Supporting Information

Dehydrogenase *versus* oxidase function: the interplay between substrate binding and flavin microenvironment

Teresa Benedetta Guerriere[1], Alessandro Vancheri[2], Ilaria Ricotti[2], Stefano A Serapian[2], Daniel Eggerichs[3], Dirk Tischler[3], Giorgio Colombo[2], Maria L. Mascotti[4], Marco W. Fraaije[5*], Andrea Mattevi[1*]

[1]Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Pavia, Italy 27100.

[2] Department of Chemistry, University of Pavia, 27100 Pavia, Italy

[3] Microbial Biotechnology, Ruhr University Bochum, 44780, Bochum, Germany.

[4] IHEM CONICET, Universidad Nacional de Cuyo, Mendoza, Argentina, M5502JMA.

[5] Molecular Enzymology Group, University of Groningen, Groningen, The Netherlands, 9747AG.

***Correspondence**: M.W.Fraaije@rug.nl; andrea.mattevi@unipv.it

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oxidases

dehydrogenases

VAO

EPO

EUGO

EUGH

PCMH

PinH

OxD

0.99/1

1/1

0.94/1

0.97/1

0.86/0.97

1/1

1/1

0.97/1

1/1

0.93/0.98

1/1

1/1

1/1

1/1

1/1

0.95/0.99

XP_025528612_Aspergillus_japonicus
PYI22211_Aspergillus_violaceofuscus
XP_025508039_Aspergillus_aculeatinus
P56216_VAO_Penicillium_simplicissimum
GAD99964_Byssochlamys_spectabilis
XP_014534267_Penicillium_digitatum
OQE40368_Penicillium_coprophilum
OQE29691_Penicillium_steckii
OQD67827_Penicillium_polonicum
KPM40738_Neonectria_ditissima
XP_033383414_Aaosphaeria_arxii
XP_030989101_Phialemoniopsis_curvata
XP_018384308_Alternaria_alternata
XP_028501271_Alternaria_arborescens
OAK97369_Stagonospora_sp
OAL52557_Pyrenochaeta_sp
EYE93387_Aspergillus_ruber
XP_022395271_Aspergillus_glaucus
XP_022395489_Aspergillus_glaucus
OJJ38082_Aspergillus_wentii
OJJ98897_Aspergillus_versicolor
GEL10034_Aspergillus_calidoustus
XP_022488900_Penicillium_arizonense
XP_013324909_Rasamsonia_emersonii
KAF3334572_Aspergillus_arachidicola
OQD77096_Penicillium_decumbens
XP_028486731_Byssochlamys_spectabilis
XP_020127612_Diplodia_corticola
EKG15142_Macrophomina_phaseolina
EPG89436_Penicillium_sp_occitanis
XP_002478276_Talaromyces_stipitatus
CEJ81907_Torrubiella_hemipterigena
XP_016644717_Scedosporium_apiospermum
XP_024725167_Amorphotheca_resinae
CZS92051_Rhynchosporium_agropyri
CVH81999_Cadophora_sp
XP_007827758_Coniosporium_apollinis
XP_018071526_Phialocephala_scopiformis
CZR50926_Phialocephala_subalpina
XP_033682823_Trematosphaeria_pertusa
OCL14615_Glonium_stellatum
TID19977_Venturia_nashicola
XP_024737822_Hyaloscypha_bicolor
PMD33321_Hyaloscypha_variabilis
PMD15297_Pezoloma_ericae
XP_018064145_Phialocephala_scopiformis
XP_001545605_Botrytis_cinerea
XP_007837557_Pestalotiopsis_fici
XP_018282209_Cutaneotrichosporon_oleaginosum
XP_029755350_Aureobasidium_pullulans
XP_018004837_Phialophora_attinorum
KEQ62306_Aureobasidium_melanogenum
XP_016758956_Sphaerulina_musiva
EME43801_Dothistroma_septosporum
XP_016648723_Scedosporium_apiospermum
XP_003043622_Nectria_haematococca
XP_018033281_Paraphaeosphaeria_sporulosa
XP_008593409_Beauveria_bassiana
XP_015403327_Aspergillus_nomiae
XP_007826789_Pestalotiopsis_fici
XP_016235707_Fonsecaea_multimorphosa
XP_013316178_Exophiala_xenobiotica
KIY84505_Exophiala_sideris
XP_022510858_Fonsecaea_monophora
XP_013258378_Exophiala_aquamarina
XP_007728563_Capronia_epimyces
XP_007722892_Capronia_coronata
XP_013267550_Rhinocladiella_mackenziei
XP_007748241_Cladophialophora_psammophila
XP_023338284_Acaromyces_ingoldii
XP_033679937_Trematosphaeria_pertusa
XP_033440574_Daldinia_childiae
XP_030995098_Phialemoniopsis_curvata
XP_024732627_Hyaloscypha_bicolor
XP_007680503_Baudoinia_panamericana
XP_033519711_Lindgomyces_ingoldianus
XP_028486771_Byssochlamys_spectabilis
XP_002483977_Talaromyces_stipitatus
XP_002569206_Penicillium_rubens
XP_013312631_Exophiala_xenobiotica
XP_007748400_Cladophialophora_psammophila
XP_013321739_Exophiala_xenobiotica
XP_025377978_Acaromyces_ingoldii
XP_009157655_Exophiala_dermatitidis
XP_013314277_Exophiala_xenobiotica
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XP_003045411_Nectria_haematococca
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WP_056728375_Agromyces_sp
WP_158051316_Gulosibacter_chungangensis
WP_058593604_Leucobacter_chromiiresistens
WP_083077797_Cnuibacter_physcomitrellae
WP_155501896_Microbacterium_sp
AZS38132_Microbacterium_lemovicicum
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WP_043059352_Enteractinococcus_helveticum
WP_115933472_Citricoccus_muralis
WP_091045255_Nocardioides_sp
WP_141796121_Nocardioides_sp
WP_126337610_Streptomyces_cavernae
WP_097207849_Geodermatophilus_sabuli
WP_069757935_Streptomyces_sp
WP_128696233_Geodermatophilaceae_bacterium
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KLJ95921_Rhodococcus_sp
WP_124765626_Gordonia_sp
WP_036369218_Mycolicibacterium_austroafricanum
Q0SBK1_EUGO_Rhodococcus_jostii
AHK34724_Rhodococcus_opacus
WP_015625223_Actinoplanes_sp
WP_106243419_Allonocardiopsis_opalescens
WP_139942235_Emcibacter_nanhaiensis
WP_104830661_Marinicaulis_flavus
PZN80562_Proteobacteria_bacterium
RPH64826_Acidobacteria_bacterium
AMK59458_uncultured_bacterium
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RLA0981_Gammaproteobacteria_bacterium
WP_105998241_Sphingopyxis_lindanitolerans
WP_088443890_Sphingobium_bauzanensis
WP_008828118_Sphingomonadaceae
WP_073023478_Cycloclasticus_sp
WP_042368081_Aeromicrobium_sp
WP_095402932_Paraburkholderia_aromaticivorans
WP_102129159_Sphingobium_yanoikuyae
WP_102634328_Paraburkholderia_rhynchosiae
Q8L388_Pseudomonas
CAB64355_Pseudomonas_sp
WP_109799174_Novosphingobium_sp
WP_124809348_Novosphingobium_sp
WP_022673211_unclassified_Novosphingobium
WP_135012638_Chakrabartia_godavariana
WP_144095967_Croceicoccus_sp
WP_093016404_Sphingobium_sp
WP_056693180_Sphingobium_sp
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WP_080263562_Pseudomonas_aeruginosa
WP_011237617_Aromatoleum_aromaticum
OJW77611_Thiobacillus_sp
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WP_056067268_Pseudomonas_sp
WP_101470071_Marinobacter_sp
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EKU30911_Alcaligenes_sp
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RPH81070_Nitrospiraceae_bacterium
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WP_191229272_Aurantiacibacter_xanthus
WP_160589268_Alteraurantiacibacter_aestuarii
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OJW61583_Sphingomonadales_bacterium
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TAJ93858_Gammaproteobacteria_bacterium
MBO9601182_Novosphingobium_sp
EIZ78193_Novosphingobium_sp
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OFW28461_Acidobacteria_bacterium
WP_081796170_Sphingomonas_paucimobilis
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WP_021239063_Sphingobium_quisquiliarum
WP_030539980_Sphingobium_sp
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WP_158897271_Sphingorhabdus_lacus
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ODU30800_Thiobacillus_sp
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MCJ97240_Proteobacteria_bacterium
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OGY79275_Sphingorhabdus_lacus
TAJ95915_Gammaproteobacteria_bacterium
HEN04094_Gammaproteobacteria_bacterium
MBX83178_Pseudomonadaceae_bacterium
MBO2485285_Gammaproteobacteria_bacterium
MBO2512751_Gammaproteobacteria_bacterium
PYV83755_Blastocatellia_bacterium
B9ZUK6_Chondromyces_crocatus
Q9KP03_uncultured_bacterium
WP_026205595_Methanococcoides_methylutens
WP_147654386_Methanomassiliicoccus_luminyensis
WP_115565255_Candidatus_Methanomethylophilus_alvus
WP_048112559_Candidatus_Methanoplasma_termitum
WP_014406728_Methanocella_conradii
WP_012035238_Methanocella_arvoryzae
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WP_014588665_Methanococcoides_burtonii
WP_013038387_Methanohalophilus_mahii
WP_159903316_Salinirussus_salinus
WP_121920850_Haloplanus_aerogenes
WP_014051603_halophilic_archaeon
WP_092802685_Halorubrum_ezzemoulense
WP_135822309_Halostella_litorea
WP_159763352_Halovenus_sp
WP_020449307_Salinarchaeum_sp
WP_129068865_Halorientalis_sp
WP_068887223_Natronolimnobius_aegyptiacus
WP_160065337_Natronorubrum_sp
WP_136592249_Natrinema_pallidum
WP_049952755_Halococcus_sediminicola
WP_007692075_Halococcus_hamelinensis
WP_149783333_Halorubrum_aquaticum
WP_129682337_Halobaculum_sp
WP_144094187_Halobellus_captivus
WP_156566944_Halorubrum_sp
WP_090506352_Natronorubrum_sediminis
WP_009972454_Natrinema_sp
WP_144144762_Candidatus_Methanoliviera
WP_066794814_Caldivirga_sp
WP_081142507_Ferroplasma_acidiphilum
WP_048101218_Acidiplasma
WP_110640545_Thermoplasma_sp
WP_015590256_Archaeoglobus_sulfaticallidus
WP_010878311_Archaeoglobus_fulgidus
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WP_013679342_Thermoproteus_uzoniensis
WP_148676291_Vulcanisaeta_distributa
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WP_012952295_Sulfolobus_islandicus
WP_048054529_Geoglobus_ahangari
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WP_049817778_haloarchaeon
WP_026436324_Thermofilum_adornatum
WP_146203977_Candidatus_Korarchaeum_cryptofilum
WP_144141943_Candidatus_Methanoliviera_sp
WP_144142933_Candidatus_Syntrophoarchaeum_sp
WP_144185090_Candidatus_Methanoliviera_sp
WP_144183801_Candidatus_Methanoliviera_sp

0.3

Supplementary Figure 1. Annotated phylogeny of the VAO/PCMH flavoprotein family. The tree was constructed in RAxML v8.2.10 using 500 rapid bootstraps and automated model selection. The Transfer Bootstrap Expectation/Poster Probability support values are shown at the nodes. Taxonomic groups are colored as follows: Fungi (red), Bacteria (white) and Archaea (blue). The accession codes and species name are given for each sequence included in the dataset. Experimentally characterized enzymes are shown in green. Clades are named as in the main text. The two sequences characterized in this work are marked with yellow stars. The scale bar indicates substitutions per site.

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PinH        -----------------------------------MLPPGIDSATFQKALSAFANVVGKEW    26
PCMH        ----------------------------MSEQNNAVLPKGVTQGEFNKAVQKFRALLGDDN    33
EUGH        ------------------------------MDTTVMLPAGVSTEQFSKAIAEFRLLLGEDN    31
nsVAO       MSGFSIDRRSLLGTGLGASLAAGLGTGASAANPAPLAPGRTKADFAGAMKAFRGVVGAEW    60
VAO         -------------------------MSKTQEFRPLTLPPKLSLSDFNEFIQDIIRIVGSEN    36
VAD         --------------------------------MAAPLPEGVSAEAMSSALDRFARIVGADW    29
EPO         -------------------------------MNFRTLPDGVSAEQFANAISEFSETIGSEY    30
EUGO        --------------------------------MTRTLPPGVSDERFDAALQRFRDVVGDKW    29
                                               *        :   :  :   :* .

PinH        VFTSDEDLKL----------YRDAYSPYMGEAEERLASAAVAPDTSEQVQEIARIANQYS    76
PCMH        VLVESDQLVP----------YNKIMMPVEN--AAHAPSAAVTATTVEQVQGVVKICNEHK    81
EUGH        VLATAERVVP----------YTKLLIPTED-DASFMPSGALTPGSVEEVQKILAICNRYK    80
nsVAO       VFGDEEAVVP----------WSKTYIPDP--AHQYKPVGAVCPQSVEEVQEIVRIANTYK    108
VAO         VEVISSKDQIVDGSYMKPTHTHDPHHVMD--QDYFLASAIVAPRNVADVQSIVGLANKFS    94
VAD         VFT-EDKITP----------YEDPYTISND-ETEHRPYAAVAPASTEEVQEIVRVANEFG    77
EPO         VRVDEATVSE----------YDDKFPVTDG--DEFKGSAVIWPGSTEDVQVIVRIANKYG    78
EUGO        VLSTADELEA----------FRDPYPVGAA--EANLPSAVVSPESTEQVQDIVRIANEYG    77
            *                     .               . :   .  :** :  :.* .

PinH        IPLYTISTGKNLGYGGSAPTYSGSVVLDL-KRMNRIIEVNEKQAYCIVEPGVSYFDMYRY    135
PCMH        IPIWTISTGRNFGYGSAAPVQRGQVILDL-KKMNKIIKIDPEMCYALVEPGVTFGQMYDY    140
EUGH        LPVWPISTGRNWGYGSASPATPGQLILDL-RKMNKIIEIDVDSCTALLEPGVTYQQLYDH    139
nsVAO       QPLWTVSTGKNMGYGMTAPATPGQVVLDL-KRMNRILEVDADLGTCLLEPGVTYQQLKDY    167
VAO         FPLWPISIGRNSGYGGAAPRVSGSVVLDMGKNMNRVLEVNVEGAYCVVEPGVTYHDLHNY    154
VAD         VPLWPVSRGKNFAYGGAAPVMSGTVVLDM-NRMNRILEVNEEFGYALVEPGVSYFELYDY    136
EPO         IPLHAFSGGRNLGYGGSSPMLTGTVLLHLGKRMNRVLEINEKLAYAVVEPGVDYKTLYEA    138
EUGO        IPLSPVSTGKNNGYGGAAPRLSGSVIVKTGERMNRILEVNEKYGYALLEPGVTYFDLYEY    137
             *:  .* *:* .** ::*   * :::.  ..**:::::: .   .::**** :  :

PinH        LQEKK--SKLWLDVPDPGW-GSMVGNAMDRGAGYTAAQFRNHFDAHCGMEVVLANGEVMR    192
PCMH        IQENN--LPVMLSFSAPSAIAGPVGNTMDRGVGYTP--YGEHFMMQCGMEVVLANGDVYR    196
EUGH        LKENN--IPLMLDVPTIGPMVGPVGNTLDRGVGYTP--YGEHFMMQCGMEVVLANGEILR    195
nsVAO       LEEHK--IPLWIDVPTVGPIASPVGNTLDRGVGYTP--YGEHFMFQCGMEVCLPDGRLMR    223
VAO         LEANNLRDKLWLDVPDLGG-GSVLGNAVERGVGYTP--YGDHWMMHSGMEVVLANGELLR    211
VAD         IQEKG--LKLWIDVPDPGW-GSVVGNALDHGIGYTP--YGDHFAMQCGMEVVLPNGEVVR    191
EPO         VRDSG--AKLMIDPAELDW-GSVMGNTMEHGVGYTP--YADHSMWRCGMEVVLADGEVLR    193
EUGO        LQSHD--SGLMLDCPDLGW-GSVVGNTLDRGVGYTP--YGDHFMWQTGLEVVLPQGEVMR    192
            :.       : :.    .   . :**::::* ***   : :*   : *:** * :* : *

PinH        TGMGAMPKS----------------KTWAMYKTGFGPAIDGIFSQSNFGIVTKMGFWLMP    236
PCMH        TGMGGVPGS----------------NTWQIFKWGYGPTLDGMFTQANYGICTKMGFWLMP    240
EUGH        TGMGSVPKA----------------KTWQAFKWGYGPYVDGIFTQSNFGVVTKMGLWLMP    239
nsVAO       TGMGSIKGS----------------TAWQAFKWGYGPYLDGLFTQSNFGIVTKMGLWLMP    267
VAO         TGMGALPDPKRPETMGLKPEDQPWSKIAHLFPYGFGPYIDGLFSQSNMGIVTKIGIWLMP    271
VAD         TGMGAMPGN----------------NTWQLFKYGYGPYVDGIFSQSNFGVVTKMGIWLMP    235
EPO         TGMGGLPGS----------------EAWHLYPGQLGPSIEGLFEQSNFGICTRMGMQLMP    237
EUGO        TGMGALPGS----------------DAWQLFPYGFGPFPDGMFTQSNLGIVTKMGIALMQ    236
            ****.:                        :    **  :*:* *:* *: *::*: **

PinH        EPEAFLKGHIHLSQYSDMVPLVELMTELENSK-IFTGYPDINSPAMGTPS-LAGLHEFLA    294
PCMH        KPPVFKPFEVIFEDEADIVEIVDALRPLRMSN-TIPNSVVIASTLWEAGSAHLTRAQYTT    299
EUGH        KPPVIKPFMVRYQNESDVVKAIDAMRPLRINQ-LIPNVVLFMHGHYETAI-CKTRAEVTS    297
nsVAO       KPPVYKPFMVRHANMEDVPKIIEAMRPLRVSN-LVANCNLMMSASYQLAM-FKRRNEIVP    325
VAO         NPRGYQSYLITLPKDGDLKQAVDIIRPLRLGM-ALQNVPTIRHILLDAAV-LGDKRSYSS    329
VAD         EPAGYRPYLITFENEDDIETVTERLRPLKVA-GVIQNGATVRSLVLDAAI-TRTKSQYYD    293
EPO         TPPEMLSFAIYFENEDDLPAIMETTLPLRIGMAPLQAAPIVRNVTFDAAC-VSKREEWQT    296
EUGO        RPPASQSFLITFDKEEDLEQIVDIMLPLRINMAPLQNVPVLRNIFMDAAA-VSKRTEWFD    295
             *       :   .  *:    :    *.     .     .               .
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PinH        HGPKEQDPEFMALLGRGAKPEEYEAYAKKKGIPFWTCALTFYGPEKVIRAQWEYAQERFK   354
PCMH        -E------------PGHTPDSVIKQMQKDTGMGAWNLYAALYGTQEQVDVNWKIVTDVFK   346
EUGH        -D------------PGPISEPDARKVFKELGIGYWNVYFALYGTAEQIAVNEKIVRSIIE   344
nsVAO       -D------------GAPLDEASLKKAASANGLGMWNTYFALYGTEQTVAGVEPIIRATLT   372
VAO         -R------------TEPLSDEELDKIAKQLNLGRWNFYGALYGPEPIRRVLWETIKDAFS   376
VAD         -G------------DGPIPPSVAKTMMADLDLGMWNFCGALYGPPPVMDTLWTAIRDSFA   340
EPO         -E------------PGPLTDEAKQRMVDELGIGHWIVYGTCYGPRWQIDKYIEMIRDAYL   343
EUGO        -G------------DGPMPAEAIERMKKDLDLGFWNFYGTLYGPPPLIEMYYGMIKEAFG   342
                                          .:  *     :**

PinH        KAFPDAKFAEHEFYKLPLTPEQA--EKVEYPAQFGIPNLRTFAIGARSNWNPAPPTHGHA   412
PCMH        KL-GKGRIVTQEEAG------DTQPFKYRAQLMSGVPNLQEFGL---YNWRG--G-GGSM   393
EUGH        PS--GGEIVTEAEAG------DNILFHHHKQLMCGEMTMEEMNI---YRWRG--AGGGAC   391
nsVAO       AS--GGEVLTAAEME------GNPWFHHHQTLMQGGLNLDEVGL---LRWRG--AGGGLA   419
VAO         AI-PGVKFYFPEDTP------ENSVLRVRDKTMQGIPTYDELKW---IDWLP--N-GAHL   423
VAD         DI-PGVKFYFPEDRR-----HKVDLLLHRAETMKGVPKLTEFNF---LNWDG--G-GGHV   388
EPO         QI-PGARFETNETLPLREGDRASELLNARHELNTGVPNRHSAAV---FDWFP--N-AGHF   396
EUGO        KI-PGARFFTHEER----DDRGGHVLQDRHKINNGIPSLDELQL---LDWVP--N-GGHI   391
                  ..                    .     *  .           *       .

PinH        WFSPVIPRDGAEVLKINEVLGTEARRLGIPLIFAMIVPVPSWERSFTFIIPLFISEDPAQ   472
PCMH        WFAPVSEARGSECKKQAAMAKRVLHKYGLDYVAEFIV---APRDMHHVIDVLYDRTNPEE   450
EUGH        WFAPVAQVKGQEAELQVKLAQRVLAKHNLDYTAGFAI---GWRDLHHIIDVLYDRNNAEE   448
nsVAO       WFAPVAAARGVEAERQTALAKEIVEKHGFDYTAAYAI---GWRDLHHIIALLFDKADPTQ   476
VAO         FFSPIAKVSGEDAMMQYAVTKKRCQEAGLDFIGTFTV---GMREMHHIVCIVFNKKDLIQ   480
VAD         GFSPVSPITGKDAIKQYNMVSSRVREYGFDYMGLLAI---GWRDLHHVTVIVYDKTDPDE   445
EPO         FYAPVSAPSGEDAAKQYEDTKRISDDHGIDYLAQFII---GLREMHHICLPLYDTADPAS   453
EUGO        GFSPVSAPDGREAMKQFEMVRNRANEYNKDYAAQFII---GLREMHHVCLFIYDTAIPEA   448
             ::*:    * :               .        :   . .  . .   ::

PinH        NKRSREVFRHLIKVAADNGWGEYRTAPTFQADVMDTYSFGDHALLRFHESIKDAVDPKGI   532
PCMH        TKRADACFNELLDEFEKEGYAVYRVNTRFQDRVAQSYGPV---KRKLEHAIKRAVDPNNI   507
EUGH        KKRAYACFDELIEVFAAEGFASYRTNIAFMDKVAKKFGST---NMHVNQQIKKALDPNGI   505
nsVAO       EQKADACYRELVTRFGAQGWASYRTGVNSMDLVAQQYGEV---NRDFNRTLKRAIDPNGI   533
VAO         KRKVQWLMRTLIDDCAANGWGEYRTHLAFMDQIMETYNWNNSSFLRFNEVLKNAVDPNGI   540
VAD         RKKLDELFNILVDEAAAEGYGEYRTHIRYMDRIAKTYSWNDNALWKMHETIKDALDPNGI   505
EPO         RKETLDMTRELIRAGAEEGYGIYRAHNVLADQVAETYSFNNHIQRRSHERIKDALDPNGI   513
EUGO        REEILQMTKVLVREAAEAGYGEYRTHNALMDDVMATFNWGDGALLKFHEKIKDALDPNGI   508
             ..        *:      *:. **.         :   :.         .. :* *:**:.*

PinH        LSPGRYGIWPKHLRDKHRYKGGDSV 557
PCMH        LAPGRSGIDLNNDF----------- 521
EUGH        LAPGKSGIHLPD------------- 517
nsVAO       LSPGKSGIHP--------------- 543
VAO         IAPGKSGVWPSQYSHVTWKL----- 560
VAD         LAPGKSGIWGKNRRKA--------- 521
EPO         LNPGKSGIWPERLRNK--------- 529
EUGO        IAPGKSGIWSQRFRGQNL------- 526
            : **: *:
```

	PinH	PCMH	EUGH	nsVAO	VAO	VAD	EPO	EUGO
PinH	100.00							
PCMH	34.71	100.00						
EUGH	32.02	53.79	100.00					
nsVAO	36.17	44.94	54.86	100.00				
VAO	37.96	32.43	35.21	36.36	100.00			
VAD	41.36	43.47	40.90	42.91	46.33	100.00		
EPO	36.85	35.47	36.45	36.27	41.84	41.92	100.00	
EUGO	41.43	39.49	38.94	39.96	46.45	51.45	51.53	100.00

Figure S2. Sequence alignment and identities of pinoresinol hydroxylase from *unclassified Pseudomonas* (PinH), *p*-cresolmethylhydroxylase from *Pseudomonas putida* (PCMH), eugenol hydroyxylase from *Pseudomonas* sp, (EUGH), vanillyl alcohol oxidase from *Novosphingobium* sp (nsVAO), vanillyl-alcohol oxidase from *Penicillium simplicissimum* (VAO), vanillyl-alcohol dehydrogenase from *Marinicaulis flavus* (VAD), 4-ethylphenol oxidase from *Gulosibacter chungangensis* (EPO) and eugenol oxidase from *Rhodococcus jostii* (EUGO). The critical residues, listed in **Table 1**, are highlighted

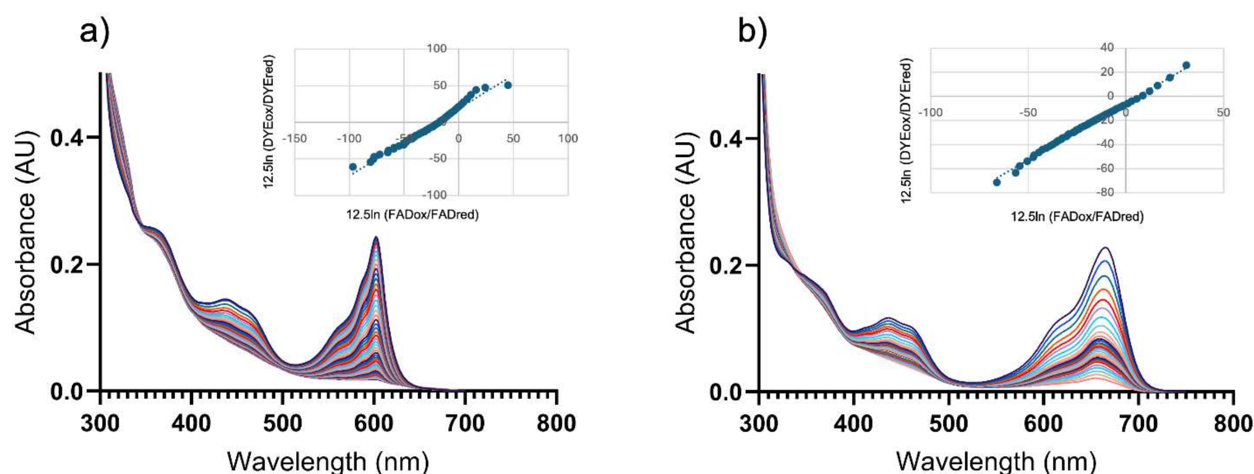
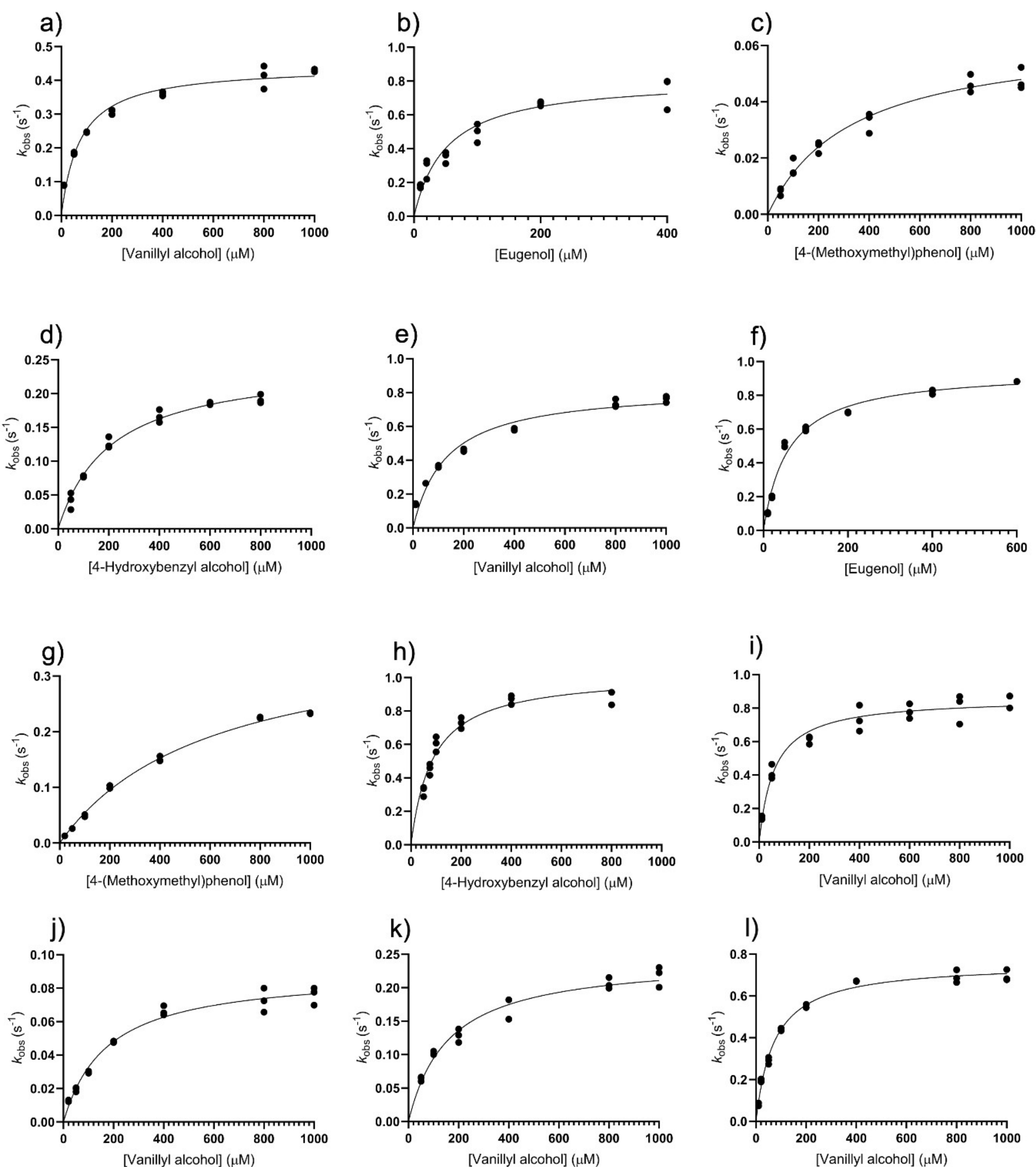


Figure S3. **Determination of the midpoint redox potential (Em) of VAD using the xanthine/xanthine oxidase method.** Spectral changes observed for the determination of the midpoint redox potential of (**a**) wild type VAD using methylene blue as dye; (**b**) P151L using thionine acetate as dye. The spectra show the simultaneous reduction of the protein and the dye. They were measured every minute for 60 minutes. The inset shows a plot in which the $\log[(Ox_{dye})/(Red_{dye})]$ is on the Y-axis, and $\log[(Ox_{enzyme})/(Red_{enzyme})]$ is on the X-axis. The redox potential of the wild-type enzyme and P151L was found to be +82±2 mV and +6±1 mV, respectively.


a)
k_{obs} (s^{-1})
[Vanillyl alcohol] (μM)
b)
k_{obs} (s^{-1})
[Eugenol] (μM)
c)
k_{obs} (s^{-1})
[4-(Methoxymethyl)phenol] (μM)
d)
k_{obs} (s^{-1})
[4-Hydroxybenzyl alcohol] (μM)
e)
k_{obs} (s^{-1})
[Vanillyl alcohol] (μM)
f)
k_{obs} (s^{-1})
[Eugenol] (μM)
g)
k_{obs} (s^{-1})
[4-(Methoxymethyl)phenol] (μM)
h)
k_{obs} (s^{-1})
[4-Hydroxybenzyl alcohol] (μM)
i)
k_{obs} (s^{-1})
[Vanillyl alcohol] (μM)
j)
k_{obs} (s^{-1})
[Vanillyl alcohol] (μM)
k)
k_{obs} (s^{-1})
[Vanillyl alcohol] (μM)
l)
k_{obs} (s^{-1})
[Vanillyl alcohol] (μM)

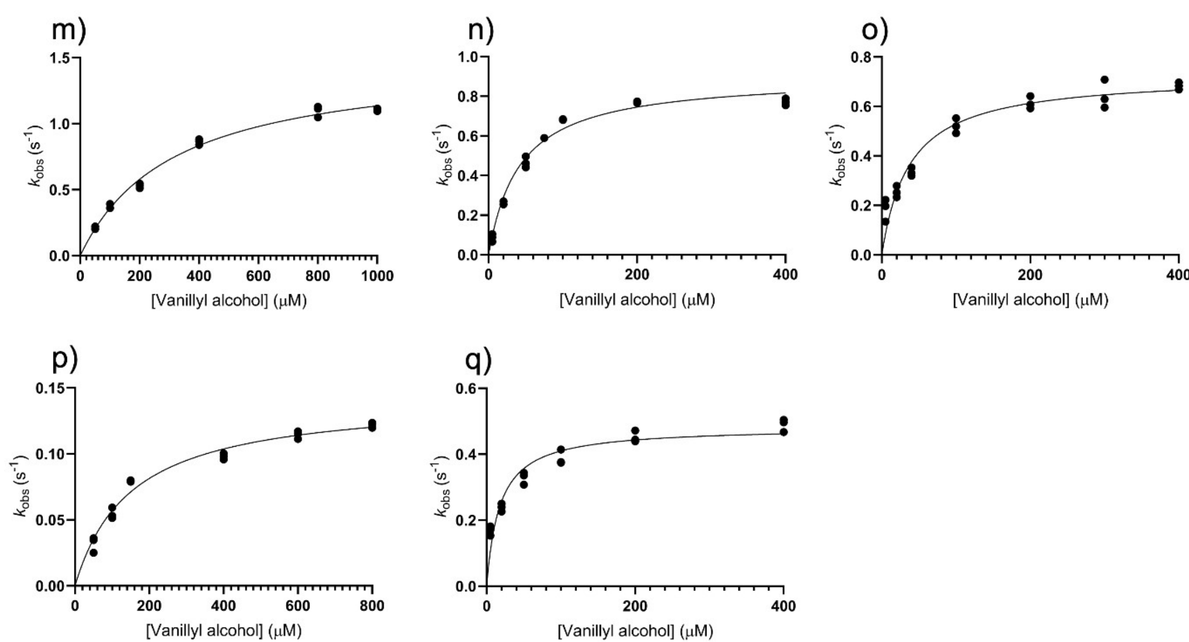


Figure S4. Steady-state kinetics experiments on VAD. Wild-type VAD with (**a**) vanillyl alcohol and 2,6-dichlorophenolindophenol (DCPIP), (**b**) eugenol and DCPIP, (**c**) 4-(methoxymethyl)phenol and DCPIP, (**d**) 4-hydroxybenzyl alcohol and DCPIP. P151L with (**e**) vanillyl alcohol, (**f**) eugenol, (**g**) 4-(methoxymethyl)phenol, (**h**) 4-hydroxybenzyl alcohol. (**i**) P151G with vanillyl alcohol. **j**) P151V with vanillyl alcohol. (**k**) P151I with vanillyl alcohol. (**l**) P151L with vanillyl alcohol and DCPIP. (**m**) P151G with vanillyl alcohol and DCPIP. (**n**) P151V with vanillyl alcohol and DCPIP. (**o**) P151I with vanillyl alcohol and DCPIP. (**p**) P151N with vanillyl alcohol and DCPIP. (**q**) P151T with vanillyl alcohol and DCPIP. n = 3 independent experiments, individually plotted as dots.

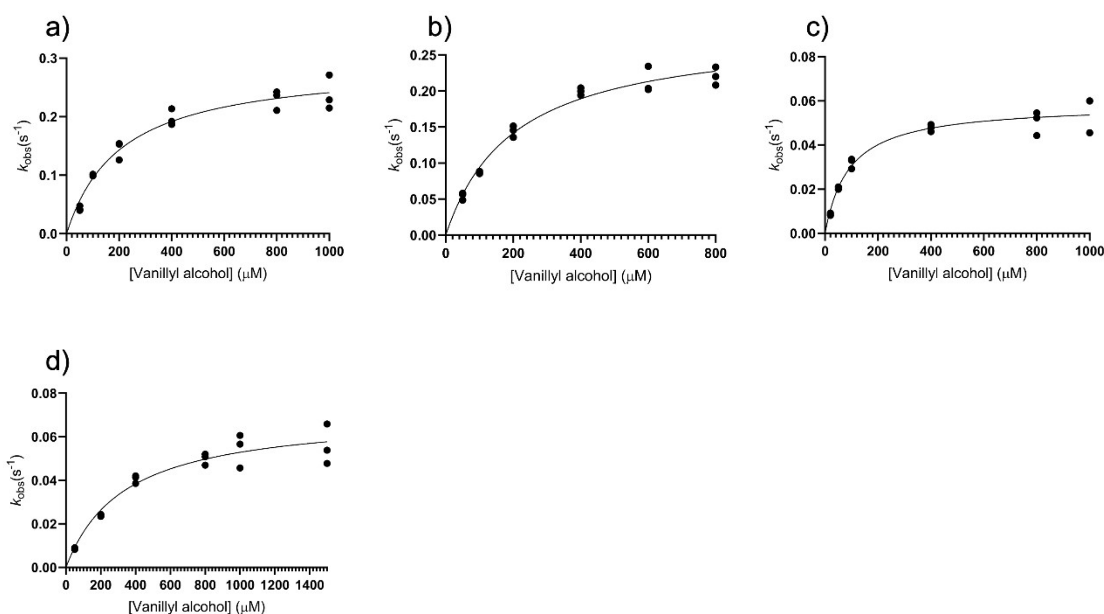


Figure S5. Steady-state kinetics experiments on nsVAO. (**a**) Wild type enzyme with vanillyl alcohol, (**b**) T181D with vanillyl alcohol, (**c**) wild type with vanillyl alcohol and cytochrome c. (**d**) T181D with vanillyl alcohol and cytochrome c. n = 3 independent experiments, individually plotted as dots.

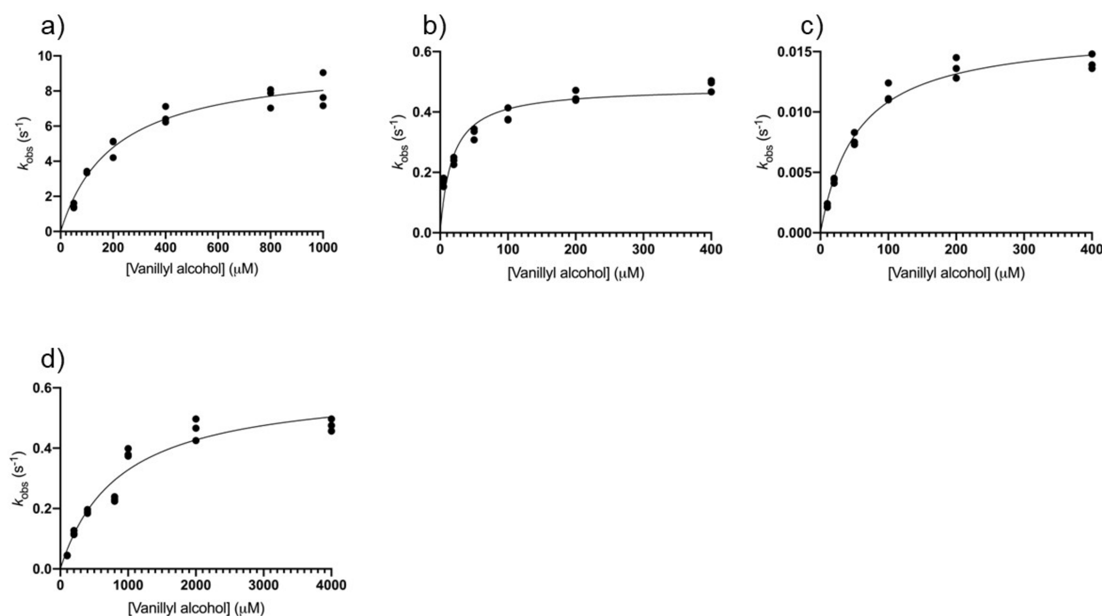


Figure S6. Steady-state kinetics experiments on EUGO from *Rhodococcus jostii* RHA1. (**a**) Wild type enzyme with vanillyl alcohol, (**b**) wild type with vanillyl alcohol and DCPIP, (**c**) EUGO L152P with vanillyl alcohol, (**d**) EUGO L152P with vanillyl alcohol and DCPIP.

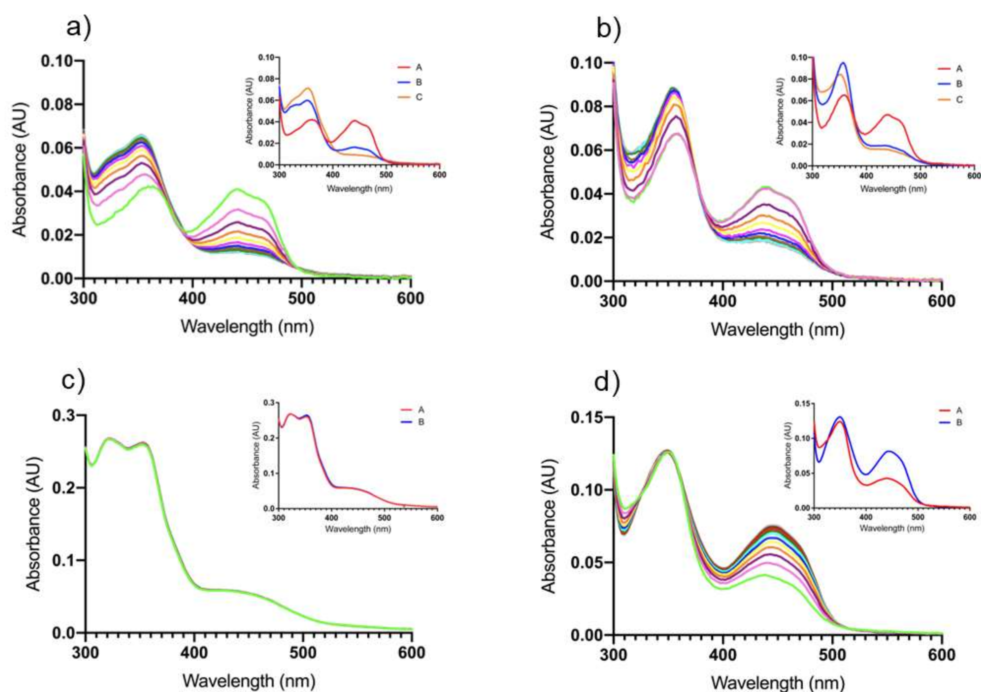


Figure S7. Spectral changes observed through stopped-flow apparatus. (**a**) Anaerobic mixing of wild-type VAD (5 µM) with vanillyl alcohol (400 µM) and **(b)** P151L (5 µM) with vanillyl alcohol (1000 µM). Spectral scans are shown from 0 to 10 s with intervals of 1 s for the wild-type enzyme, and from 0 to 20 ms with intervals of 2 ms for P151L. The data were fit with two exponential functions (*A→B, B→C)*. The *insets* show the deconvolution of the spectral scans for *A, B* and *C*. In the *B* state, the enzyme is almost completely reduced and the k_{red} values listed in **Table 3** refer to the *A→B* conversion rates. (**c**) Vanillyl alcohol-reduced wild-type VAD (20 µM) upon mixing with oxygen and (**d**) vanillyl alcohol-reduced P151L (10 µM) upon mixing with oxygen. Spectral scans are shown from 0 to 10 s with intervals of 1 s for the wild-type enzyme, and from 0 to 2 s with intervals of 0,2 s for P151L. The data were fit with one exponential function (*A→B)* as listed in **Table 4**. The *insets* show the deconvolution of the spectral scans for *A* and *B*.

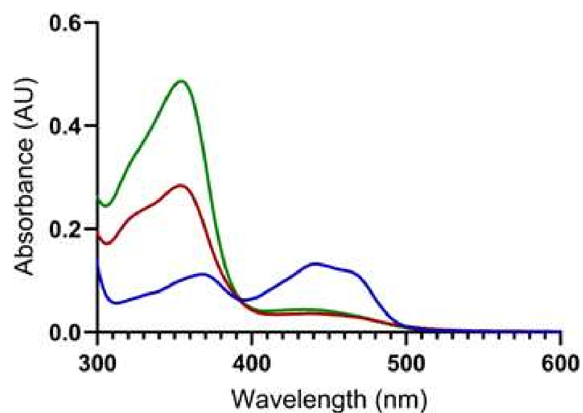


Figure S8. Enzyme-monitored turnover experiment. Spectral changes observed upon aerobic mixing wild type and P151L VAD (12 μM) with 2.5 mM vanillyl alcohol. The spectrum of the initial oxidized wild-type enzyme is in blue. The spectrum of P151L would be indistinguishable and is not shown for clarity. The spectra of the wild-type and P151L proteins after 10 s incubation are in red and blue, respectively.

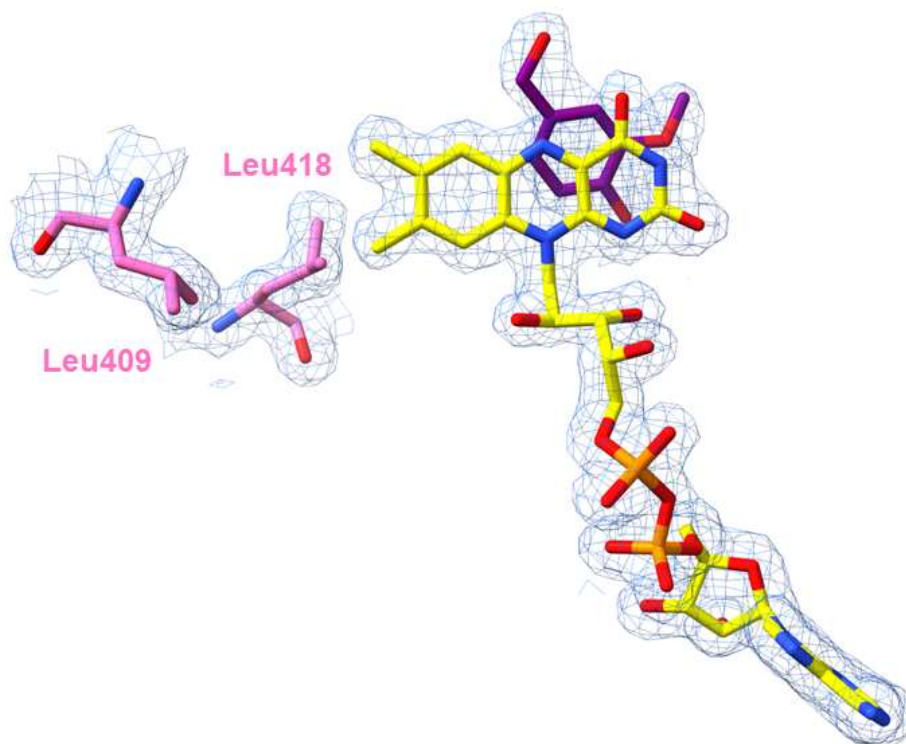


Figure S9. The electron density of the flavin and surronding residues in nsVAO. The weighted 2Fo-Fc map is contoured at 1.2 σ level.

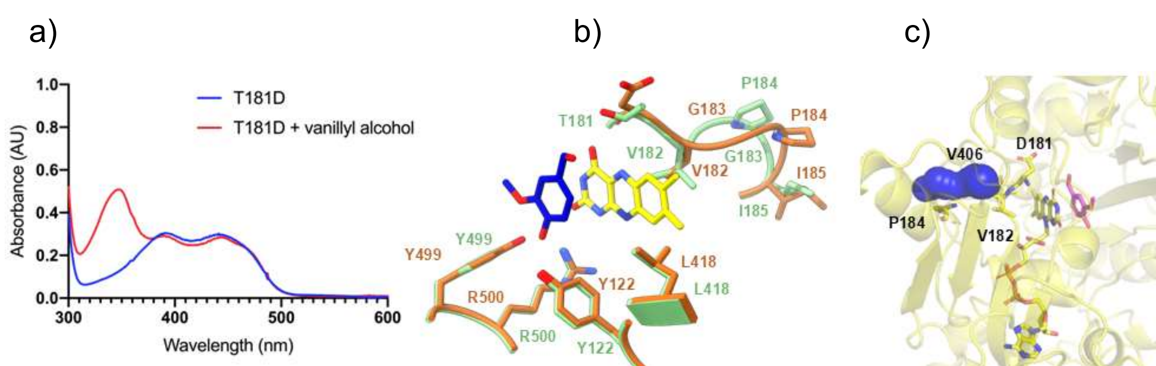


Figure S10. **Properties of nsVAO T181D mutant**. **(a**) Incubation of T181D (30 μM) with vanillyl alcohol (45 μM). The spectra before substrate addition and after 30-minute incubation are in blue and red, respectively. Vanillin, formed upon vanillyl alcohol oxidation, has an absorbance peak at 340 nm (ε=14000 M−1·cm−1). (**b**) Structural comparison between wild type (green carbons) and T181D (dark orange carbons) nsVAOs. (**c**) Accessibility of the flavin N5-C4a locus. Compared to the wild-type nsVAO (**Figure 8c**), there is a narrowing of the tunnel leading to the flavin re side, as revealed by the mutant's crystal structure

Table S1a. Data collection and refinement statistics of VAD: wild type, P151L, P151I and VAD P151V.

	Wild Type	**P151L**	**P151I**	**P151V**
PDB code	8S7P	8S7U	8S7Q	8S7W
Space group	$C222_1$	$P4_12_12$	$P2_12_12_1$	$P2_12_12_1$
Unit cell axes (Å)	90.14, 143.22, 287.75	138.495, 138.495, 180.947	100.443, 113.338, 130.168	100.62, 114.64, 130.71
Unit cell angles (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	67.49– 2.35	47.31– 1.80	52.01– 2.40	49.86– 2.10
R_{merge}	0.166 (3.295)	0.146 (2.885)	0.198 (1.032)	0.159 (1.132)
$CC_{1/2}$	0.998 (0.488)	0.999 (0.390)	0.994 (0.664)	0.989 (0.394)
Completeness (%)	100.0 (99.6)	99.7 (100.0)	99.7 (96.9)	98.8 (99.1)
Unique reflections	77772 (4521)	161652 (7961)	58641 (4368)	87358 (4434)
Multiplicity	21.3 (16.1)	13.5 (13.4)	8.8 (7.8)	4.3 (4.1)
Overall I/σ (I)	12.6 (0.8)	11.8 (0.9)	9.1 (2.1)	8.4 (1.6)
Protein residues	1556	1038	1038	1038
FAD molecules	3	2	2	2
Ligand	-	-	-	-
Water molecules	48	718	358	589
R/R_{free} (%)	22.8 /27.9	15.5/18.0	17.8/23.1	17.2/20.9
Rms bond length	0.001	0.001	0.001	0.001
Rms bond angle	0.518	1.497	0.488	1.343
Ramachandran outliers (%)	0.2	0.1	0.2	0.0
Average B-factor	57.0	30.0	34.0	28.0

Table S1b. Data collection and refinement statistics of VAD: P151L, P151T, P151N and P151G.

	P151L with eugenol	**P151T**	**P151N**	**P151G**
PDB code	8S7N	8S7R	8S7T	8S7S
Space group	$P4_12_12$	$C222_1$	$C222_1$	$C222_1$
Unit cell axes (Å)	139.02, 139,02, 181.06	91.949 144.451 286.785	89.525 142.426 286.436	90.804 144.145 289.452
Unit cell angles (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	49.20– 1.80	143.39– 1.90	143.22-2.10	64.60 – 2.50
R_{merge}	0.194 (3.735)	0.066 (1.253)	0.139 (2.349)	0.202 (3.274)
$CC_{1/2}$	0.998 (0.388)	0.991 (0.423)	0.999 (0.553)	0.997 (0.605)
Completeness (%)	100.0 (100.0)	97.8 (98.7)	100.0 (100.0)	100.0 (100.0)
Unique reflections	163464 (8025)	146187 (7204)	106762 (5214)	66056 (4381)
Multiplicity	13.5 (13.3)	3.7 (3.8)	20.1 (20.7)	22.8 (23.6)
Overall I/σ (I)	11.0 (0.9)	9.2 (1.1)	14.3 (1.5)	12.5 (1.2)
Protein residues	1038	1554	1553	1554
FAD molecules	2	3	3	3
Ligand molecules	2	-	-	-
Water molecules	917	468	292	242
R/R_{free} (%)	15.8 /18.4	19.4/ 23.5	18.4/23.2	19.2/25.2
Rms bond length	0.001	0.001	0.001	0.001
Rms bond angle	1.485	0.456	0.463	0.506
Ramachandran outliers (%)	0.0	0.0	0.1	0.2
Average B-factor	27.0	46.0	50.0	57.0

Table S1c. Data collection and refinement statistics of nsVAO.

	nsVAO with vanillyl alcohol	**T181D with vanillin**
PDB code	9FFK	9FGE
Space group	$P22_12_1$	$P22_12_1$
Unit cell axes (Å)	90.84, 96.77, 129.11	91.33 97.82 129.61
Unit cell angles (°)	90, 90, 90	90, 90, 90
Resolution (Å)	90.85 – 1.70	78.20 – 1.60
R_{merge}	0.162 (2.466)	0.15 (1.476)
$CC_{1/2}$	0.999 (0.707)	0.998 (0.793)
Completeness (%)	99.7 (99.7)	99.6 (99.3)
Unique reflections	125029 (6098)	152440 (7444)
Multiplicity	20.0 (18.6)	9.3 (9.1)
Overall $I/\sigma (I)$	10.1 (1.3)	8.8 (1.6)
Protein residues	1002	1002
FAD molecules	2	2
Ligand molecules	2	2
Water molecules	558	772
R/R_{free} (%)	22.3 /25.4	17.5/20.4
Rms bond length	0.002	0.001
Rms bond angle	1.449	0.579
Ramachandran outliers (%)	1	0.2
Average B-factor	27.0	18.0

Table S2. Genetic context of VAD from *Marinicaulis flavus* and VAO from *Novosphingobium* sp.

	GenBank Accession Code	Annotation
Upstream genes	WP_104830656.1	UbiD family decarboxylase
	WP_104830657.1	Hypothetical protein
	WP_104830658.1	Pilus assembly protein TadG-related protein
	WP_104830659.1	Pilus assembly protein
	WP_104830660.1	Pilus assembly protein
	WP_207764815.1	Putative cytochrome c
VAD	WP_104830661.1	FAD-binding oxidoreductase
Downstream genes	WP_133162311.1	Hypothetical protein
	WP_104830663.1	ATPase
	WP_104830664.1	Hypothetical protein
	WP_104830665.1	Hypothetical protein
	WP_104830666.1	Glutathione S-transferase family protein
	WP_104830667.1	Glutathione-dependent disulfide-bond oxidoreductase

	GenBank Accession Code	**Annotation**
Upstream genes	WP_124809343.1	Glycogen/starch/alpha-glucan phosphorylase
	WP_124809344.1	1,4-alpha-glucan branching protein GlgB
	WP_124809345.1	Glucose-1-phosphate adenylyltransferase
	WP_124809346.1	Glycogen synthase GlgA
	WP_124809347.1	Alpha-D-glucose phosphate-specific phosphoglucomutase
	WP_223806624.1	Glycogen debranching protein GlgX
nsVAO	WP_124809348.1	FAD-binding oxidoreductase
Downstream genes	WP_124809349.1	Putative cytochrome c
	WP_190287233.1	TetR/AcrR family transcriptional regulator
	WP_124809351.1	Alpha/beta hydrolase
	WP_124809352.1	DUF2334 domain-containing protein
	WP_223806625.1	Hypothetical protein
	WP_124809353.1	Glycosyltransferase

Table S3. **Steady state kinetic parameters of P151L VAD**

VAD	Substrate	O_2 k_{cat} (s^{-1})	K_M (µM)	k_{cat}/K_M (s^{-1} mM^{-1})
P151L	Vanillyl alcohol	0.83±0.05	131.9±30.4	6
P151L	Eugenol	0.92±0.04	55±8.8	17
P151L	4-Hydroxy benzylalcohol	1.02±0.03	86.74±9.1	12
P151L	4-Methoxy methylphenol	0.38±0.02	600±70	0.6

Measured at 25 °C in 50 mM potassium phosphate pH 7.5, 150 mM NaCl. Data are shown as average values ± standard deviation. n = 3 independent experiments. No activity was detected on *p*-cresol.

Supplementary Table 4. Primers used for quick change mutagenesis

Primer name	Sequence
VAD_P151Lfw	gtgccggacctgggctggggtagtgtggtcgg
VAD_P15Lrv	accccagcccaggtccggcacatcgatccata
VAD_P151Vfw	ccggacgtgggctggggtagtgtg
VAD_P151Vrv	ccagcccacgtccggcacatcgatc
VAD_P151Ifw	ccggacattggctggggtagtgtg
VAD_P151Irw	ccagccaatgtccggcacatcgatc
VAD_P151Gfd	gtgccggacggcggctggggtagtgtggtcgg
VAD_P151Grv	accccagccgccgtccggcacatcgatccata
VAD_P151Nfw	gtgccggacaacggctggggtagtgtggtcgg
VAD_P151Nrv	accccagccttggtccggcacatcgatccata
VAD_P151Tfw	ccggacaccggctggggtagtgtg
VAD_P151Trv	ccagccggtgtccggcacatcgatc
nsVAO_T181Dfw	acgtcccagatgtcggcccgattgcatctccag
nsVAO_T181Drv	cgggccgacatctgggacgtcaatccaaagggga

EUGO_L152Pfw	gacccgggatggggcagcgtg
EUGO_L152Prv	ccatcccgggtcgggacagtc