Supplemental material

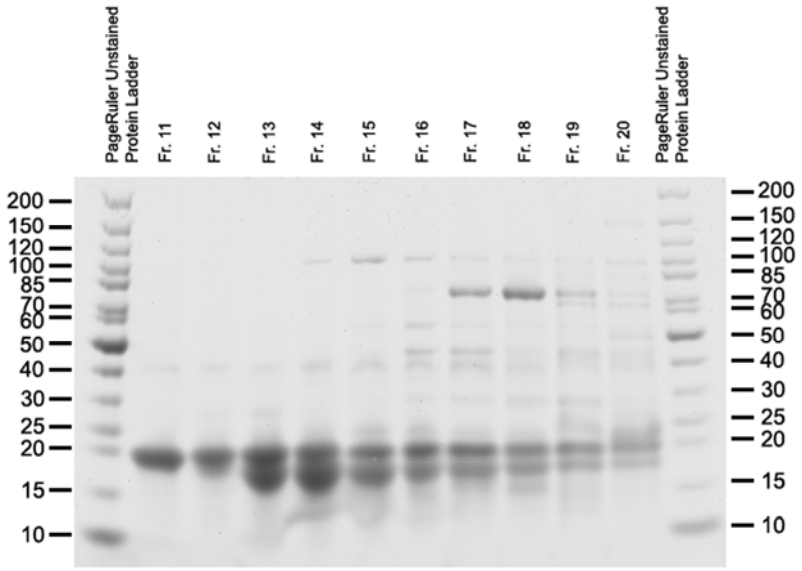

FIG S1 Purification of AlnH: SDS-PAGE of AlnH fractions after final anion exchange chromatography (RESOURCE Q). AlnH from fraction 11 was used.

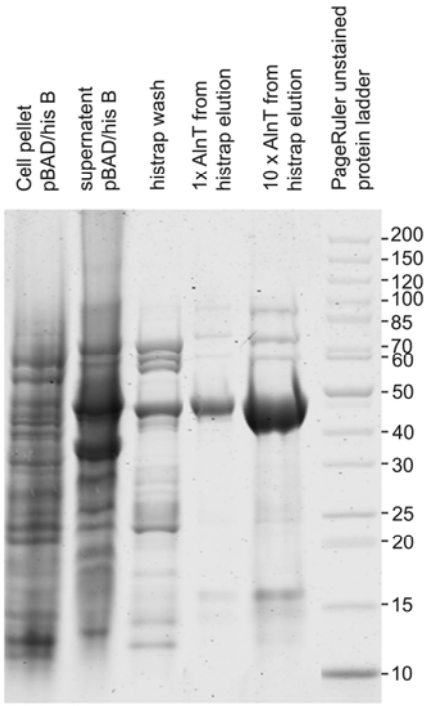

FIG S2 Purification of AlnT: SDS-PAGE of AlnT fractions after Ni-affinity purification.

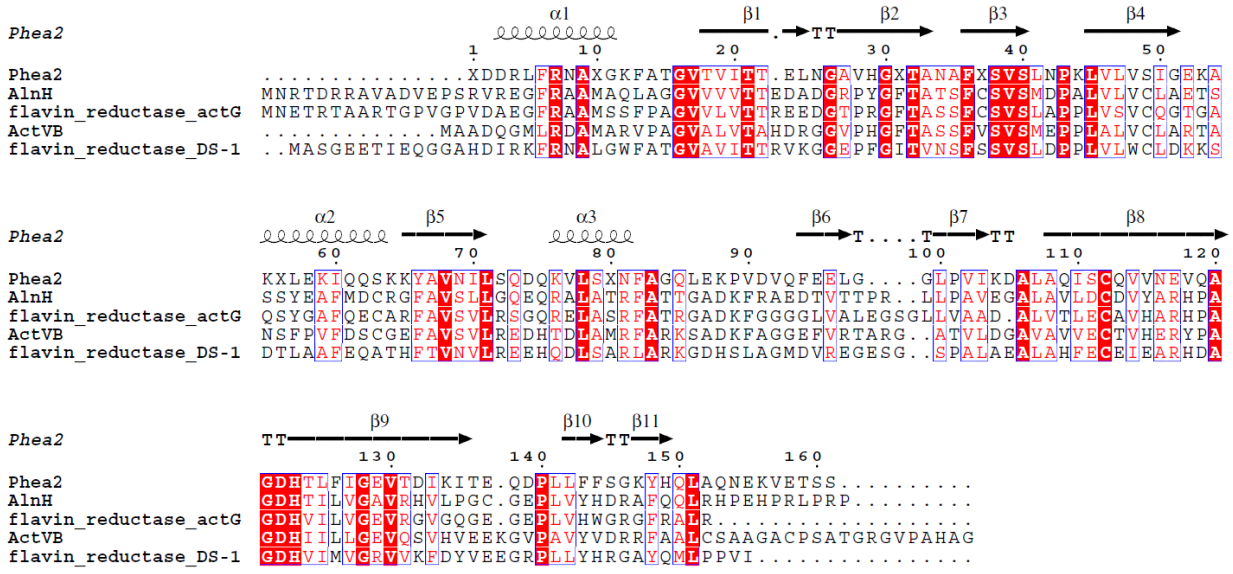

FIG S3. Multiple sequence alignment of AlnH with selected homologous proteins. The accession numbers for the proteins above are as follows: Phea2, Flavin Reductase Phea2 (PDB: 1RZ0) (Van Den Heuvel *et al*, 2004); AlnH flavin reductase from *Streptomyces sp. CM020* (GenBank: ACI88884); flavin_reductase _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 α1, Arg7 is conserved and forms a hydrogen bond with the adenine ring (N3). In addition, the conserved motif SVS (β3,38-40 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.

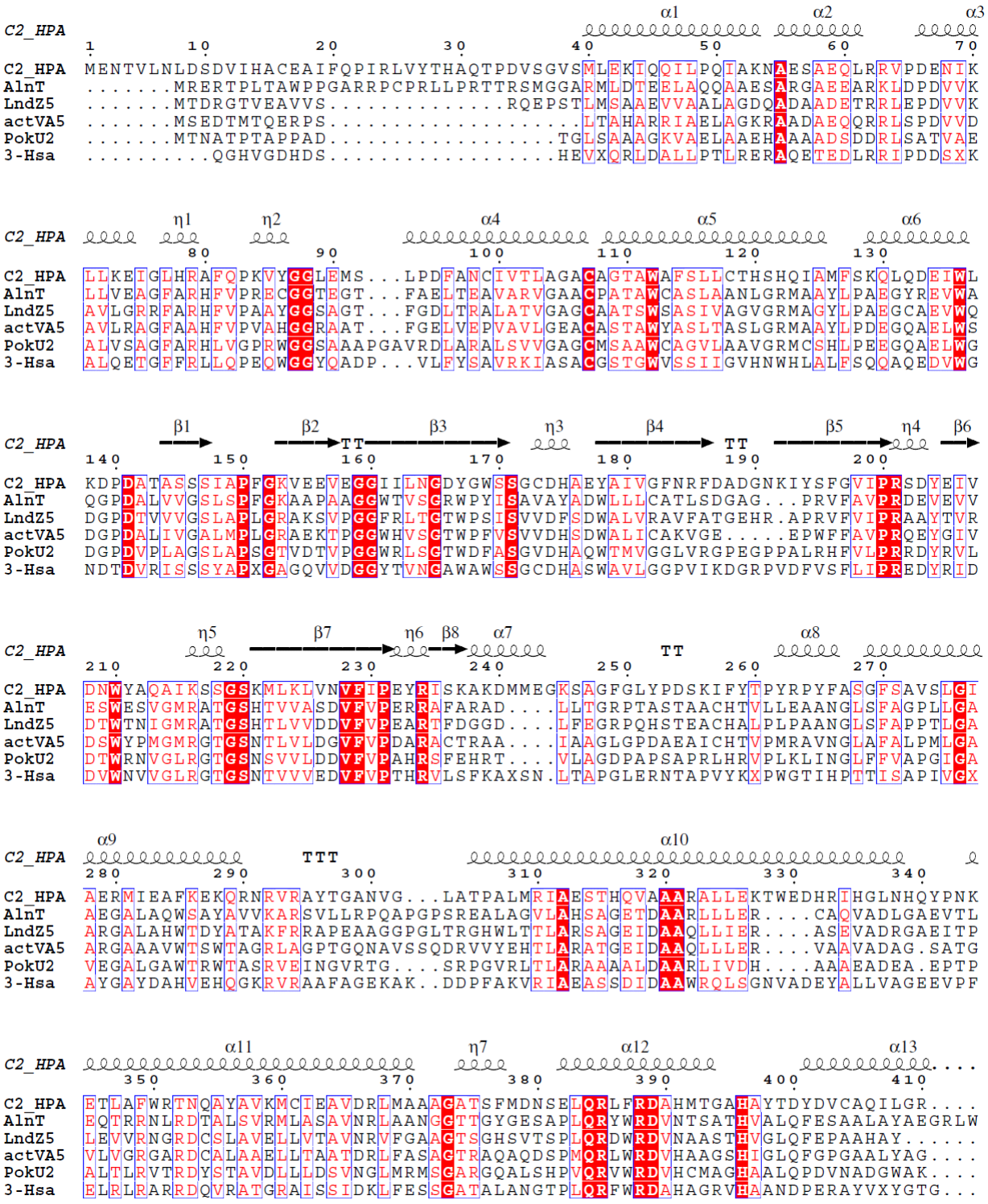

FIG S4. Multiple sequence alignment of AlnT with selected homologous proteins. The accession numbers for the proteins above are as follows: C2_HPA, Monooxygenase Component C_2 Of P-Hydroxyphenylacetate Hydroxylase from *Acinetobacter Baumanni* (PDB: 2JBT, Alfieri *et al*, 2007); AlnT, hydroxylase from

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 C_2 HPA with only HPA containing bound substrate and cofactor. The secondary structure of C_2 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 C_2 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 C_2 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.

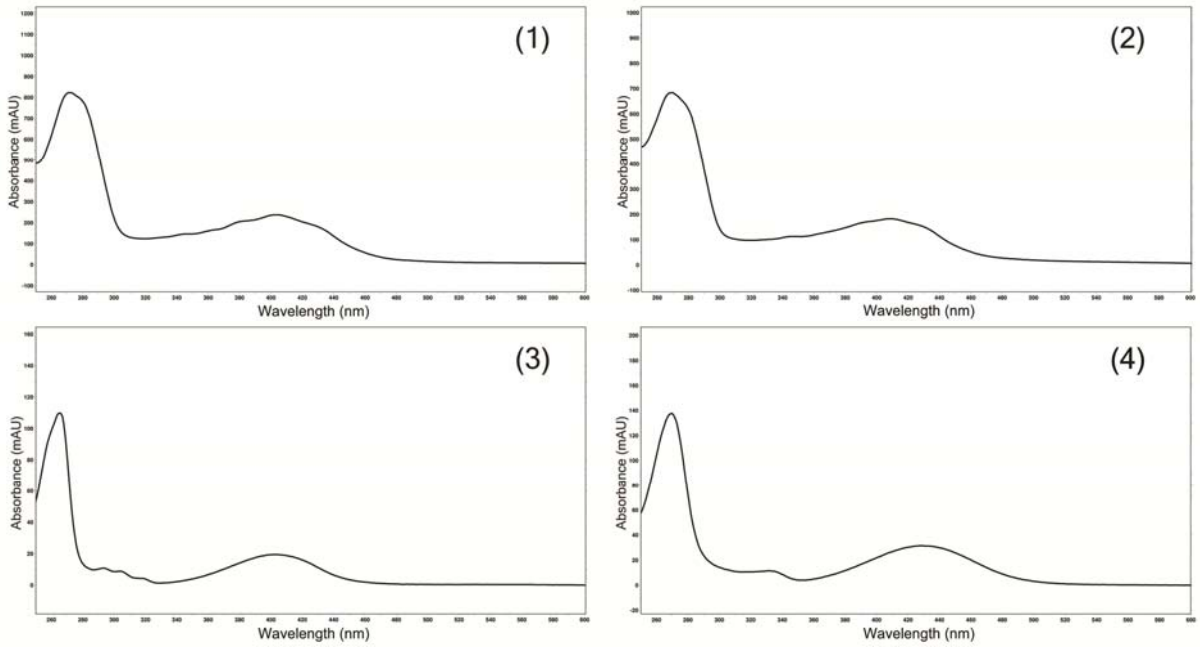

FIG S5 UV/Vis spectra of K1115 A (1), DHPA (2), ThA (3), ThB (4)

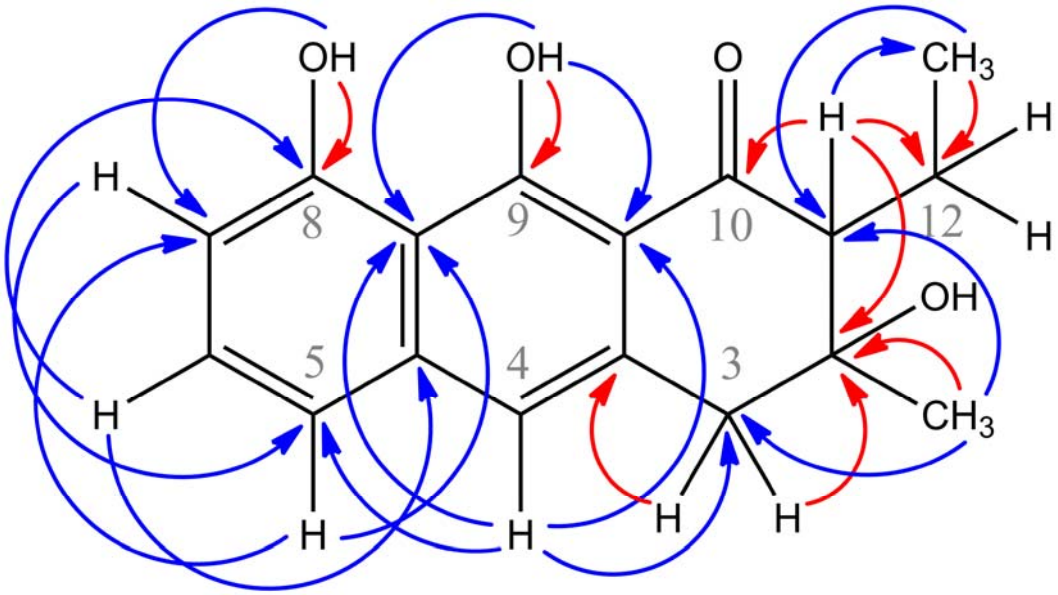

FIG S6 Observed NMR heteronuclear multiple bond correlations for ThA. Signals for nuclei connected through 3 bonds are shown in blue, and 2-bond correlations are shown in red.

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
2. **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