Supplemental material



FIG S1 Purification of AlnH: SDS-PAGE of AlnH fractions after final anion exchange chromatography (RESOURCE Q). AlnH from fraction 11 was used.



FIG S2 Purification of AInT: SDS-PAGE of AInT fractions after Ni-affinity purification.



FIG S3. Multiple sequence alignment of AlnH with selected homologous proteins. The accession numbers for the proteins above are as follows: Phea2, Flavin Reductase Phea2 (PDB: 1RZO) (Van Den Heuvel et al, 2004); AlnH flavin reductase from Streptomyces sp. CM020 (GenBank: ACI88884); flavin reductase actG, flavin reductase domain-containing protein FMN-binding protein from Streptomyces sp.SA3_actG (GenBank:ZP 07976974); ActVB, flavin reductase ActVB from Streptomyces coelicolor A3(2) (GenBank:NP_629242); Flavin_reductase_DS-1, flavin reductase domain-containing protein from Parvibaculum lavamentivorans DS-1 (GenBank:YP 001414152). PheA2 is currently the most similar protein to AlnT whose structure has been solved. The secondary structure of PheA2, is illustrated with α -helices, β sheets and turns. From the sequence alignment, though patches of amino acids are conserved, few of the highly conserved amino acids are involved in binding either the FAD or the NAD in the PheA2 structure. From $\alpha 1$, Arg7 is conserved and forms a hydrogen bond with the adenine ring (N3). In addition, the conserved motif SVS (β 3,38-40 of the second subunit) seems to be involved in binding NAD through hydrophobic interaction and hydrogen bonding of Ser38. Other than side chain (N81 which is not conserved) the remaining hydrogen bonds occur between the main chain of PheA2 and the FAD. Of these, only Thr32 is highly conserved. Like with AlnT, hydrogen bonds may occur between the main chain and flavin as well as NADH. The conserved amino acids visible within the sequence alignment may be important for fold formation.

C2_HPA	1 10	2 0	3 0	αl 200000000 40 5	α2 2000 200000 0 60	α3 2000 70
C2_HPA AlnT LndZ5 actVA5 PokU2 3-Hsa	MENTVLNLDSDV MRERT MTDRG MSEDT MSEDT QG	THACEAIFQPIRL PLTAWPPGARRPC TVEAVVS MTQERPS PTAPPAD HVGDHDS	VYTHAQTPDVS PRLLPRTTRSM RQE	GVSMLEKIQQILE GGARMLDTEELAQ PSTLMSAAEVVAA LTAHARRIAE .TGLSAAAGKVAE .HEVXQRLDALE	QIAKNAESAEQL QAAESARGAEEA LAGDQADAADET LAGKRAADAEQ LAAEHAAAADSD TLRERAQETEDL	R V P D E N I K R K L D P D V V K R L E P D V V K R L S P D V V D D R L S A T V A E R I P D D S X K
	n1	n2	α4	α	5	α6
C2_HPA	2222 222 80	222 90	100	2222 2222222 110	222222222 120 <u>1</u>	20202020
C2_HPA AlnT LndZ5 actVA5 PokU2 3-Hsa	LIKEIGLHRAFQ LLVEAGFARHFV AVLGRRFARHFV AVLRAGFAAHFV ALVSAGFARHFV ALVSAGFARHLV ALQETGFFRLLQ	PKVYGGLEMS PRECGGTEGT PAAYGGSAGT PVAHGGRAAT GPRWGGSAAAPGA PEQWGGYQADP	L PDFANCIVTL FAELTEAVARV FGDLTRALATV FGELVEPVAVL VRDLARALSVV .VLFYSAVRKI	AGACAGTAWAFSI GAACPATAWCASI GAGCAATSWSASI GEACASTAWYASI GAGCMSAAWCAGV ASACGSTGWVSSI	L C TH S HQ I AMFS AANLGRMAAYLP VAGVGRMAGYLP TASLGRMAGYLP LAAVGRMCSHLP IGVHNWHLALFS	KQLQDEIWL AEGYREVWA AEGCAEVWQ DEGQAELWS EEGQAELWG QQAQEDVWG
	β1	β2	β3	η3 β4	β5	η4 <mark>β</mark> 6
C2_HPA	140 15	0 TT	170	180	TT 2	• • •
C2_HPA AlnT LndZ5 actVA5 PokU2 3-Hsa	KDPDATASSSIA QGPDALVVGSLS DGPDTVVVGSLA DGPDALIVGALM DGPDVPLAGSLA NDTDVRISSYA	PFGKVEEVEGGII PFGKAAPAAGGWT PLGRAKSVPGGFF PLGRAEKTPGGWF PSGTVDTVPGGWF PXGAGQVVDGGYT	LNGDYGWSSGC VSGRWPYISAV LTGTWPSISVV VSGTWPFVSVV LSGTWDFASGV VNGAWAWSSGC	DHAEYAIVGFNRF AYADWLLLCATLS DFSDWALVRAVFA DHSDWALICAKVG DHAQWTMVGGLVR DHASWAVLGGPVI	DADGNKIYSFGV DGAGPRVFA TGEHR.APRVFV EEPWFFA GPEGPPALRHFV KDGRPVDFVSFL	IPRSDYEIV VPRDEVEVV IPRAAYTVR VPRQEYGIV LPRRDYRVL IPREDYRID
	25	87	n6 B8 a7		a g	
C2_HPA	رار 210 22	0 230	10 p8 07 22-→222222 240	250 TT	رمه المراجع 260 2	<u>0000000000000000000000000000000000000</u>
C2_HPA AlnT LndZ5 actVA5 PokU2 3-Hsa	DNWYAQAIKSSG ESWESVGMRATG DTWTNIGMRATG DSWYPMGMRGTG DTWRNVGLRGTG DVWNVVGLRGTG	SKMLKLVNVFIPE SHTVVASDVFVPE SHTLVVDDVFVPE SNTLVLDGVFVPE SNSVVLDDVFVPA SNTVVVEDVFVPA	YRISKAKDMME RRAFARAD ARTFDGGD ARACTRAA HRSFEHRT HRVLSFKAXSN	GKSAGFGLYPDSK LLTGRPTASTAA LFEGRPQHSTEA IAAGLGPDAEAI VLAGDPAPSAPR LTAPGLERNTAP	IFYTPYRPYFAS CHTVLLEAANGL CHTVLLEAANGL CHTVPMRAVNGL LHRVPLKLINGL VYKXPWGTIHPT	GFSAVSLGI SFAGPLLGA SFAPPTLGA AFALPMLGA FFVAPGIGA TISAPIVGX
	95			g10		
$C2_HPA$	280 29	Q TTT 0 300	202020	320	<u>2222222222</u> 330	2000 Q 340
C2_HPA AlnT LndZ5 actVA5 PokU2 3-Hsa	AERMIEAFKEKQ AEGALAQWSAYA ARGALAHWTDYA ARGALAHWTDYA VEGALGAWTRWT AYGAYDAHVEHQ	RN <mark>R VR</mark> AYTGANVG VVKARSVLLRPQA TAKFRRAPEAAGG AGRLAGPTGQNAV ASRVEINGVRTG. GK <mark>RVR</mark> AAFAGEKA	LATPALM [,] PGPSREALAGV PGLTRGHWLT SSQDRVVYEHT SRPGVRLT KDDPFAKV <mark>R</mark>	IAESTHQVAAARA LAHSAGETDAARL LARSAGEIDAAQL LARATGEIDAAQL LARATGEIDAAQL LARAAAALDAARL IAEASSDIDAAWR	LLEKTWEDHRIH LLERCAQV LIERASEV LLERVAAV IVDHAAAE QLSGNVADEYAL	GLNHQYPNK ADLGAEVTL ADRGAEITP ADAG.SATG ADEA.EPTP LVAGEEVPF
			-	10		12
$C2_HPA$	α 222222222222 359	11 <u>00000000000000000000000000000000000</u>	η/ & 2222 ο 380	α12 <u>000000000000000000000000000000000000</u>	2 <u>2222</u> 400	α13 20000 410
C2_HPA AlnT LndZ5 actVA5 PokU2 3-Hsa	ETLAFWRTNQAY EQTRRNLRDTAL LEVVRNGRDCSL VLVGRGARDCAL ALTLRVTRDYST ELRLRARRDQVR	AVKMCIEAVDRLM SVRMLASAVNRLA AVELLVTAVNRVF AAELLTAATDRLF AVDLLLDSVNGLM ATGRAISSIDKLF	AAAGATSFMDN ANGGTTGYGES GAAGTSGHSVT ASAGTRAQAQD IRM <mark>SGA</mark> RGQALS 'ES <mark>SGAT</mark> ALANG	SELQRLFRDAHMT APLQRYWRDVNTS SPLQRDWRDVNAA SPMQRLWRDVHAA HPVQRVWRDVHAA TPLQRFWRDAHAG	GAHAYTOYDVCA ATHVALQFESAA STHVGLQFEPAA GSHIGLQFGPGA AGHAALQPDVNA RVHAANDPERAY	QILGR LAYAEGRLW HAY ALYAG DGWAK VXYGTG

FIG S4. Multiple sequence alignment of AlnT with selected homologous proteins. The accession numbers for the proteins above are as follows: C2_HPA, Monooxygenase Component C₂ Of P-Hydroxyphenylacetate Hydroxylase from *Acinetobacter Baumanni* (PDB: 2JBT, Alfieri *et al*, 2007); AlnT, hydroxylase from

Streptomyces sp. CM020 (GenBank: ACI88867); LndZ5, oxygenase LndZ5 from Streptomyces globisporus (GenBank: AAR16420); actVA5, actVA5 from Streptomyces coelicolor A3(2) (GenBank: CAA41641); PokU2, PokU2 from Streptomyces diastatochromogenes (GenBank: ACN64826); and 3-Has, 3-Hsa Hydroxylase from Rhodococcus Sp.Rha1. (PDB:2RFQ). The two closest structures to AlnT solved are 3-Has and C₂ HPA with only HPA containing bound substrate and cofactor. The secondary structure of C₂ HPA, determined from the structure, is illustrated with α -helices, β -sheets and turns. Of the highly conserved amino acids (highlighted in red) in the sequence alignment, only amino acids W112, S171,W210,G219,S220, G374, H396 in the structure of C₂ HPA are involved in protein scaffold embedding FMNH-. Of these amino acids, only one is suggested to form hydrophobic interactions with the flavin (W210), one is involved in forming two hydrogen bonds with flavin (S171) and one has been suggested to play an active role in catalysis (H396). In many flavoenzymes, the flavin is held in its position through hydrophobic interactions and hydrogen bonds networks. From the structure of C₂ HPA, many of the suggested hydrogen bonds occur between the main chain and the flavin. This may be reflected in the low degree of conserved amino acids within these flavoenzymes despite similar reaction mechanisms.



FIG S5 UV/Vis spectra of K1115 A (1), DHPA (2), ThA (3), ThB (4)



FIG S6 Observed NMR heteronuclear multiple bond correlations for ThA. Signals for nuclei connected through 3 bonds are shown in blue, and 2-bond correlations are shown in red.

Supplemental references

- 1. Alfieri, A., Fersini, F., Ruangchan, N., Prongjit, M., Chaiyen, P., Mattevi, A., (2007) Structure of the monooxygenase component of a two-component flavoprotein monooxygenase. Proc.Natl.Acad.Sci.(USA) **104**: 1177
- Van Den Heuvel, R.H., Westphal, A.H., Heck, A.J., Walsh, M.A., Rovida, S., Van Berkel, W.J., Mattevi, A., (2004) Structural studies on flavin reductase PheA2 reveal binding of NAD in an unusual folded conformation and support novel mechanism of action. J.Biol.Chem. 279: 12860-12867