKSASLPL----- 426
T.th  RHFKDQYLALAREKRPVPRPSLWR----- 438
E.co  KYFREEFEAGIKQPFSENTHLINGIQPNLLKERW 445
      . * : : : : : .
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Supplementary Figure 1 | Sequence alignment of subunits NuoE (A) and NuoF (B) from *A. aeolicus* (A.ae), *T. thermophilus* (T.th) and *E. coli* (E.co). a) Alignment of NuoF orthologues. The sequence identity between *A. aeolicus* and *T. thermophilus* is 32%, that between *E. coli* and *A. aeolicus* 31%

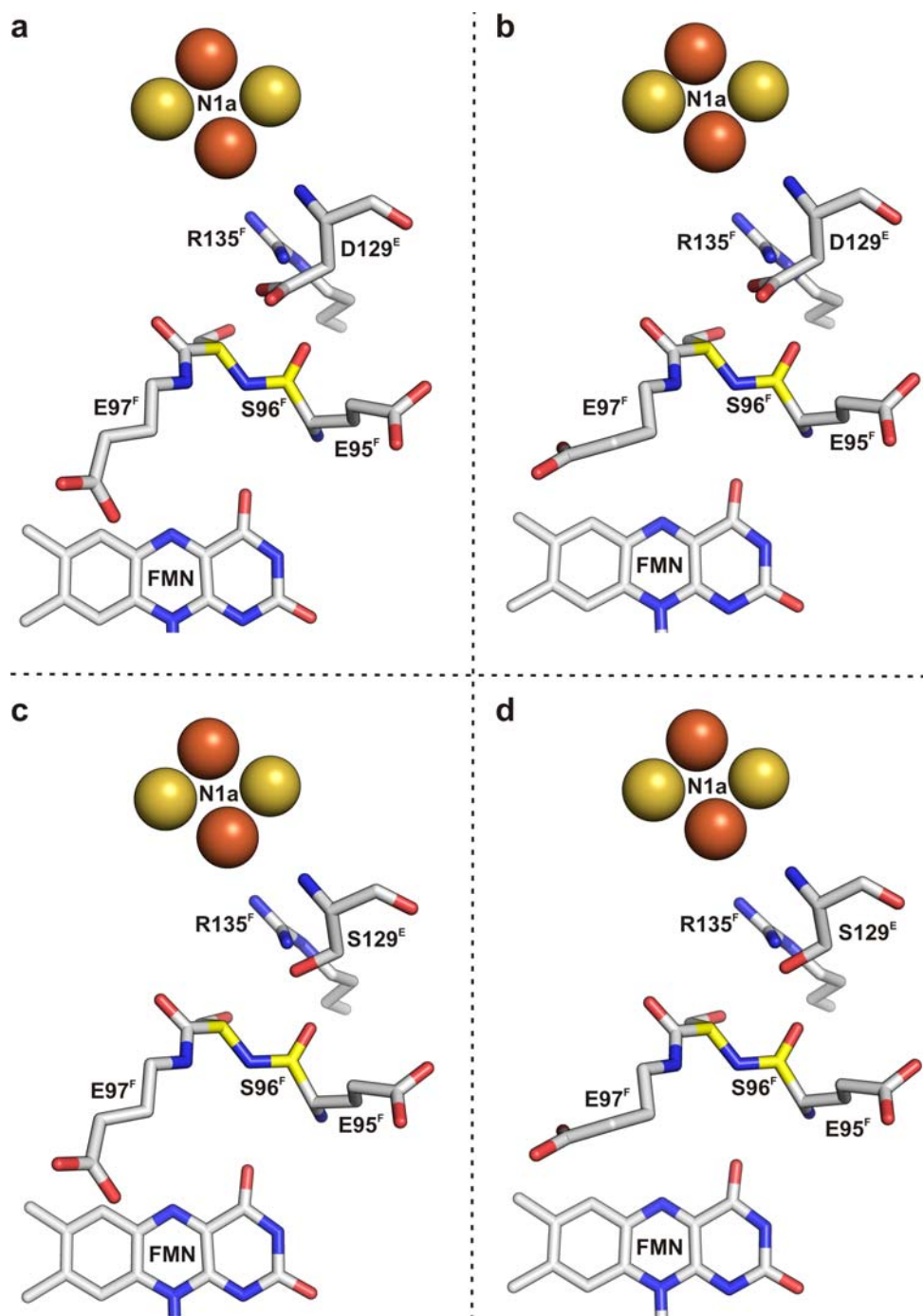
and that between *E. coli* and *T. thermophilus* 24%. **b)** Alignment of NuoE orthologues. The sequence identity between *A. aeolicus* and *T. thermophilus* is 45%, that between *E. coli* and *A. aeolicus* 43% and that between *E. coli* and *T. thermophilus* 45%.



Supplementary Figure 2 | Backbone superposition of *A. aeolicus* NuoEF and *T. thermophilus* Nqo1 and Nqo2.

a) Stereo representation of a superposition of C α -traces for *A. aeolicus* NuoEF and *T. thermophilus* Nqo1 and 2. For *A. aeolicus*, NuoF is shown in black and NuoE in blue, for *T. thermophilus* Nqo1 is shown in red and Nqo2 in grey.

b) Superposition of *A. aeolicus* NuoF with *T. thermophilus* Nqo1. In spite of a slight difference in the positioning of FMN the overall structure of the two orthologs is very similar (rmsd: 1.2 Å for all atoms). **c)** Overlay of *A. aeolicus* NuoE with *T. thermophilus* Nqo2 highlighting the two-domain architecture. The rmsd is 1.8 Å for all atoms.

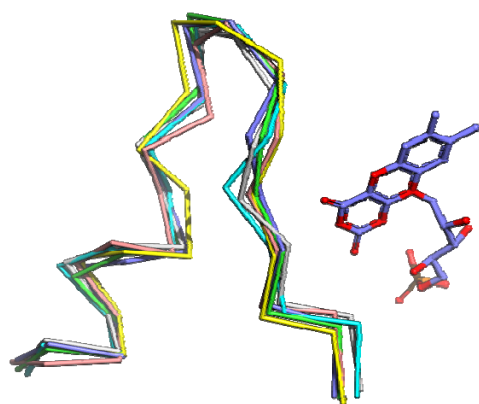


Supplementary Figure 3 | Structure of the *A. aeolicus* G129^E variants in the reduced and oxidized state. **a)** Structure of the G129D^E variant reduced by NADH and dithionite. **b)** Structure of the G129D^E variant in the oxidized state. **c)** Structure of the G129S^E variant reduced by NADH and dithionite. **d)** Structure of the G129S^E variant in the oxidized state. The position of the Fe-S cluster N1a and the FMN isoalloxazine ring is given. The position of the flipping peptide bond indicated by the yellow colored carbon atoms does not change in dependence of the redox state of NuoEF.

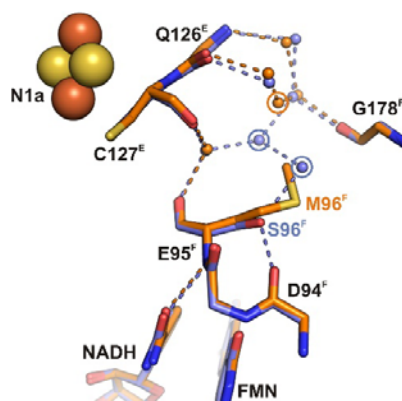
a)

Bovine	KYLVVNADE	G	EPGTCKDREI	IRHDPH
Mouse	KYLVVNADE	G	EPGTCKDREI	MRHDPH
Rat	KYLVVNADE	G	EPGTCKDREI	MRHDPH
Human	KYLVVNADE	G	EPGTCKDREI	IRHDPH
<i>Y. lipolytica</i>	RYLVVNADE	G	EPGTCKDREI	MRKDPH
<i>Paracoccus</i>	SYLVINADES	S	EPATCKDREI	MRHDPH
<i>E. coli</i>	RYLLCNADEM	M	EPGTYKDRLL	MEQLPH
<i>T. thermophilus</i>	HYLICNADES	S	EPGSFKDRII	LEDVPH
<i>A. aeolicus</i>	RYFICNADES	S	EPGTFKDRII	IERDPH
Consensus	*	*****	**	***

b)

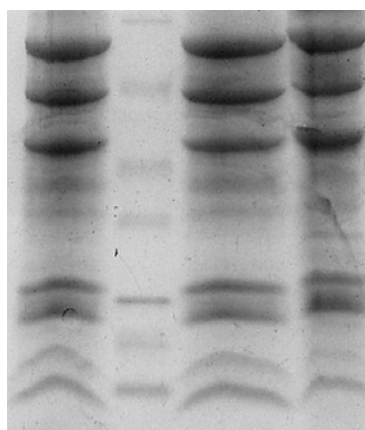


c)

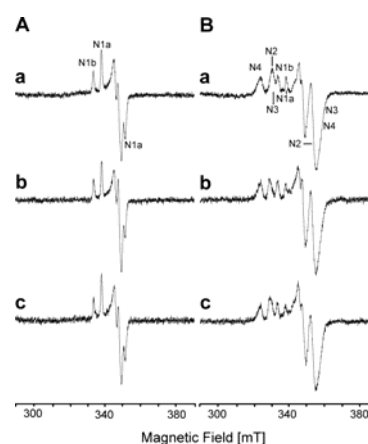


Supplementary Figure 4 | Conservation of the residues establishing the flipping peptide bond. **a)** Sequence alignment of NuoF from different organisms in the region containing the flipping peptide bond. The sequence from R87 to H112 (*A. aeolicus* numbering) is shown. The not conserved position 96 is marked in yellow. **b)** Superposition of the NuoF protein backbone of bovine, human, mouse and rat mitochondrial complex I and of the *T. thermophilus* and *A. aeolicus* homologues from C91 to R109 (*A. aeolicus* numbering). The position of the FMN is shown for orientation. The rmsd between the individual structures ranges from 0.42 to 1.36 Å, the mean value is 0.7 Å. Thus, the structures are considered to be identical. **c)** Structure of the reduced NuoEF (blue) and S96M^F variant (orange). The water molecules displaced by the methionine residue are circled in blue, the water molecule not present in the original protein is circled in orange. The position of the other amino acid side chain did not change.

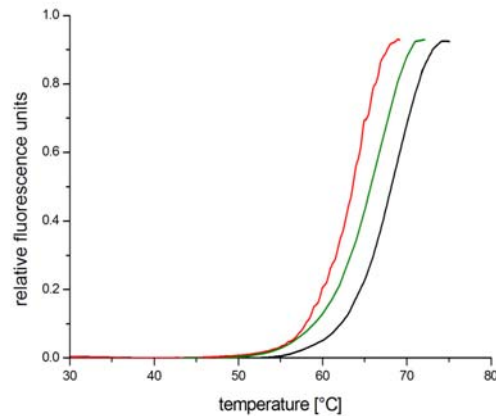
a) (A) (M) (B) (C)



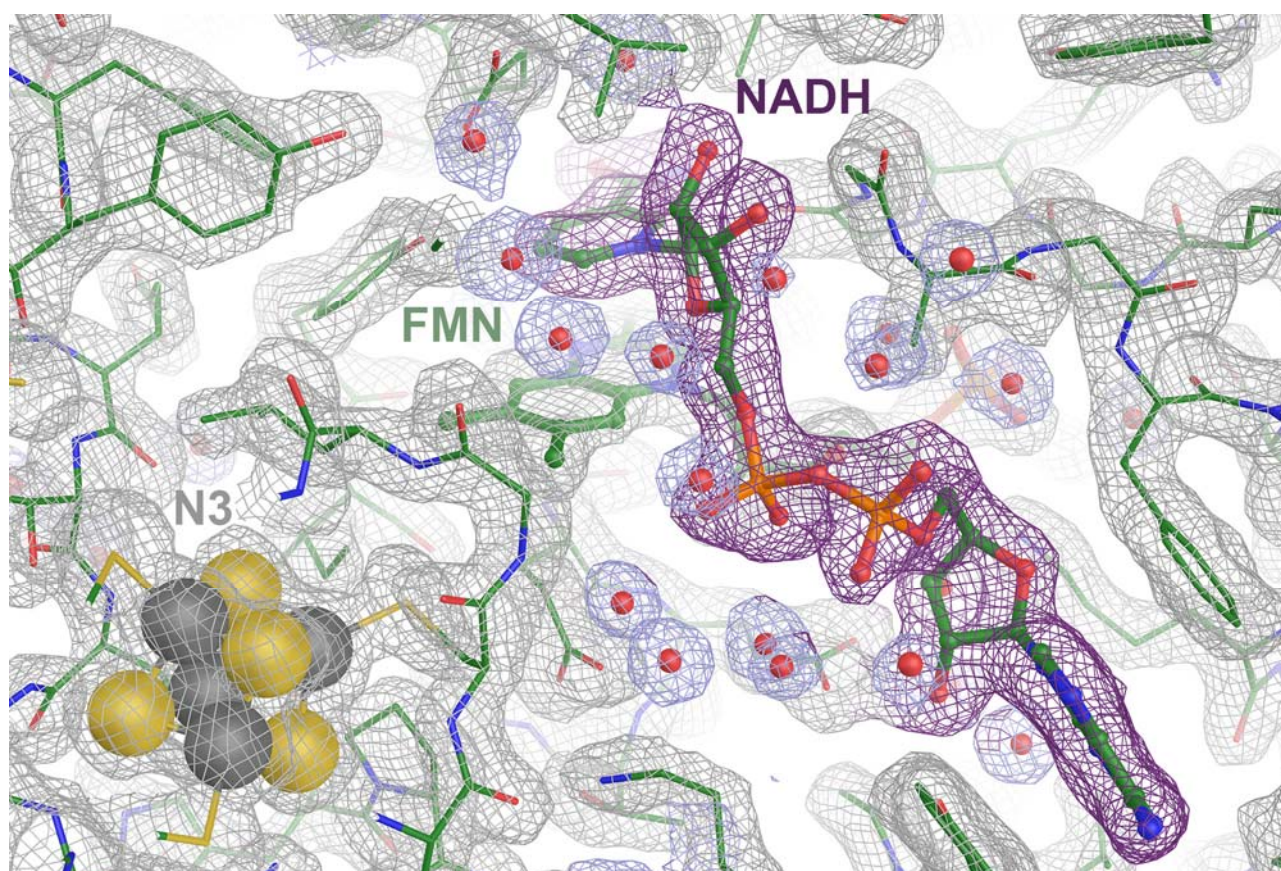
b)



Supplementary Figure 5 | Characterization of the G135^E variant preparations. **a)** SDS-PAGE of the preparations from the parental strain (A), the G135S^E (B) and the G135D^E mutant strain (C). Each lane was loaded with 50 µg protein. The gel was stained with Coomassie brilliant blue leading to a weaker staining of the hydrophobic proteins. Lane (M) shows the pattern of the Unstained Protein Molecular Weight Marker. **b)** EPR spectra of the preparation of the parental protein (a), the G135S^E variant (b), and the G135D^E variant (c) at 40 K and 2 mW (A) and 13 K and 5 mW (B). The samples were reduced with a 1000-fold excess NADH under anoxic conditions. The absorbances of the individual clusters are indicated in the spectra of the parental protein. Source data are provided as a Source Data file.



Supplementary Figure 6 | Thermal stability of the flavin site in the parental protein (black) and the G135S^E (green) and G135D^E (red) variants of complex I. The fluorescence of free FMN was recorded with a real-time PCR machine while the temperature was constantly increased. The curves show the average data points of three measurements. The standard error per data point was less than 1%. The calculated 'melting points' were $68.6 \pm 0.1^{\circ}\text{C}$ for the parental complex, $64.0 \pm 0.1^{\circ}\text{C}$ for the G135S^E variant and $63.2 \pm 0.1^{\circ}\text{C}$ for the G135D^E variant. Assay conditions: 1 mg/ml protein in 50 mM MES/NaOH, 50 mM NaCl, 0.1% (w/v) dodecyl-maltoside, pH 6.0. Source data are provided as a Source Data file.



Supplementary Figure 7 | Example of electron density of the NADH oxidation site with bound NADH. $2F_o - F_c$ electron density is contoured at 1σ . Final atomic models are shown in the element colors. Sulfur of the Fe-S clusters is shown in yellow, iron grey. Water is shown in red. The electron density of NADH is shown in purple.

Supplementary Table 1 | Data collection and refinement statistics.

Data Set	Oxidized (Ox)	Ox plus NADH (reduced)	Ox plus NAD ⁺ (oxidized)	Ox plus Dithionite (reduced)
space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
wavelength [Å]	1.65308	1.00000	1.54179	1.54179
cell constants <i>a</i> , <i>b</i> , <i>c</i> [Å]	63.2, 116.4, 189.4	63.3, 115.8, 189.8	63.4, 115.8, 189.6	59.7, 108.1, 188.2
α, β, γ [°]	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 91.7, 90.0
resolution limits [Å]	99.2 – 1.95 (1.98 – 1.95)	49.44 – 1.78 (1.81 – 1.78)	73.35 – 2.04 (2.15 – 2.04)	188.2 – 2.06 (2.18 – 2.06)
completeness (%)	99.9 (97.7)	100.0 (100.0)	97.6 (95.6)	86.7 (71.1)
unique reflections	102,591 (4,912)	134,290 (6,611)	88,168 (12,408)	65,727 (7,715)
multiplicity (%)	15.9 (10.0)	13.7 (13.8)	7.4 (7.5)	4.9 (3.1)
<i>R</i> _{merge} ^{a)}	0.162 (1.561)	0.190 (1.436)	0.123 (0.904)	0.110 (0.390)
<i>R</i> _{p.i.m.}	0.044 (0.53)	0.077 (0.577)	0.065 (0.486)	0.060 (0.242)
mean <i>I</i> / σ(<i>I</i>)	12.9 (1.5)	7.2 (1.6)	14.4 (2.3)	9.3 (2.7)
CC _{1/2}	0.998 (0.662)	0.995 (0.813)	0.998 (0.780)	0.994 (0.795)
refinement statistics				
<i>R</i> _{cryst} ^{b)}	0.169	0.180	0.173	0.200
<i>R</i> _{free} (%)	0.195	0.207	0.203	0.242
non-hydrogen atoms	9084	10,355	9073	9054
solvent molecules	693	961	530	127
r.m.s. deviations from ideal values				
bond lengths (Å)	0.009	0.004	0.008	0.010
bond angles (°)	0.93	1.22	0.93	1.08
average <i>B</i> values (Å²)				
protein main chain atoms	29.1	30.0	32.2	29.5
protein all atoms	32.8	31.0	35.8	33.2
FMN	20.6	20.3	22.0	21.6
NAD	-	26.1	43.6	-
FES	22.1	22.6	25.0	24.7
SF4	23.6	21.1	29.7	25.1
solvent	34.8	40.6	37.1	23.4
Wilson plot	25.1	29.3	30.0	24.2

^a $R_{\text{merge}} = \sum_{hkl} [(\sum_i |I_i - \langle I \rangle|) / \sum_i I_i]$

^b $R_{\text{cryst}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$

*R*_{free} is the cross-validation *R* value for a test set of 5 % of unique reflections.

Supplementary Table 1 | Data collection and refinement statistics (continued).

Data Set	Reduced (Dithionite + NADH)	Reduced (Dithionite + NAD ⁺)	G129D oxidized plus NAD ⁺	G129D reduced (Dithionite + NADH)
space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
wavelength [Å]	1.00000	1.54179	1.54179	1.54187
cell constants <i>a</i> , <i>b</i> , <i>c</i> [Å]	63.6, 116.4, 189.5	63.6, 116.0, 189.9	92.7, 63.3, 124.2	63.3, 116.2, 189.8
α, β, γ [°]	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 107.2, 90.0	90.0, 90.0, 90.0
resolution limits [Å]	189.45 – 1.90 (1.93 – 1.90)	116.0 – 2.38 (2.51 – 2.38)	118.7 – 1.80 (1.83 – 1.80)	26.40 – 2.10 (2.14 – 2.10)
completeness (%)	98.7 (83.7)	100.0 (100.0)	90.1 (92.8)	99.8 (99.5)
unique reflections	110,071 (4,524)	57,523 (8,284)	114,854 (5,845)	82,411 (4,411)
multiplicity	7.0 (5.5)	6.4 (6.4)	2.7 (2.5)	6.4 (5.6)
<i>R</i> _{merge} ^{a)}	0.143 (0.743)	0.105 (0.757)	0.035 (0.508)	0.185 (0.835)
<i>R</i> _{p.i.m.}	0.086 (0.499)	0.051 (0.351)	0.037 (0.509)	0.118 (0.562)
mean <i>I</i> / σ(<i>I</i>)	9.5 (2.3)	13.2 (2.2)	14.7 (1.4)	6.5 (1.6)
CC _{1/2}	0.995 (0.577)	0.998 (0.778)	0.998 (0.602)	0.993 (0.658)
refinement statistics				
<i>R</i> _{cryst} ^{b)}	0.215	0.206	0.177	0.191
<i>R</i> _{free} (%)	0.240	0.238	0.197	0.231
non-hydrogen atoms	9101	9095	9090	10,337
solvent molecules	670	248	497	961
r.m.s. deviations from ideal values				
bond lengths (Å)	0.009	0.010	0.010	0.005
bond angles (°)	1.00	1.04	0.95	1.30
average <i>B</i> values (Å²)				
protein main chain atoms	26.3	47.3	30.8	25.4
protein all atoms	29.6	50.9	34.1	26.3
FMN	15.6	37.6	21.1	15.5
NAD	22.5	45.5	43.8	20.7
FES	20.9	38.8	24.8	16.3
SF4	22.2	48.2	26.6	15.8
solvent	30.1	39.7	36.6	32.7
Wilson plot	21.1	45.7	27.3	24.7

^a $R_{\text{merge}} = \sum_{hkl} [(\sum_i |I_i - \langle I \rangle|) / \sum_i I_i]$

^b $R_{\text{cryst}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$

*R*_{free} is the cross-validation *R* value for a test set of 5 % of unique reflections.

Supplementary Table S1 | Data collection and refinement statistics (continued).

Data Set	G129S oxidized	G129S reduced (Dithionite + NADH)	S96M oxidized	S96M reduced (Dithionite + NADH)
space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
wavelength [Å]	1.54179	1.00000	1.54187	1.54187
cell constants <i>a</i> , <i>b</i> , <i>c</i> [Å]	62.6, 115.8, 189.9	63.6, 116.3, 190.3	63.4, 114.6, 187.9	63.4, 116.0, 190.0
α, β, γ [°]	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
resolution limits [Å]	98.9 – 2.10 (2.14 – 2.10)	48.2 – 1.83 (1.86 – 1.83)	38.2 – 3.22 (3.48 – 3.22)	21.3 – 1.99 (2.02 – 1.99)
completeness (%)	99.9 (99.9)	99.6 (99.5)	99.8 (100.0)	99.8 (99.3)
unique reflections	81,432 (4,421)	124,614 (6,107)	22,909 (4,655)	96,646 (4,699)
multiplicity (%)	6.4 (6.4)	13.7 (14.1)	6.7 (6.8)	5.8 (5.0)
<i>R</i> _{merge} ^{a)}	0.128 (0.987)	0.148 (1.707)	0.248 (0.822)	0.162 (0.776)
<i>R</i> _{p.i.m.}	0.061 (0.463)	0.059 (0.681)	0.151 (0.505)	0.108 (0.528)
mean <i>I</i> / σ(<i>I</i>)	10.5 (1.8)	11.8 (1.7)	6.8 (2.0)	6.9 (1.6)
CC _{1/2}	0.998 (0.698)	0.999 (0.761)	0.991 (0.795)	0.992 (0.699)
refinement statistics				
<i>R</i> _{cryst} ^{b)}	0.179	0.172	0.208	0.188
<i>R</i> _{free} (%)	0.218	0.197	0.257	0.219
non-hydrogen atoms	9097	10,377	9,380	10,338
solvent molecules	544	981	113	959
r.m.s. deviations from ideal values				
bond lengths (Å)	0.009	0.004	0.005	0.005
bond angles (°)	0.99	1.22	1.32	1.27
average <i>B</i> values (Å ²)				
protein main chain atoms	36.0	28.9	64.2	22.2
protein all atoms	39.3	30.0	66.0	23.0
FMN	26.9	19.3	67.2	13.4
NAD	-	24.1	-	18.1
FES	26.0	21.1	35.4	14.4
SF4	27.5	20.6	83.6	12.9
solvent	34.8	39.6	32.2	30.3
Wilson plot	29.7	28.4	60.3	21.7

^a $R_{\text{merge}} = \sum_{hkl} [(\sum_i |I_i - \langle I \rangle|) / \sum_i I_i]$

^b $R_{\text{cryst}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$

*R*_{free} is the cross-validation *R* value for a test set of 5 % of unique reflections.

Supplementary Table 2 | Interactions of NADH with reduced NuoEF.

First coordination sphere			Second coordination sphere		
Atom NADH	Residue Atom	Length [Å]	Residue Atom	Residue Atom	Length [Å]
AO3'	Lys76 ^F NZ	H _B 3.0	Lys76 ^F N	Pro72 ^F O	H _B 3.3
			Lys76 ^F NZ	Val218 ^F O	H _B 2.7
			Lys76 ^F O	Ala80 ^F N	H _B 3.4
			Lys76 ^F NZ	FMN O2P	H _B 2.7
AO2'	Glu185 ^F OE1	H _B 3.3	Glu185 ^F OE2	HOH 540	H _B 2.7
AO2'	Glu185 ^F OE2	H _B 2.5	Glu185 ^F O	Ile189 ^F N	H _B 3.1
AO3'	Glu185 ^F OE1	H _B 2.6	Glu185 ^F N	FMN O2'	H _B 3.0
			Glu185 ^F OE1	HOH 103	H _B 3.2
AO2'	HOH 528	H _B 2.7	HOH 528	HOH 103	H _B 2.9
			HOH 528	AN3	H _B 3.0
NO2'	HOH 545	H _B 2.7	HOH 545	Glu97 ^F OE2	H _B 2.9
			HOH 545	HOH 541	H _B 3.2
NO7	Glu97 ^F N	H _B 3.0	Glu97 ^F O	Thr100 ^F N	H _B 3.0
			Glu97 ^F O	Thr100 ^F OG1	H _B 3.2
			Glu97 ^F OE2	Tyr180 ^F OH	H _B 2.7
			Glu97 ^F OE1	Ala296 ^F N	H _B 2.9
			Glu97 ^F OE2	HOH 545	H _B 2.9
			Glu97 ^F O	HOH 32	H _B 3.1
			Glu97 ^F OE1	HOH 82	H _B 2.7
			Glu97 ^F OE2	HOH 82	H _B 3.3
NN7	Gly67 ^F O	H _B 3.2			
NN7	HOH 526	H _B 2.9	HOH 526	Glu95 ^F OE2	H _B 2.7
			HOH 526	Asp103 ^F OD2	H _B 2.8
NO1	HOH 534	H _B 2.7	HOH 534	Gly394 ^F N	H _B 3.1
			HOH 534	Glu184 ^F OE2	H _B 2.7
			HOH 534	HOH 514	H _B 2.7
NO1	HOH 567	H _B 2.7	HOH 567	HOH 261	H _B 2.7
NO2	FMN O2'	H _B 3.1			
NO2	FMN O3'	H _B 2.7			
NO2	HOH 540	H _B 2.7	HOH 540	Glu185 ^F OE2	H _B 2.7
AN3	HOH 528	H _B 3.0	HOH 528	AO2'	H _B 2.7
			HOH 528	HOH 103	H _B 2.9

Supplementary Table 3 | Interactions of NAD⁺ with NuoEF in the oxidized state.

First coordination sphere			Second coordination sphere		
Atom NAD ⁺	Residue Atom	Length [Å]	Residue Atom	Residue Atom	Length [Å]
AO3'	Lys76 ^F NZ	H _B 2.9	Lys76 ^F N	Pro72 ^F O	H _B 3.2
			Lys76 ^F NZ	Val218 ^F O	H _B 2.9
			Lys76 ^F O	Ala80 ^F N	H _B 3.2
			Lys76 ^F NZ	FMN O2P	H _B 3.2
AO2'	Glu185 ^F OE1	H _B 3.3	Glu185 ^F OE2	Lys202 ^F NZ	H _B 2.9
AO2'	Glu185 ^F OE2	H _B 2.6	Glu185 ^F O	Ile189 ^F N	H _B 3.1
AO3'	Glu185 ^F OE1	H _B 2.6	Glu185 ^F N	FMN O2'	H _B 3.0
			Glu185 ^F OE1	HOH 308 (103)	H _B 3.4
AO2'	HOH 483 (528)	H _B 2.4	HOH 483 (528)	HOH 308 (103)	H _B 2.8
NO2'	HOH 648	H _B 2.8	HOH 648	Glu97 ^F OE1	H _B 3.0
NO2'	Gly68 ^F O	H _B 3.3	Gly68 ^F N	HOH 129	H _B 2.7
NO3'	Gly68 ^F O	H _B 3.4			
NO7	Glu95 ^F O	E _R 2.9	Glu95 ^F O	HOH 150 (6)	H _B 2.9
			Glu95 ^F N	HOH 79 (527)	H _B 3.1
			Glu95 ^F OE2	Asp103 ^F N	H _B 2.8
			Glu95 ^F OE1	Arg104 ^F N	H _B 3.2
			Glu95 ^F OE1	Tyr138 ^F OH	H _B 2.7
			Glu95 ^F OE2	HOH 61 (526)	H _B 2.7
NO7	HOH 545	H _B 2.5	HOH 545	HOH 61 (526)	H _B 2.8
			HOH 545	HOH 241	H _B 2.9
NO1	HOH 145 (534)	H _B 2.5	HOH 145 (534)	Glu184 ^F OE2	H _B 3.0
			HOH 483 (528)	Gly394 ^F N	H _B 2.9
NO2	FMN O2'	H _B 2.8			
NO2	FMN O3'	H _B 2.6			
AN3	HOH 483 (528)	H _B 2.5			

Supplementary Table 4 | Preparation of the *A. aeolicus* G129S^E and G129D^E and the *E. coli* G135S^E and G135D^E variants.

15 g (wet weight) Rosetta/pETBlue-1*nuoEF_{his} nuoE* G129S cells were used.

Preparation	Volume	Protein	NADH/ferricyanide-oxidoreductase activity		Yield
			total	specific	
	[mL]	[mg]	[$\mu\text{mol}\cdot\text{min}^{-1}$]	[$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$]	[%]
Cytosol	88	1074	3846	3.6	100
Probond Ni ²⁺ -IDA	22.5	180	1706	9.5	44
Superdex 200 16/60	24	90	1064	11.8	28
SQ-15	5	29	538	20.1	14
Superdex 200 16/60	2	22	466	21.2	12

10 g (wet weight) Rosetta/pETBlue-1*nuoEF_{his} nuoE* G129D cells were used.

Preparation	Volume	Protein	NADH/ferricyanide-oxidoreductase activity		Yield
			total	specific	
			[mL]	[mg]	
Cytosol	62	742	2283	3.1	100
Probond Ni ²⁺ -IDA	21	110	1105	10.0	48
Superdex 200 16/60	10	40	567	14.2	25
SQ-15	5	21	401	19.1	18
Superdex 200 16/60	1.2	13.5	292	21.6	13

31 g (wet weight) BW25113 Δ *nuo ndh::nptI*/pBAD*nuo nuoF_{his} nuoE* G135S cells were used.

Preparation	Volume	Protein	NADH/ferricyanide-oxidoreductase activity		Yield
			total	specific	
	[mL]	[mg]	[$\mu\text{mol}\cdot\text{min}^{-1}$]	[$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$]	[%]
Membrane	19.0	1210	1551	1.3	100
Detergentextract	58.0	913	1272	1.4	82
Fractogel EMD	60.0	210	612	2.9	39
ProBond Ni ²⁺ -IDA	0.8	4.3	202	47.0	13

42 g (wet weight) BW25113 Δ *nuo ndh::nptI*/pBAD*nuo nuoF_{his} nuoE* G135D cells were used.

Preparation	Volume	Protein	NADH/ferricyanide-oxidoreductase activity		Yield
			total	specific	
	[mL]	[mg]	[$\mu\text{mol}\cdot\text{min}^{-1}$]	[$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$]	[%]
Membrane	28.7	1828	2011	1.1	100
Detergentextract	125.0	1409	1690	1.2	84
Fractogel EMD	60.0	358	718	2.0	36
ProBond Ni ²⁺ -IDA	0.7	7.0	315	45.0	16

Supplementary Table 5 | Mutagenic primer to construct the *A. aeolicus* G129S^E, G129D^E and S96M^F and the *E. coli* G135S^E and G135D^E variants.

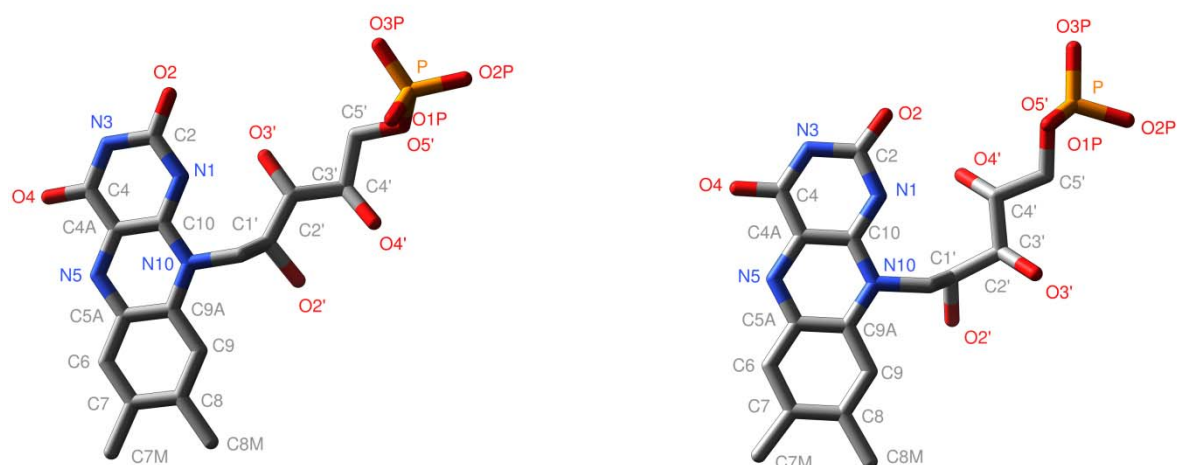
Oligonucleotide	Sequence	New restriction site
<i>nuoE</i> G135S_fwd	5'-CAACTTGCTGCTTA AGT AACTGTGAT AAAG-3'	AflII
<i>nuoE</i> G135S_rev	5'-CTTTATCACAGTT ACTTA AGCAGCAA GTTG-3'	AflII
<i>nuoE</i> G135D_fwd	5'-CTTGCTGCCTGG ACA ACTGTGATAA AGGGCCCAACATGATGATC-3'	Apal
<i>nuoE</i> G135D_rev	5'-GATCATCATGTTGGGCCCTTTATCAC AGTT GTCC AGGCAGCAAG-3'	Apal
<i>nuoE_{axa}</i> G129S_fwd	5'-CGTTCAGTGTCTG AGCGC <u>CTGCAGT</u> GAAGCTCCCGTG-3'	PstI
<i>nuoE_{axa}</i> G129S_rev	5'-CACGGGAGCTTC <u>ACTGC</u> AGGC GCTC AGACACTGAACG-3'	PstI
<i>nuoE_{axa}</i> G129D_fwd	5'-CGTTCAGTGTCTG GATGC <u>CTGCAGT</u> GAAGCTCCCGTG-3'	PstI
<i>nuoE_{axa}</i> G129D_rev	5'-CACGGGAGCTTC <u>ACTGC</u> AGGC ATCC AGACACTGAACG-3'	PstI
<i>nuoF_{axa}</i> S96M_fwd	5'-GCAACGCGGACGAG ATG GAACCGG <u>GTACCTTTA</u> AGGACAGG	KpnI
<i>nuoF_{axa}</i> S96M_rev	5'- CCTGTCCTTAAAG GTAC CCGGTTCC ATCTCGTCCGCGTTGC	KpnI

Supplementary Note

Binding of NAD⁺ and NADH to *A. aeolicus* NuoEF

In the presence of 10 mM NAD⁺ an extra electron density feature appeared that was modeled accordingly (**Fig. 2**). The electron density of the adenosine-ribose moiety was clearly visible while that of the nicotinamide was only weakly defined. In our model and in that described by Berrisford and Sazanov¹⁷ the nicotinamide and the isoalloxazine ring of FMN interact by π -stacking in a distance of about 3.0 Å. The nicotinamide N7 forms hydrogen bonds with the backbone amides of E97^F and S96^F. The side chain of E97^F tilts upon NADH binding and forms a hydrogen bond with the hydroxyl-group of Y180^F to arrange the nicotinamide in an optimal position for hydride transfer. NO2 of the nicotinamide is hydrogen bonded to the ribityl O2' and O3' of the FMN. The adenine ring is stabilized by π -stacking interactions with the conserved F71^F (F70¹ in *T. t.*) and the variable Y205^F (F205¹ in *T. t.*). The variable F79^F does not interact with the adenine ring while a stacking interaction of this position (F78¹ in *T. t.*) with the adenine moiety was described by Berrisford and Sazanov¹⁷. The conserved E185^F formed two hydrogen bonds to adenosyl-ribose, as described for other dehydrogenases⁴⁶. Common to both structures the loop formed by residues 201–206 moves 0.9 Å towards the binding site upon NADH binding. Accordingly, the side chain of Y205^F re-orientates such that it exerts stacking interactions with the adenine. The glycine-rich loop of complex I (residues 65–70) moves 0.6–1.3 Å apart opening up the binding site, assisted by a movement of the preceding α -helix by about 0.5 Å. In both structures, no hydrogen bonds between the glycine-rich loop and the pyrophosphate occur. The carbonyl group of G67^F (G66¹ in *T. t.*) forms a hydrogen bond to the adenine N7. However, the interaction of G68^F (G67¹ in *T. t.*) and N71 reported for the *T. thermophilus* complex is not present in the *A. aeolicus* enzyme as well as the described interaction of G67^F and G68^F with NO3' of the nicotinamide ribose¹⁷. In contrast, an interaction of G68^F (G67¹ in *T. t.*) with NO3' of NAD⁺ is observed in the oxidized NuoEF. In both redox states the backbone amide of G67^F (G66¹ in *T. t.*) is hydrogen bonded to the O1P of the FMN. In addition, K76^F (K75¹ in *T. t.*) forms a hydrogen bond with the FMN O2P. Upon NADH binding, K76^F does not lose this bond as described¹⁷, but it forms an additional bond to the AO3' of the adenosine ribose. The described interaction of K76^F with FMN O5' was not detected. In contrast to the *T. thermophilus* structure¹⁶, K202^F is hydrogen-bonded to the carboxylate of E184^F and the carbonyls of R200^F and I392^F. In the oxidized NuoEF a hydrogen bond is formed by K202^F and E185^F. The backbone amide of E185^F also forms a hydrogen bond with the FMN O2'. This bond is not contained within the *T. thermophilus* structure. This difference may be caused by the different orientation of the ribityl moiety of FMN in both structures (see below). E185^F (E185¹ in *T. t.*) makes two strong hydrogen bonds with the AO2' and AO3' of the adenosine ribose. However, as the nicotinamide moiety of

NAD⁺ is not stringently defined in the electron density maps, and the position of the vicinal ribose sugar indicates that it points away from the actual binding pocket, unable to stack onto the isoalloxazine system of FMN. In the model, the ligand was consequently only included as ADP-ribose. The nicotinamide moiety was modeled according to the positions with a defined electron density. We rationalize the observed ejection of nicotinamide from the binding pocket with the observed peptide flip at residue G95^F.



Structural model of the FMN cofactor in *T. thermophilus* (left; pdb-entry: 3IAM16) and *A. aeolicus* (right) complex I (this work).

46. Lesk, A.M. NAD-binding domains of dehydrogenases. *Curr. Biol.* **5**, 775-783 (1995).