SUPPORTING INFORMATION

Electron Paramagnetic Resonance Characterization of Three Iron-Sulfur Clusters Present in the Nitrogenase Cofactor Maturase NifB from *Methanocaldococcus infernus*

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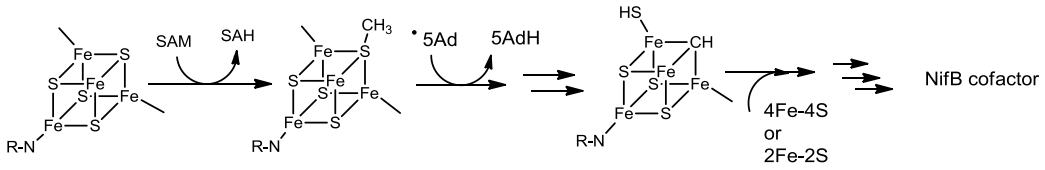

Scheme S1. Minimal mechanism of NifB-co synthesis in NifB

Table S1. Fe and S content of as-isolated and reconstituted *Mi*NifB

WT *Mi*NifB Preparation	**Fe**[a]	**S**
Anaerobically purified *Mi*NifB		
As isolated	3.3 ± 0.1	4.0 ± 0.2
Reconstituted with Fe and S	9.2 ± 0.3	8.0 ± 0.2
apo-*Mi*NifB	< 0.1	< 0.1
apo-*Mi*NifB reconstituted with Fe and S	10 ± 0.2	11.2 ± 0.4
Aerobically purified *Mi*NifB	0.8 ± 0.1	1.2 ± 0.3
***Mi*NifB Variant**[b]	**Fe**	**S**
As isolated		
SM	3.3 ± 0.3	4.5 ± 0.4
AM	5.3 ± 0.4	6.3 ± 0.1
DM	2.6 ± 0.2	3.5 ± 0.3
Reconstituted with Fe and S		
SM	6.1 ± 0.3	7.4 ± 0.4
AM	5.5 ± 0.2	6.4 ± 0.2
DM	4.6 ± 0.4	5.5 ± 0.3

[a] Data represent mean ± SD.
[b] Proteins purified under anaerobic conditions

Table S2. Estimated molecular weights of WT *Mi*NifB and the site-directed variants AM, SM and DM. Table includes the molecular weight standards used to calibrate the Superdex 200 used for size exclusion chromatography.

Sample	Elution volume (ml)	Kav	Measured MW (Da)[a]	Theoretical MW (Da)	Probable oligomeric state
Aprotenin	103.75	0.7930	6,843	6,500	
Ribonuclease A	95.21	0.6843	13,559	13,700	
Carbonic anhydrase	87.27	0.5832	25,613	29,000	
Ovalbumin	78.71	0.4742	50,849	43,000	
Conalbumin	74.74	0.4237	69,865	75,000	
***Mi*NifB WT**	85.29	0.5581	29,995	37,543	monomer
***Mi*NifB SM variant**	83.59	0.5364	34,383	37,446	monomer
***Mi*NifB AM variant**	85.25	0.5575	30,108	37,478	monomer
***Mi*NifB DM variant**	85.30	0.5582	29,976	37,382	monomer

[a] Kav= 2.1967-0.366 * log MW; R^2 = 0.989. Column Geometric volume = 120 ml. Column void volume = 41.46 ml.

Table S3. EPR Simulation Values

	g_1	g_2	g_3	g_{av}
RS	2.038	1.931	1.916	1.962
AC1	2.062	1.917	1.875	1.951
AC2	2.039	1.923	1.886	1.949
ACx	2.058	1.985	1.909	1.984

	WT		
	σ_1	σ_2	σ_3
RS	0.028	0.015	0.04
AC1	0.015	0.015	0.018
AC2	0.03	0.03	0.05
ACx	-	-	-

	AM			SM			DM		
	σ_1	σ_2	σ_3	σ_1	σ_2	σ_3	σ_1	σ_2	σ_3
RS	0.028	0.018	0.04	-	-	-	-	-	-
AC1	-	-	-	0.025	0.018	0.018	-	-	-
AC2	0.03	0.04	0.08	0.03	0.03	0.08	0.03	0.04	0.08
ACx	-	-	-	0.017	0.018	0.03	-	-	-

Table S4. Spin quantitation of *Mi*NifB variants in the absence or presence of SAM

	-SAM (μM)	+SAM (μM)
WT	52	111
SM	65	82
AM	66	92
DM	53	59

Table S5. NifB amino acid sequences used to determine strictly conserved residues

Entry	Organism	Sequence length (Amino acids)
P10390	*Klebsiella pneumoniae*	468
P17434	*Rhodobacter capsulatus*	452
P27714	*Herbaspirillum seropedicae*	525
P24427	*Rhizobium leguminosarum*	490
P11067	*Azotobacter vinelandii*	502
P46044	*Frankia alni*	510
Q43883	*Trichormus azollae* *(Anabaena azollae)*	475
P06770	*Bradyrhizobium japonicum* (strain USDA 110)	499
P20627	*Nostoc* sp. (strain PCC 7120 / UTEX 2576)	475
Q53205	*Rhizobium* sp. (strain NGR234)	493
P09824	*Rhizobium meliloti* (strain 1021)	490
Q7MRF5	*Wolinella succinogenes*	449
B6IXL5	*Rhodospirillum centenum* (strain ATCC 51521 / SW)	492
D5ANH7	*Rhodobacter capsulatus* (strain ATCC BAA-309 / NBRC 16581 / SB1003)	497
B5YH11	*Thermodesulfovibrio yellowstonii* (strain ATCC 51303 / DSM 11347 / YP87)	293
A0B690	*Methanosaeta thermophila* (strain DSM 6194 / PT)	299
D5VRM1	*Methanocaldococcus infernus* (strain DSM 11812 / JCM 15783 / ME)	302
A6UUJ9	*Methanococcus aeolicu*s (strain Nankai-3 / ATCC BAA-1280)	318
Q58493	*Methanocaldococcus jannaschii* (strain ATCC 43067 / DSM 2661 / JAL-1 / JCM 10045 / NBRC 100440)	300
Q8TIF7	*Methanosarcina acetivorans* (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A)	323
Q6LZH0	*Methanococcus maripaludis* (strain S2 / LL)	297
O27899	*Methanothermobacter thermautotrophicus* (strain ATCC 29096 / DSM 1053 / JCM 10044 / NBRC 100330 / Delta H)	288
Q46G74	*Methanosarcina barkeri* (strain Fusaro / DSM 804)	321
Q8PYU4	*Methanosarcina mazei* (strain ATCC BAA-159/ DSM 3647 / Goe1 / Go1 / JCM 11833 / OCM 88)	328
B3EH81	*Chlorobium limicola* (strain DSM 245 / NBRC 103803)	424
A0LH03	*Syntrophobacter fumaroxidans* (strain DSM 10017 / MPOB)	423
Q72WT2	*Desulfovibrio vulgaris* (strain Hildenborough / ATCC 29579 / NCIMB 8303)	514
A4J8B5	*Desulfotomaculum reducens* (strain MI-1)	299
A6TTX6	*Alkaliphilus metalliredigens* (strain QYMF)	415
D6Z0A8	*Desulfurivibrio alkaliphilus* (strain DSM 19089 / UNIQEM U267 / AHT2)	424
C0GKD1	*Dethiobacter alkaliphilus* AHT 1	418
A5N6Z7	*Clostridium kluyveri* (strain ATCC 8527 / DSM 555 / NCIMB 10680)	423
Q44481	*Anabaena variabilis*	484
A1KYD1	*Cyanothece* sp. (strain ATCC 51142)	490
B5ER81	*Acidithiobacillus ferrooxidans* (strain ATCC 53993)	522
A8IIJ5	*Azorhizobium caulinodans* (strain ATCC 43989 / DSM 5975 / ORS 571)	519
Q6N0X9	*Rhodopseudomonas palustris* (strain ATCC BAA-98 / CGA009)	518
Q2J1J1	*Rhodopseudomonas palustris* (strain HaA2)	518
G7DEY1	*Bradyrhizobium japonicum* USDA 6	527
B2JYC0	*Burkholderia phymatum* (strain DSM 17167 / STM815)	535
A4WRX8	*Rhodobacter sphaeroides* (strain ATCC 17025 / ATH 2.4.3)	491
A6UMF6	*Sinorhizobium medicae* (strain WSM419)	490

Strictly conserved NifB residues. *M. infernus* NifB (*Mi*NifB) amino acid sequence showing in **Bold** those amino acid residues that are conserved in all 42 NifB sequences. Regions where deletion variants were generated are underlined.

MEKMSKFSHLLKA**HPC**FNEKVHDKYG**R**V**H**LPV**A**PR**CN**IA**C**KF**C**K**R**SVSKE**C**C**E**H**RP**G**V**SLG**V**LKPEDVEDY
LKKILKEMPNIKVVGI**AGPG**DSLFNKE**T**FETLKIIDEKFPNLIK**C**IST**NG**LLLSKYYKDLANLNVRTI**T**V**T**
V**N**AIKPEILEKIVDWVYYDKKLYRGLEGAKLLIEK**Q**IEGIKKASEEDFII**K**I**N**TVLIPEI**N**MDHVVEIAKF
FKDYAYVQ**N**IIPLIPQYKMKELRAPTCEEIKKVRKE**C**EKYIPQFRA**C**GQ**CR**A**DA**V**G**LIKEKELLKEFFKEK
NKEKNIKLEVFDLKHFSH

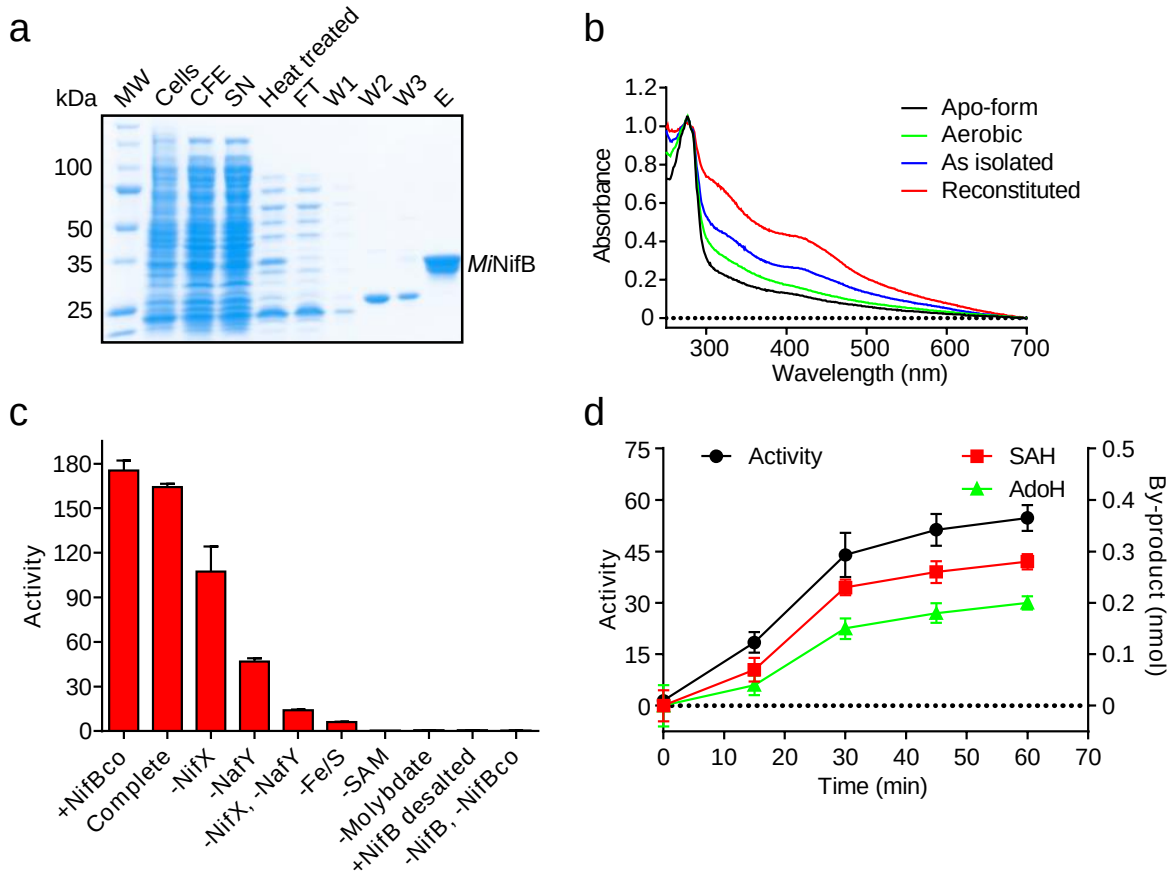

Figure S1. Biochemical characterization of *Mi*NifB. (a) SDS-PAGE analysis of *Mi*NifB purification steps. CFE, cell-free extract; SN, supernatant after removing cell debris; Heat treated, fraction obtained after heating the SN at 75˚C for 30 min to precipitate most *E. coli* proteins; W1-3, 30-100 mM imidazole washing fractions; E, 250 mM imidazole eluted fraction containing pure *Mi*NifB. **(b)** UV-visible spectra of anaerobically as-isolated *Mi*NifB (blue, 19.9 μM), aerobically as-isolated *Mi*NifB (green, 19.9 μM), [Fe-S] cluster stripped apo-*Mi*NifB (black, 21.9 μM), and [Fe-S] cluster reconstituted *Mi*NifB (red, 13 μM). **(c)** Protein and substrate requirements for *Mi*NifB-dependent *in vitro* reconstitution of apo-NifDK. Assays consisted of a 60ºC NifB activity phase (that in the complete version contained Fe, S, SAM, DTH, and MiNifB) followed by a 30ºC FeMo-co synthesis and apo-NifDK reconstitution phase (that in the complete version contained Mo, homocitrate, DTH, NifX, apo-NifEN, NifH, NafY, and apo-NifDK). Detailed assay conditions are described in the Methods Section. A reaction containing NifB-co in place of MiNifB (+NifB-co) was carried out as control of pathway functionality downstream NifB. Protein and substrate requirements were analyzed by removing one or more substrates/proteins at a time from the reaction mixtures, as indicated in the x-axis. **(d)** Time course of SAH and AdoH by-product formation during *Mi*NifB-dependent FeMo-co synthesis and apo-NifDK reconstitution. For (c) and (d) activity refers to nmol ethylene formed per min and mg of NifDK. Date are the mean ± SD of at least two independent experiments.

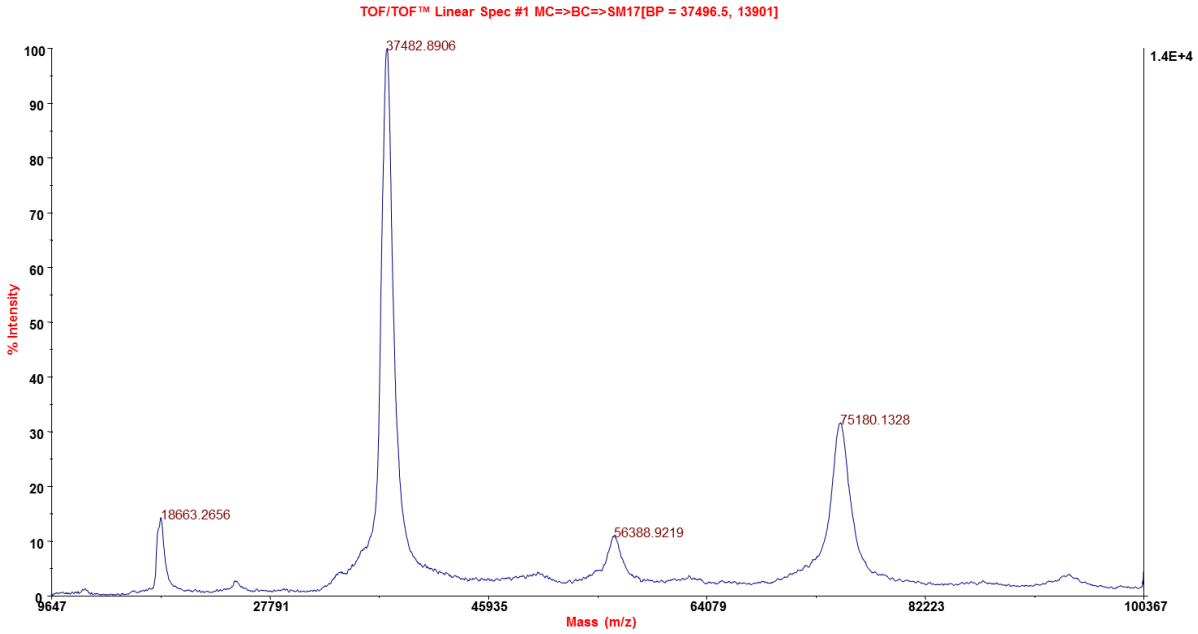

Figure S2. **TOF/TOF analysis of *Mi*NifB**. Analysis was carried out by using a 4800 Proteomics Analyzer. As-isolated *Mi*NifB yielded a main peak corresponding to a molecular weight of 37,483 Da and a second peak corresponding to a molecular weight of 75,180 Da that could account for a NifB dimer.

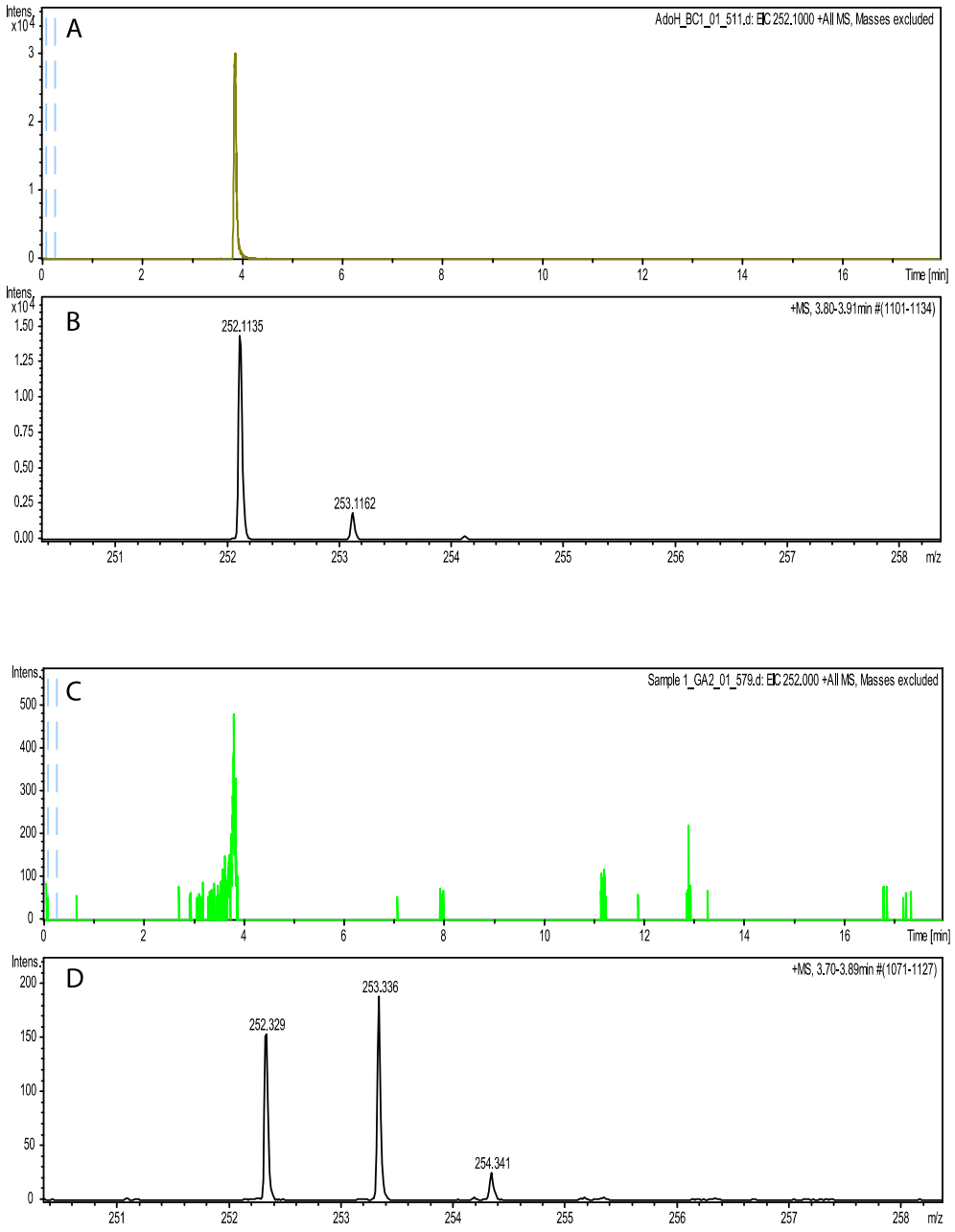

Figure S3: Electrospray Ionization Mass Spectrometry (ESI-MS) analysis showing the shift in m/z 253 abundance between the 5′-dAdoH control and 5′-dAdoD generated by incubating *Mi*NifB with CD_3-SAM. 5′-dAdoH elution profile (A) and MS analysis (B). Panel (C) shows the products formed by incubation of *Mi*NifB with CD_3-SAM . (D) MS analysis of the peak eluted at 3.8 min corresponding to 5′-dAdoD.

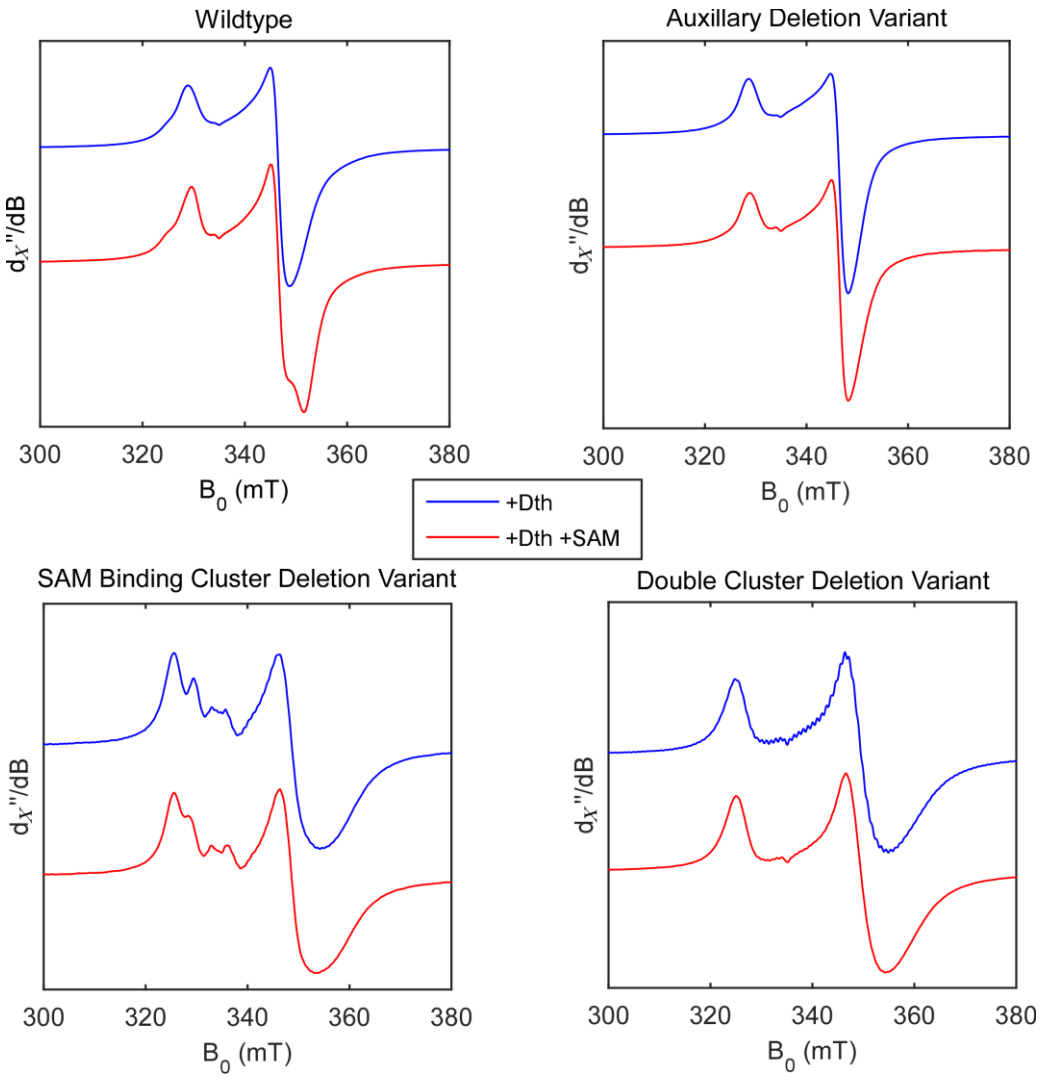

Figure S4. EPR spectra of [Fe-S] clusters reconstituted *Mi*NifB variants. X-band CW EPR measurements of *Mi*NifB protein samples prepared anaerobically with 10 mM DTH in the absence (Blue) or Presence (Red) of 3 mM SAM. **Top Left**: Wild type *Mi*NifB **Top Right**: Auxiliary cluster substituted variant (AM); **Bottom Left**: SAM Binding cluster substituted variant (SM); **Bottom Right**: Double cluster substituted variant (DM). Typical conditions for recording spectra were 9.40 GHz microwave frequency, 1 mW power, 10 G modulation amplitude, and temperature of 15K.

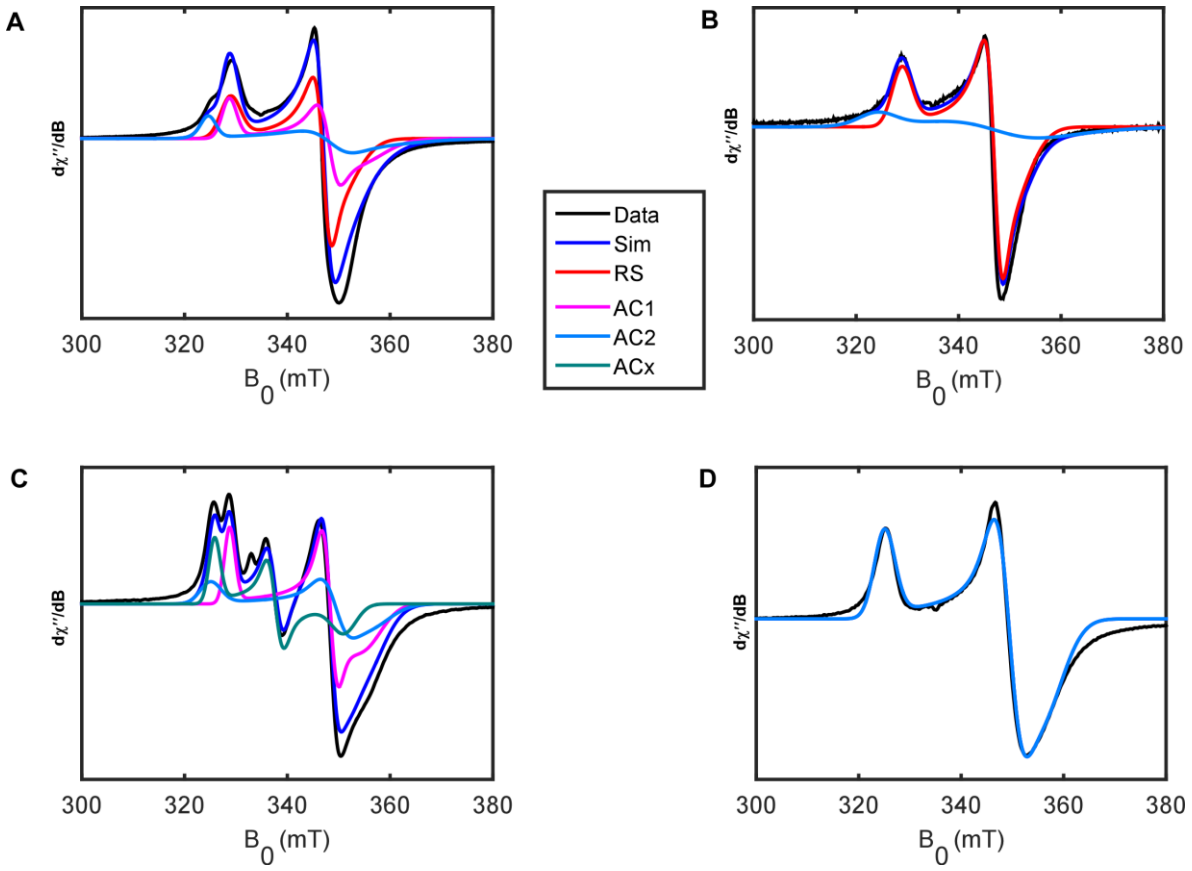

Figure S5. Components of simulations for [Fe-S] cluster reconstituted *Mi*NifB. X-band CW EPR measurements of *Mi*NifB protein samples prepared anaerobically with 10 mM DTH and frozen in an acetone/dry ice bath. (**A**) WT (274 μM), (**B**) AM (159 μM), (**C**) SM (159 μM), and (**D**) DM (252 μM). Data are presented in Black with composite spectral simulations in Blue for the WT *Mi*NifB and each of the variants. Contributions of each of the individual component clusters are included with RS (Red), AC1 (Fuchsia), AC2 (Light Blue) and degradation cluster, ACx, (Dark Green). Typical conditions for recording spectra were 9.40 GHz microwave frequency, 1 mW power, 5G modulation amplitude, and temperature of 15K.

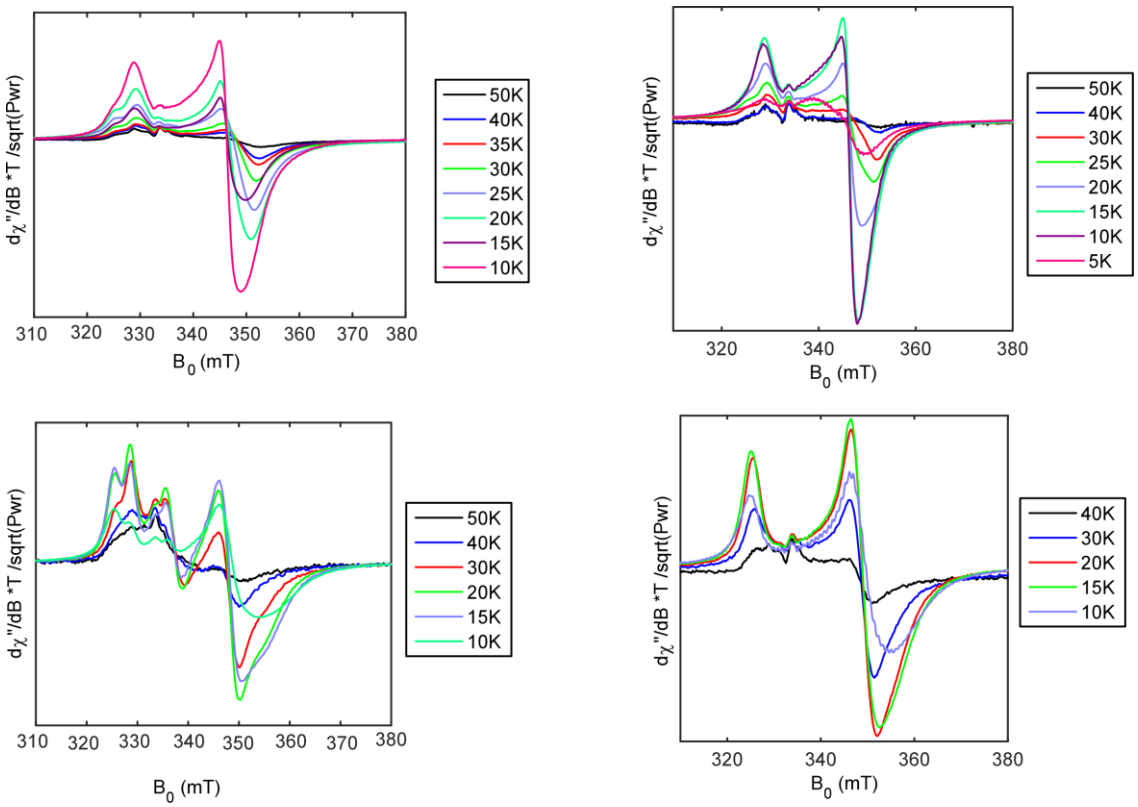

Figure S6. Temperature dependence and spectral subtractions of WT and site-directed variants of *Mi*NifB. All MiNifB samples were reduced with 10 mM DTH. **(Top Left)** Temperature dependence of 274 μM WT *Mi*NifB. **(Top Right)** Temperature dependence of 159 μM AM *Mi*NifB. **(Bottom Left)** Temperature dependence of 159 μM SM *Mi*NifB. **(Bottom Right)** Temperature dependence of 252 μM DM *Mi*NifB. Typical conditions for recording spectra are 9.40 GHz microwave frequency, 1 mW power, and 5G modulation amplitude. Spectra were recorded at various temperatures indicated to the right of each panel.

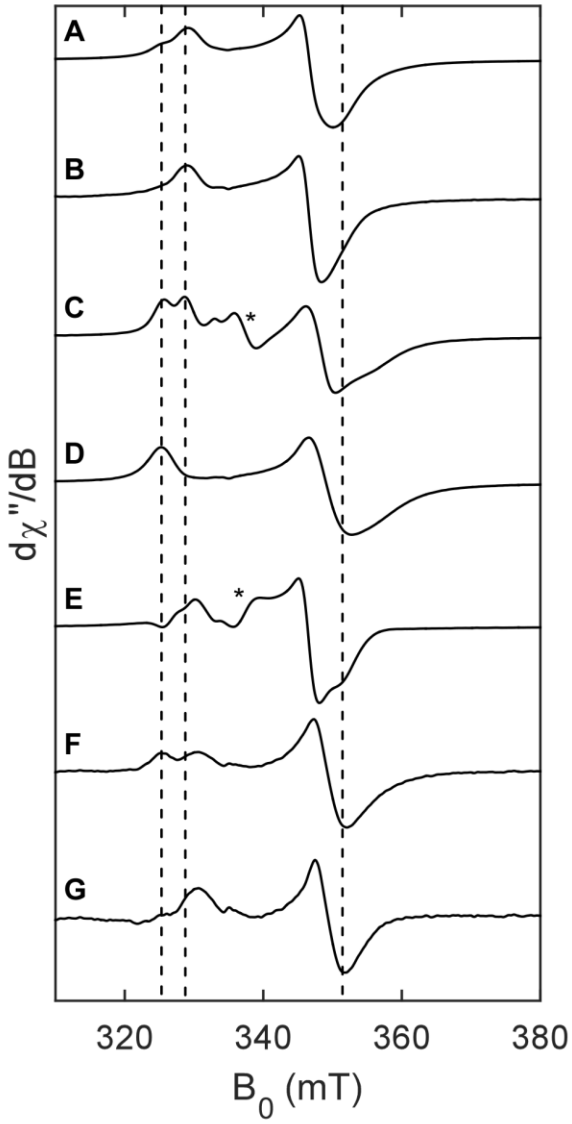

Figure S7. Spectral subtractions of WT *Mi*NifB and variants utilized in determining the clusters present by differences in the spectral composition of the variants. (**A**) WT *Mi*NifB; (**B**) AM variant; (**C**) SM variant; (**D**) DM variant; (**E**) WT minus AM; (**F**) WT minus SM; (**G**) Difference spectrum **F** minus DM to remove unsubtracted component and highlight the EPR signal from AC1 cluster. Asterisk marks g2 of ACx degradation cluster. Dashed lines are included to indicate defining $\mathbf{g_1}$ and $\mathbf{g_3}$ spectral features used in deconvolution. Typical conditions for recording spectra are 9.40 GHz microwave frequency, 1 mW power, 5G modulation amplitude, and a temperature of 15 K.

Figure S8. Codon optimized and amino acid sequences of WT *Mi*NifB and site-directed variants generated in this work

Codon optimized sequence (*E. coli*) of *Methanocaldococcus infernus Mi*NifB (metin_0554):

```
CATATGGAAAAAATGTCAAAATTCTCGCATCTGCTGAAAGCGCACCCGTGCTTCAACGAAAAAGTC
CACGATAAATACGGTCGCGTCCACCTGCCGGTTGCACCGCGTTGCAACATCGCTTGCAAATTTTGT
AAACGCAGCGTCTCTAAAGAATGCTGTGAACATCGTCCGGGCGTCTCACTGGGTGTGCTGAAACCG
GAAGATGTGGAAGACTACCTGAAGAAAATTCTGAAAGAAATGCCGAACATCAAAGTGGTTGGCATT
GCGGGCCCGGGTGATTCGCTGTTTAATAAAGAAACGTTCGAAACCCTGAAAATTATCGACGAAAAA
TTCCCGAACCTGATTAAATGTATCAGTACGAATGGTCTGCTGCTGTCCAAATATTACAAAGATCTG
GCAAACCTGAATGTTCGCACCATCACGGTTACCGTCAACGCTATTAAACCGGAAATCCTGGAAAAA
ATTGTGGATTGGGTTTATTACGACAAAAAACTGTACCGTGGCCTGGAAGGTGCGAAACTGCTGATC
GAAAAACAGATCGAAGGCATCAAAAAAGCCTCTGAAGAAGACTTCATCATCAAAATCAACACGGTC
CTGATTCCGGAAATCAATATGGATCACGTCGTGGAAATTGCGAAATTTTTCAAAGACTATGCCTAC
GTTCAGAATATTATCCCGCTGATTCCGCAATATAAAATGAAAGAACTGCGCGCACCGACCTGCGAA
GAAATCAAAAAAGTGCGTAAAGAATGTGAAAAATACATTCCGCAGTTTCGCGCCTGTGGTCAATGT
CGTGCCGATGCCGTTGGTCTGATTAAAGAAAAAGAACTGCTGAAAGAATTTTTCAAAGAAAAGAAT
AAAGAAAAGAACATTAAACTGGAAGTCTTTGACCTGAAACACTTCTCCCACTGACTCGAG
```

***Mi*NifB wild-type (WT) amino acid sequence:**

```
MEKMSKFSHLLKAHPCFNEKVHDKYGRVHLPVAPRCNIACKFCKRSVSKECCEHRPGVSLGVLKPE
DVEDYLKKILKEMPNIKVVGIAGPGDSLFNKETFETLKIIDEKFPNLIKCISTNGLLLSKYYKDLA
NLNVRTITVTVNAIKPEILEKIVDWVYYDKKLYRGLEGAKLLIEKQIEGIKKASEEDFIIKINTVL
IPEINMDHVVEIAKFFKDYAYVQNIIPLIPQYKMKELRAPTCEEIKKVRKECEKYIPQFRACGQCR
ADAVGLIKEKELLKEFFKEKNKEKNIKLEVFDLKHFSH
```

Codon optimized sequence of the C36A/C40A/C43A (SM) *Mi*NifB variant:

```
CATATGGAAAAAATGTCAAAATTCTCGCATCTGCTGAAAGCGCACCCGTGCTTCAACGAAAAAGTC
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AAACGCAGCGTCTCTAAAGAATGCTGTGAACATCGTCCGGGCGTCTCACTGGGTGTGCTGAAACCG
GAAGATGTGGAAGACTACCTGAAGAAAATTCTGAAAGAAATGCCGAACATCAAAGTGGTTGGCATT
GCGGGCCCGGGTGATTCGCTGTTTAATAAAGAAACGTTCGAAACCCTGAAAATTATCGACGAAAAA
TTCCCGAACCTGATTAAATGTATCAGTACGAATGGTCTGCTGCTGTCCAAATATTACAAAGATCTG
GCAAACCTGAATGTTCGCACCATCACGGTTACCGTCAACGCTATTAAACCGGAAATCCTGGAAAAA
ATTGTGGATTGGGTTTATTACGACAAAAAACTGTACCGTGGCCTGGAAGGTGCGAAACTGCTGATC
GAAAAACAGATCGAAGGCATCAAAAAAGCCTCTGAAGAAGACTTCATCATCAAAATCAACACGGTC
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GTTCAGAATATTATCCCGCTGATTCCGCAATATAAAATGAAAGAACTGCGCGCACCGACCTGCGAA
GAAATCAAAAAAGTGCGTAAAGAATGTGAAAAATACATTCCGCAGTTTCGCGCCTGTGGTCAATGT
CGTGCCGATGCCGTTGGTCTGATTAAAGAAAAAGAACTGCTGAAAGAATTTTTCAAAGAAAAGAAT
AAAGAAAAGAACATTAAACTGGAAGTCTTTGACCTGAAACACTTCTCCCACTGACTCGAG
```

Amino acid sequence of *Mi*NifB SM variant:

```
MEKMSKFSHLLKAHPCFNEKVHDKYGRVHLPVAPRANIAAKFAKRSVSKECCEHRPGVSLGVLKPE
DVEDYLKKILKEMPNIKVVGIAGPGDSLFNKETFETLKIIDEKFPNLIKCISTNGLLLSKYYKDLA
NLNVRTITVTVNAIKPEILEKIVDWVYYDKKLYRGLEGAKLLIEKQIEGIKKASEEDFIIKINTVL
IPEINMDHVVEIAKFFKDYAYVQNIIPLIPQYKMKELRAPTCEEIKKVRKECEKYIPQFRACGQCR
ADAVGLIKEKELLKEFFKEKNKEKNIKLEVFDLKHFSH
```

Codon optimized sequence of the C260A/C263A (AM) *Mi*NifB variant:

```
CATATGGAAAAAATGTCAAAATTCTCGCATCTGCTGAAAGCGCACCCGTGCTTCAACGAAAAAGTC
CACGATAAATACGGTCGCGTCCACCTGCCGGTTGCACCGCGTTGCAACATCGCTTGCAAATTTTGT
```

AAACGCAGCGTCTCTAAAGAATGCTGTGAACATCGTCCGGGCGTCTCACTGGGTGTGCTGAAACCG
GAAGATGTGGAAGACTACCTGAAGAAAATTCTGAAAGAAATGCCGAACATCAAAGTGGTTGGCATT
GCGGGCCCGGGTGATTCGCTGTTTAATAAAGAAACGTTCGAAACCCTGAAAATTATCGACGAAAAA
TTCCCGAACCTGATTAAATGTATCAGTACGAATGGTCTGCTGCTGTCCAAATATTACAAAGATCTG
GCAAACCTGAATGTTCGCACCATCACGGTTACCGTCAACGCTATTAAACCGGAAATCCTGGAAAAA
ATTGTGGATTGGGTTTATTACGACAAAAAACTGTACCGTGGCCTGGAAGGTGCGAAACTGCTGATC
GAAAAACAGATCGAAGGCATCAAAAAAGCCTCTGAAGAAGACTTCATCATCAAAATCAACACGGTC
CTGATTCCGGAAATCAATATGGATCACGTCGTGGAAATTGCGAAATTTTTCAAAGACTATGCCTAC
GTTCAGAATATTATCCCGCTGATTCCGCAATATAAAATGAAAGAACTGCGCGCACCGACCTGCGAA
GAAATCAAAAAAGTGCGTAAAGAATGTGAAAAATACATTCCGCAGTTTCGCGCCGCGGGTCAAGCG
CGTGCCGATGCCGTTGGTCTGATTAAAGAAAAAGAACTGCTGAAAGAATTTTTCAAAGAAAAGAAT
AAAGAAAAGAACATTAAACTGGAAGTCTTTGACCTGAAACACTTCTCCCACTGACTCGAG

Amino acid sequence of *Mi*NifB AM variant:

MEKMSKFSHLLKAHPCFNEKVHDKYGRVHLPVAPRCNIACKFCKRSVSKECCEHRPGVSLGVLKPE
DVEDYLKKILKEMPNIKVVGIAGPGDSLFNKETFETLKIIDEKFPNLIKCISTNGLLLSKYYKDLA
NLNVRTITVTVNAIKPEILEKIVDWVYYDKKLYRGLEGAKLLIEKQIEGIKKASEEDFIIKINTVL
IPEINMDHVVEIAKFFKDYAYVQNIIPLIPQYKMKELRAPTCEEIKKVRKECEKYIPQFRAAGQAR
ADAVGLIKEKELLKEFFKEKNKEKNIKLEVFDLKHFSH

Codon optimized sequence of the C36A/C40A/C43A/C260A/C263A (DM) *Mi*NifB variant:

CATATGGAAAAAATGTCAAAATTCTCGCATCTGCTGAAAGCGCACCCGTGCTTCAACGAAAAAGTC
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GCAAACCTGAATGTTCGCACCATCACGGTTACCGTCAACGCTATTAAACCGGAAATCCTGGAAAAA
ATTGTGGATTGGGTTTATTACGACAAAAAACTGTACCGTGGCCTGGAAGGTGCGAAACTGCTGATC
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GTTCAGAATATTATCCCGCTGATTCCGCAATATAAAATGAAAGAACTGCGCGCACCGACCTGCGAA
GAAATCAAAAAAGTGCGTAAAGAATGTGAAAAATACATTCCGCAGTTTCGCGCCGCGGGTCAAGCG
CGTGCCGATGCCGTTGGTCTGATTAAAGAAAAAGAACTGCTGAAAGAATTTTTCAAAGAAAAGAAT
AAAGAAAAGAACATTAAACTGGAAGTCTTTGACCTGAAACACTTCTCCCACTGACTCGAG

Amino acid sequence of *Mi*NifB DM variant::

MEKMSKFSHLLKAHPCFNEKVHDKYGRVHLPVAPRANIAAKFAKRSVSKECCEHRPGVSLGVLKPE
DVEDYLKKILKEMPNIKVVGIAGPGDSLFNKETFETLKIIDEKFPNLIKCISTNGLLLSKYYKDLA
NLNVRTITVTVNAIKPEILEKIVDWVYYDKKLYRGLEGAKLLIEKQIEGIKKASEEDFIIKINTVL
IPEINMDHVVEIAKFFKDYAYVQNIIPLIPQYKMKELRAPTCEEIKKVRKECEKYIPQFRAAGQAR
ADAVGLIKEKELLKEFFKEKNKEKNIKLEVFDLKHFSH