Figure S1. PROMALS3D (1) structure-based sequence alignment of NqrC and RnfG flavinylation target proteins. The first line in each block shows conservation indices for positions with a conservation index above 5. Each representative sequence has a magenta name and is colored according to PSIPRED (2) secondary structure predictions (red: alpha-helix, blue: beta-strand). A representative sequence and the immediate sequences below it with black names, if there are any, form a closely related group. The consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss) are labeled. Consensus predicted secondary structure symbols: alpha-helix: h; beta-strand: e. Functionally important conserved residues are highlighted in yellow and labeled above the sequence alignment with the following codes: residues that contact the FMN in the NqrC_So structure are labeled with an asterisk (*); the putative catalytically important lysine residue is labeled with a dagger (†); and the conserved hydrogen-bonding residue in the loop preceding the threonine target of flavinylation is labeled with an ampersand (&).

Figure S2. Flavin site geometry of NqrC_So structure. Ligplot+ (3) schematic of residues involved in interactions with the phosphoester-threonyl-FMN residue. The bonds of the FMN modification are purple, the bonds of protein side chains are brown, carbon atoms are black spheres, nitrogens are blue, oxygens are red, and sulfurs are yellow. Green dotted lines represent hydrogen-bonding interactions with interatomic distances labeled. Hydrophobic interactions of protein residues with the FMN ligand are represented as red arcs with the name of the residue labeled. Corresponding atoms of both ligand and protein residues involved in hydrophobic contacts are shown with a halo of short red lines radiating from the atom sphere.

1. **Pei J, Kim BH, Grishin NV.** 2008. PROMALS3D: a tool for multiple protein sequence and structure alignments. Nucleic Acids Res **36:**2295-2300.
2. **Jones DT.** 1999. Protein secondary structure prediction based on position-specific scoring matrices. J Mol Biol **292:**195-202.
3. **Laskowski RA, Swindells MB.** 2011. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. J Chem Inf Model **51:**2778-2786.

Figure S1.

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Conservation:                       8
RnfG_Tm            1  M-------------------------------------------------KDILKTGLILMVFTAISGLFLGL   24
RnfG_Tm coords.       ----------------------------------------------------------------------
NqrC_Pd            1  MAKYKCKVCGYVHEGNKAPDTCPVCSAPASEFEEIKEEGAAKKGINRDSNVYTVVYAAVMVVLVAVVLAF   70
NqrC_Pd coords.       ----------------------------------------------------------------------
NqrC_Vc            1  MAS---------------------------------------------NNDSIKKTLFVVIALSLVCSIIVSA   28
NqrC_Vc coords.       ----------------------------------------------------------------------
NqrC_Vh            1  MAS---------------------------------------------NNDSIKKTLGVVIGLSLVCSIIVST   28
NqrC_Hd            1  MAK----------------------------------------------FNKDSVSGTLTVVVLLSLICSLIVAS   29
NqrC_So            1  MVF------------------------------------------------KKDTVVGTMIFTITLCLLCSFMITG   28
NqrC_So coords.       ----------------------------------------------------------------------
NqrC_Td            1  M------------------------------------------------KQMIKLALTLSAYAVIACLALAA   24
RnfG_Vc            1  ML-----------------------------------------------TAIRKNGLILAVFACVSTGLVAL   25
RnfG_Ec            1  ML-----------------------------------------------KTIRKHGITLALFAAGSTGLTAA   25
NqrC_Ef            1  MK------------------------------------------------VNK---------FVKGF   10
Consensus_aa:         M.....................................................................
Consensus_ss:                                                          hhhhhhhhhhhhhhhhhhhhh


Conservation:                     5    5              5
RnfG_Tm           25  VYVGVKGKIQEADNAAKLSAIKFVLKDPLTGDYLVDEKE----IEEIVKKT-----------------GI   73
RnfG_Tm coords.   37  ------------DNAAKLSAIKFVLKDPLTGDYLVDEKE----IEEIVKKT-----------------GI   73
NqrC_Pd           71  TSQSLRSFQKQNEDNDKRQQILRSINVNVSS---SEAETKYNELIKEAFLVNENGEKVE--GD---AFAT   132
NqrC_Pd coords.   73  --QSLRSFQKQNEDNDKRQQILRSINVNVSS---SEAETKYNELIKEAFLVNENGEKVE--GD---AFAT   132
NqrC_Vc           29  AAVGLRDKQKENAALDKQSKILQVAGIEAKGS--KQIVELFNKSIEPRLVDFNTGDFVE--GD---AANY   91
NqrC_Vc coords.   33  ----LR---------DKQSKILQVAGIEAKGS--KQIVELFNKSIEPRLVDFNTGDFVE--GD---AANY   91
NqrC_Vh           29  AAVGLRDKQKANAVLDKQSKIVEVAGIEADG---KKVPELFAEYIEPRLVDFKTGDFVETAGDGSTAANY   95
NqrC_Hd           30  AAVLLKPTQDIQKQLDKQKNILQAAGLMHEN---TNVQETYAKFIEPKIVDLATGDYVE--D----VANF   90
NqrC_So           29  TAGVLKERKLAKKRDELQRYVLMAADVNLGQG--NEFRDIFAKSVKPLLINLDTGKVDS--DAN--VLDF   92
NqrC_So coords.   32  ---SLKERKLAKKRDELQRYVLMAADVNLGQG--NEFRDIFAKSVKPLLINLDTGKVDS--DAN--VLDF   92
NqrC_Td           25  VYNFTAPRIAEVKIEKTNRALKAVFPEAEDF---KEIS------------------------------   59
RnfG_Vc           26  TYALTAEQIQQQEQKQLLQVLNQVIPHKYHD---NPLA--------QACTLV----------------   66
RnfG_Ec           26  INQMTKTTIAEQASLQQKALFDQVLPAERYN---NALA--------QSCYLV----------------   66
NqrC_Ef           11  AAIALSSL-----------VLAACGADKKD---------------------NTTNS--SS---AASS   40
Consensus_aa:         ....hp..b......pb.p.l..hhs...p....pph.........................sh
Consensus_ss:         hhhhhhhhhhhhhhhhhhhhhhhhhhh          hhhh        hh
```

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                                                                     **
Conservation:       6                    55 5                     6  5              5
RnfG_Tm           74 ETVV-------LKE----YKEG-----VVLGPLYEFVTKD-GRNAYVLSG-YAPGFGGNVTVVACFIKTE 125
RnfG_Tm coords.   74 ETVV-------LKE----YKEG-----VVLGPLYEFVTKD-GRNAYVLSG-YAPGF-GNVTVVACFIKTE 125
NqrC_Pd          133 DVVKAA-------------------TEHQYPVFVANVDG--QPKYIMAL-HGAGLWGPLWGYISVDSD- 178
NqrC_Pd coords.  133 DVVKAA-------------------TEHQYPVFVANVDG--QPKYIMAL-HGAGLWGPLWGYISVDSD- 178
NqrC_Vc           92 DQRKAAKEASESIKLTAEQDKAKIQRRANGVVYLVKDGD-KTSKVILPV-HGNGLWSMMYAFVAVETD- 158
NqrC_Vc coords.   92 DQRKAAKEASESIKLTAEQDKAKIQRRANGVVYLVKDGD-KTSKVILPV-HGNGLWSMMYAFVAVETD- 158
NqrC_Vh           96 DQRKAAKDPAESIKLTAEEDKAKILRRANTGIVYLVKSGD-EVSKVIIPV-HGTGLWSMMYAFVAVETD- 162
NqrC_Hd           91 DAKAFAKDPATSVAIKPEDDKANIRMRAKYAEVYLVKDEMGQTTQVVLPM-YGNGLWSMMYGFVAVQPD- 158
NqrC_So           93 DERMAAINPETSSTPK--KDIAKIKTRANDARVFKVFDDSGKLSSVVVPF-YGKGLWSMIYGYVAVEPD- 158
NqrC_So coords.   93 DERMAAINPETSSTPK--KDIAKIKTRANDARVFKVFDDSGKLSSVVVPF-YGKGLWSMIYGYVAVEPD- 158
NqrC_Td           60 -----------------AEIPEGLNKTTFLNAYTAIKNG-KTVGLTITA-KGPTYAS-ATILIAMDLN- 107
RnfG_Vc           67 --------------------NDDKLGTAKTMHAYLAQRDG-QPTAIAIETIAPDGYNGEIKLIVGIANN- 114
RnfG_Ec           67 --------------------TAP-ELGKGEHRVYIAKQDD-KPVAAVLEATAPDGYSGAIQLLVGADFN- 113
NqrC_Ef           41 ETKK-------------------STESSAPAKKVAGGDLKDGTYKLEE-KNEKNGYRAVFEMTVKD--  86
Consensus_aa:        -.....................s....p.....h@.h...s.p.s.hhl....s.sh.t.hh.hhth..s.
Consensus_ss:         hh                       eeeeeee   eeeeeee e    eeeeeee


                      *    *
Conservation:       65    5     676667 5                   5 6
RnfG_Tm          126 DGFMLNSVRVIDYSQETPGLGAKIG------------EESIQRRFFPVPPEGLKN-------------- 168
RnfG_Tm coords.  126 DGFMLNSVRVIDYS------------------------QESIQRRFFPVPPEGLKN-------------- 168
NqrC_Pd          179 -KNTIYGADFSHQG-ETPGLGAEIS------------KPVFSNEFKGKKIFMSGEF------------- 220
NqrC_Pd coords.  179 -KNTIYGADFSHQG-ETPGLGAEIS------------KPVFSNEFKGKKIFMSGEF------------- 220
NqrC_Vc          159 -GNTVSGLTYYEQG-ETPGLGGEVE------------NPAWRAQWVGKKLFDENHK------------- 200
NqrC_Vc coords.  159 -GNTVSGLTYYEQG-ETPGLGGEVE------------NPAWRAQWVGKKLFDENHK------------- 200
NqrC_Vh          163 -GDTVSGITYYEQG-ETPGLGGEVE------------NPAWRAQFVGKKLFDENHK------------- 204
NqrC_Hd          159 -ANTVNGITYYEQG-ETAGLGGEIA------------NPNWQKSFVGKKLFNANNE------------- 200
NqrC_So          159 -FNTIKGVVVYEHG-ETPGIGDFVT------------DPHWLSLWKGKQLFDDKGK------------- 200
NqrC_So coords.  159 -FNTIKGVVVYEHG-ETPGIGDFVT------------DPHWLSLWKGKQLFDDKGK------------- 200
NqrC_Td          108 --KTIRKIEFLELT-DTPSLGSKAA------------DEPFAGQFNGKALDSAF--------------- 146
RnfG_Vc          115 -G-TVLGVRVLAHQ-ETPGLGDKIDLR----------ISNWVLGFNGQQVTADNQ-------------- 156
RnfG_Ec          114 -G-TVLGTRVTEHH-ETPGLGDKIELR----------LSDWITHFAGKKISGADD-------------- 155
NqrC_Ef           87 -G-KITESKYDNIN-ADGKSKTEDTKYEESMKAKSGVGPKEYIKQLNDSFVKAQSASGVEVVTGATHSSE 153
Consensus_aa:        ...hl..hph.p.s.-Tst.lGsch..................@..p@.sp.l.s................
Consensus_ss:         eeeeeeeeee              hhhhhh
```

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                                                                        †
Conservation:                                                           7
RnfG_Tm              169 ----------------------------------------------GLRVDKDAG------------ 177
RnfG_Tm coords.      169 ----------------------------------------------GLRVDKDAG------------ 177
NqrC_Pd              221 ----------------------------------------------KSVAVVK-------------- 227
NqrC_Pd coords.      221 ----------------------------------------------KSVAVVK-------------- 227
NqrC_Vc              201 ----------------------------------------------PAIKIVK--G----------- 208
NqrC_Vc coords.      201 ----------------------------------------------PAIKIVK--G----------- 208
NqrC_Vh              205 ----------------------------------------------PAIKIVK--G----------- 212
NqrC_Hd              201 ----------------------------------------------VALTIGK--G----------- 208
NqrC_So              201 ----------------------------------------------FAMRLVK--G----------- 208
NqrC_So coords.      201 ----------------------------------------------FAMRLVK--G----------- 208
NqrC_Td                  ---------------------------------------------------------------------
RnfG_Vc              157 ----------------------------------------------DDWKVRK-------------- 163
RnfG_Ec              156 ----------------------------------------------AHWAVKK-------------- 162
NqrC_Ef              154 SFQNYAQQLIQAAQAGNTDTIEIDNGATLKDGTYSLKEKNDSNGYHTTFSMTVKDGKVTESNYDNVNADG 223
Consensus_aa:            ...............................................sh.lsK................
Consensus_ss:                                                                    eeee
```

```
                                                                        &     *****
Conservation:                                                           6     5979 7  57
RnfG_Tm              178 ----------------------------------LPKGSPEELKKQGIVKVSDVMTGATITPRAVVTAL 212
RnfG_Tm coords.      178 ----------------------------------LPKGSPEELKKQGIVKVSDV------TPRAVVTAL 212
NqrC_Pd              228 ----------------------------------PGKS----VAGQ---DYVDGISGGTITSKGVDEML 255
NqrC_Pd coords.      228 ----------------------------------PGKS----VAGQ---DYVDGISGGTITSKGVDEML 255
NqrC_Vc              209 ----------------------------------GAP----QGSE---HGVDGLSGATLTSNGVQNTF 235
NqrC_Vc coords.      209 ----------------------------------GAP----QGSE---HGVDGLSGATLTSNGVQNTF 235
NqrC_Vh              213 ----------------------------------GAP----EGSE---HGVDGLSGATLTGNGVQGTF 239
NqrC_Hd              209 ----------------------------------AS----ADKE---HGVDGLSGATLTSKGVDNSF 234
NqrC_So              209 ----------------------------------GVK----EGDI---HGVDAVSGATMTGRGVQRAM 235
NqrC_So coords.      209 ----------------------------------GVK----EGDI---HGVDAVSGATMTGRGVQRAM 235
NqrC_Td              147 ----------------------------------------EVN---ADINAIGGATITSRGVAAIV 169
RnfG_Vc              164 ----------------------------------------DG---GQFDQFTGATITPRAVVLAV 185
RnfG_Ec              163 ----------------------------------------DG---GDFDQFTGATITPRAVVNAV 184
NqrC_Ef              224 KSKKDDTEYESKMKDVAGVGPKEYIETLNKEFVKAMGEE----DGSP---AGVEVVTGATHSTHSFINYA 286
Consensus_aa:            .................................................h-shoGAThTs+tV..hh
Consensus_ss:                                                             eeeee  hhhhhhhh
```

5

```
Conservation:                                    5
RnfG_Tm              213  NLMYR--YLEEVSK------------------  224
RnfG_Tm coords.      213  NLMYR--YLEEVSK------------------  224
NqrC_Pd              256  FNSLS--GYVKFLTSQN---------------  270
NqrC_Pd coords.      256  FNSLS--GYVKFLTSQN---------------  270
NqrC_Vc              236  DFWLGDMGFGPFLTKVRDGGLN----------  257
NqrC_Vc coords.      236  DFWLGDMGFGPFLTKVRDGGLN----------  257
NqrC_Vh              240  DFWLGDMGFGPFLAKVRDGGLN----------  261
NqrC_Hd              235  KYWFGTNGFGPYLAKFKATAGAN---------  257
NqrC_So              236  EFWFGVEGFQTFFNQLKASADQGELGGAK---  264
NqrC_So coords.      226  EFWFGVEGFQTFFNQLKAS-------------  253
NqrC_Td              170  KDASV--TAIEYMAKNNLEEGK----------  189
RnfG_Vc              186  KKAVE--YVNQHQQQLHNQPNPCEGQ------  209
RnfG_Ec              185  KRAGL--YAQTLPAQLSQLPACGE--------  206
NqrC_Ef              287  QQLVN--AAEKGDT-------TEIVVDNIVTK  309
Consensus_aa:             p.hh....h..h..p.p...............
Consensus_ss:             hhhhh  hhhhhhhhhh
```

6

Figure S2.