ADDITIONAL FILE 1: Supplemental Figures S1-S4

A

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CaPo       VTCSDGVSTASNAACCAWFAVLDDIQANLFDGGQCGEEAHESLRLTFHDAIGFSPALAAQGKFGGGGADGSIITFADIETNFHANNGLDDIVDALKPFAD 100
CaPo-bis   VTCSDGVSTATNAACCAWFAVLDDIQANLFDGGQCGEEARESALRLTFHDAIGFSPALAAQGKFTGGGADGSIITFADIETSFHANNGLDDIVEALKHFAD 100
CaPo-tris  ATCSDGIRTATNAACCAWFAVLDDIQANLFDGGQCGDQARESALRLTFHDAIGRSPALARQGKFTGGGADGSIITFADIELSYHANNGLEEIVEAQKPFAD 100
           .*****: **:**************************::*:*:********** ***** **** *************** .:******::**:* * ***
CaPo       KHNVSYGDFIQFAGAVGVSNCPGAPRLEFLAGRPNATAPSPDGLVPEPSDSVDKILARMADAGGFSPDEVVALLASHSVAAQEHVDPTIPGTPFDSTPST 200
CaPo-bis   KHNVSYGDFIQFAGAVGVSNCPGAPRLEFHAGRPNATAPSPDGLVPEPSDSVDKILARMSDAGGFTPDETVALLASHSVAAQRHVDPTIPGTPFDSTPGT 200
CaPo-tris  KHNVSFGDFIQFAGAVGLSNCPGAPRLEFHAGRPNAIAPSPDGLVPEPSDSVDKILARMSDAGGFTPDDTVALLASHSVAAQETVDPTIPGTPFDSTPGT 200
           *****:***********:********** ****** ********************:*****:**:.************  **************.*
CaPo       FTTQFFLETLLKGTAFPGTGANSGEVKSPLKGEFRLQSDAAIARDPRTACEWQSFVNNQELMQSSFRAAMAKLANLGHDRSDLIDCSEVIPVPKPLAASA 300
CaPo-bis   FTTQFFVETLLKGTAFPGKGANSGEVKSPSPGEFRLASDAAIARDPRTACEWQSFVNNQEKMQSSFRAAMLKLAIQGHDRSDLIDCSEVIPVPKPLASSA 300
CaPo-tris  FTTQFFVETLLKGTAFPGTGANTGEVKSPLKGEFRLASDAAIARHPRTACEWQSFVNNQEKMQASFRAAMAKLANQGHDRSDLVDCSEVIPVPKPLAASA 300
           ******:***********.***:******  ***** ******.*************** **:****** ***  *******:*************:**
CaPo       TFPAGKTRSDIEQSCRSTPFPTLPTDPGPATSIPPV 336
CaPo-bis   TFPAGKTRKDIEQSCPSTPFPTLPTDPGPETSVPPV 336
CaPo-tris  TFPAGKSRKDIEQSCPSTPFPTLPSDPGPATSIPPV 336
           ******:*.****** ********:**** **:***
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B

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CaCD       VTCPDGVNTATNAACCALFAVLDDIQENLFDGGECGEEAHESLRLTFHDAIGFSPALARQGKFGGGGADGSIITFSDIETNFHANGGIDEIVEVQKPFVA 100
CaCD-bis   VTCPDGVNTATNAACCALFAVVDDIQENLFDGGECGEEVHESLRLTFHDAIGFSPALARQGKFGGGGADGSIITFSDIETNFHANGGIDEIVEVQKPFVA 100
CaCD-tris  VTCPDGVNTATNAACCALFAVVDDIQENLFDGGECGEEAHESLRLTFHDAIGFSPALARQGKFGGGGADGSIITFSDIETNFHANGGIDDIVEVQKPFVA 100
           ********************:***************.**************************************************:********** 
CaCD       KHNMTAGDFIQFAGAVGVSNCPGAPRLEFLLGRPAATAPSPDGLVPEPFDSVDKILARFADAGGFSPDEVVALLASHSVAAAEHVDPTIPGTPFDSTPST 200
CaCD-bis   KHNMTAGDFIQFAGAVGVSNCPGAPRLEFLLGRPAATAPSPDGLVPEPFDSVDKILARFADAGGFSPDEVVALLASHSVAAAEHVDPTIPGTPFDSTPST 200
CaCD-tris  KHNMTAGDFIQFAGAVGVSNCPGAPRLEFLLGRPAATAPSPDGLVPEPFDSVDKILARFADAGGFSPDEVVALLASHSVAAAEHVDPTIPGTPFDSTPST 200
           ********************************************************************************************** 
CaCD       FTTQFFVEVLLRGTLFPGTGGNQGEVKSALRGEIRLQSDHEVARDPRTACEWQSFVNNQAKMQKSFRAAMAKLAILGHDRSDLIDCSEVIPVPPPLAATA 300
CaCD-bis   FTTQFFVEVLLRGTLFPGTGGNQGEVKSALPGEIRLQSDHEVARDPRTACEWQSFVNNQAKMQKSFRAAMAKLAILGHDRSDLVDCSEVIPVPPPLAATA 300
CaCD-tris  FTTQFFVEVLLRGTLFPGTGGNQGEVKSPLRGEIRLQSDNEVARDPRTACEWQSFVNNQAKMQKSFRAAMAKLAILGHDRSDLIDCSEVIPVPPPLAATA 300
           *************************** * ********.****************************************:**************** 
CaCD       HFPAGLTRKDIEQSCRSTPFPTLSTDPGPATSVPPV 336
CaCD-bis   HFPAGLTRKDIEQSCRSTPFPTLSTDPGPATSVPPV 336
CaCD-tris  HFPAGLTRKDIEQSCRSTPFPTLSTDPGPATSVAPV 336
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C

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AVP        VACPDGVNTATNAACCALFAVRDDIQQNLFDGGECGEEVHESLRLTFHDAIAFSPALEAQGQFGGGGADGSIAIFEDIETNFHANLGLDEIVNEQKPFIA 100
AVP-bis    VACPDGVNTATNAACCALFAVRDDIQQNLFDGGECGEEVHESLRLTFHDAIAFSPALEAQGQFGGGGADGSIAIFEDIETNFHANLGLDEIVNEQKPFIA 100
AVP-tris   VACPDGVNTATNAACCALFAVRDDIQQNLFDGGECGEEVHESLRLTFHDAIAFSPALEAQGQFGGGGADGSIAIFEDIETNFHANLGLDEIVNEQKPFIA 100
           ********************************************************************************************** 
AVP        RHNMTTADFIQFAGAVGVSNCPGAPQLDFFLGRPDATQPAPDGLVPEPFDTVDQILARMADAGGFDPIETVWLLTSHTIAAAEHVDPTIPGTPFDSTPEL 200
AVP-bis    RHNMTTADFIQFAGAVGVSNCPGAPQLDFFLGRPDATQPAPDGLVPEPFDTVDQILARMADAGGFDPIETVWLLTSHTVAAAEHVDPTIPGTPFDSTPEL 200
AVP-tris   RHNMTTADFIQFAGAVGVSNCPGAPQLDFFLGRPDATQPAPDGLVPEPFDTVDQILARMADAGGFDPIETVWLLASHTIAAAEHVDPTIPGTPFDSTPEL 200
           ************************************************************************:***:********************** 
AVP        FTTQFFIETQLRGTLFPGTGGNQGEVESPLRGEIRLQSDHLLARDSRTACEWQSFVNNQPKLQKSFQAAFHDLSMLGHDVNDLIDCSEVIPIPPPPTSTA 300
AVP-bis    FTTQFFIETQLRGTLFPGTGGNQGEVESPLRGEIRLQSDHLLARDSRTACEWQSFVNNQPKLQKNFQAAFHDLSMLGHDVNDLIDCSEVIPIPPPPASTA 300
AVP-tris   FTTQFFIETQLRGTLFPGTGGNQGEVESPLRGEIRLQSDHLLARDSRTACEWQSFVNNQPKLQKNFQAAFHDLSVLGHDVNDLIDCSEVIPIPPPPTSTA 300
           ************************************************************.*********:**********************:*** 
AVP        HFPAGLTNADVEQACAETPFPTLPTDPGPATSVAPV 336
AVP-bis    HFPAGLTNADVEQACAETPFPTLPTDPGPATSVAPV 336
AVP-tris   HFPAGLTNADVEQACAETPFPTLPTDPGPATSVAPV 336
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D

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ALiP       VACPDGVNTATNAACCALFAVRDDIQQNLFNGGQCGEEAHESLRLTFHDAIAFSPALEAQGQFGGGGADGSIVIFSDIETNFHANIGLDEIVAIQKPFIA 100
ALiP-bis   VACPDGVNTATNAACCALFAVRDDIQQNLFNGGQCGEEAHESLRLTFHDAIAFSPALEAQGQFGGGGADGSIVIFSDIETNFHANIGLDEIVAIQKPFIA 100
ALiP-tris  VACPDGVNTATNAACCALFAVRDDIQQNLFNGGQCGEEAHESLRLTFHDAIAFSPALEAQGQFGGGGADGSIVIFSDIETNFHANIGLDEIVAIQKPFIA 100
           ********************************************************************************************** 
ALiP       RHNMTVADFIQFAGAVGVSNCPGAPQLNFFLGRPDATQPAPDGLVPEPFDTVDQILARMADAGEFDELETVWLLIAHTVAAANDVDPTIPGTPFDSTPEL 200
ALiP-bis   RHNMTVADFIQFAGAVGVSNCPGAPQLNFFLGRPDATQPAPDGLVPEPFDTVDQILARMADAGEFDELETVWLLIAHTVAAANDVDPTIPRTPFDSTPEL 200
ALiP-tris  RHNMTVADFIQFAGAVGVSNCPGAPQLNFFLGRPDATQPAPDGLVPEPFDTVDQILARMADAGEFDELETVWLLIAHTVAAANDVDPTIPRTPFDSTPEL 200
           ********************************************************************************************** ********* 
ALiP       FTSQFFIETQLRGTLFPGTGGNQGEVESPLKGEMRLQSDHLLARDSRTACEWQSFVNNQPKLQKNFQFVFEALSMLGQDPNDLIDCSEVIPIPPPLTLTP 300
ALiP-bis   FTSQFFIETQLRGTAFPGTGGNQGEVESPLKGEMRLQSDHLLARDSRTACEWQSFVNNQPKLQKNFQFIFEALSMLGQDPNNLIDCSEVIPIPPPLTLTP 300
ALiP-tris  FTSQFFIETQLRGTLFPGTGGNQGEVESPLKGEIRLQSDHLLARDSRTACEWQSFVNNQPKLQKNFQFVFEALSMLGQDPNDLIDCSEVIPIPPPLTLTP 300
           ************** ******************:************************************:************:****************** 
ALiP       AASHFPAGKTNKDVEQACAETPFPTLPTDPGPATSVAPVPPSPAA 345
ALiP-bis   AFSHFPAGKTNADVEQACAETPFPTLPTDPGPATSVAPVPPSPAA 345
ALiP-tris  GAAHFPAGKTNKDVEQACAETPFPTLPTDPGPATSVAPVPPSPAA 345
           . :******** ********************************
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Figure S1. Twelve reconstructed sequences representative of the different nodes. Most probable and alternative (bis/tris) sequences (mature proteins) for CaPo (**A**, 88-92% identity), CaCD (**B**, 98-99%), AVP (**C**, 99%) and ALiP (**D**, 98-99%) nodes were obtained by Monte Carlo sampling (yielding 5000 sequences/node using 0.5 threshold) followed by random sampling (see Methods for sampling strategy) yielding 3 sequences/node (red and blue, typical Mn-binding site and catalytic tryptophan, respectively; other colors, see **Fig. 2**).

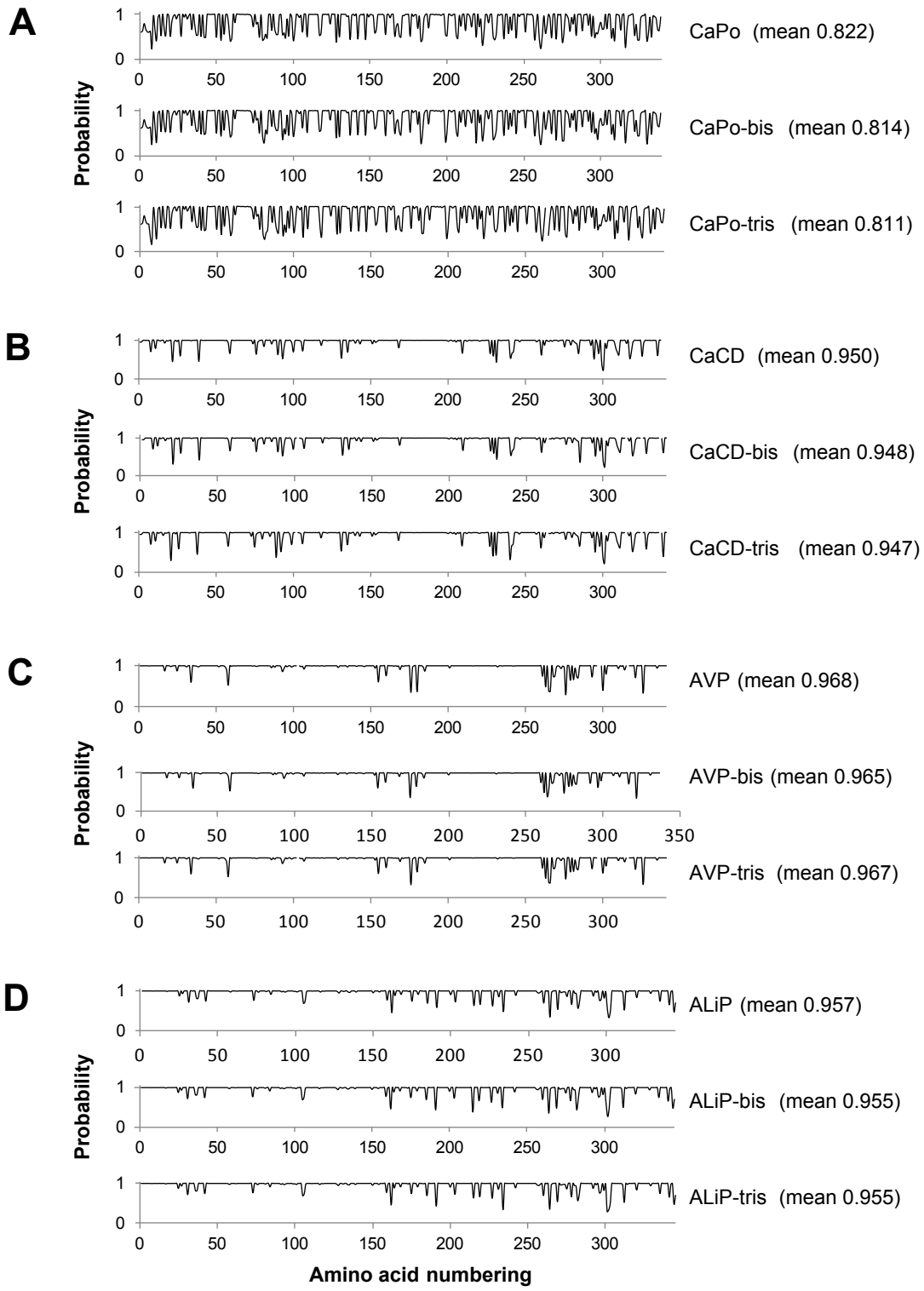

Figure S2. Posterior probability for each amino acid of the 12 reconstructed ancestral sequences shown in **Fig. S1**. The most probable sequence for nodes CaPo (**A**), CaCD (**B**), AVP (**C**) and ALiP (**D**), and two alternative sequences (bis and tris) for each of them (see Methods for sampling strategy) are included. The probabilities of Trp172 in AVP and ALiP sequences are 0.999 and 1.000, respectively; and that of Asn183 in ALiP sequences is 0.993. The mean probabilities for every reconstructed sequence are also shown.

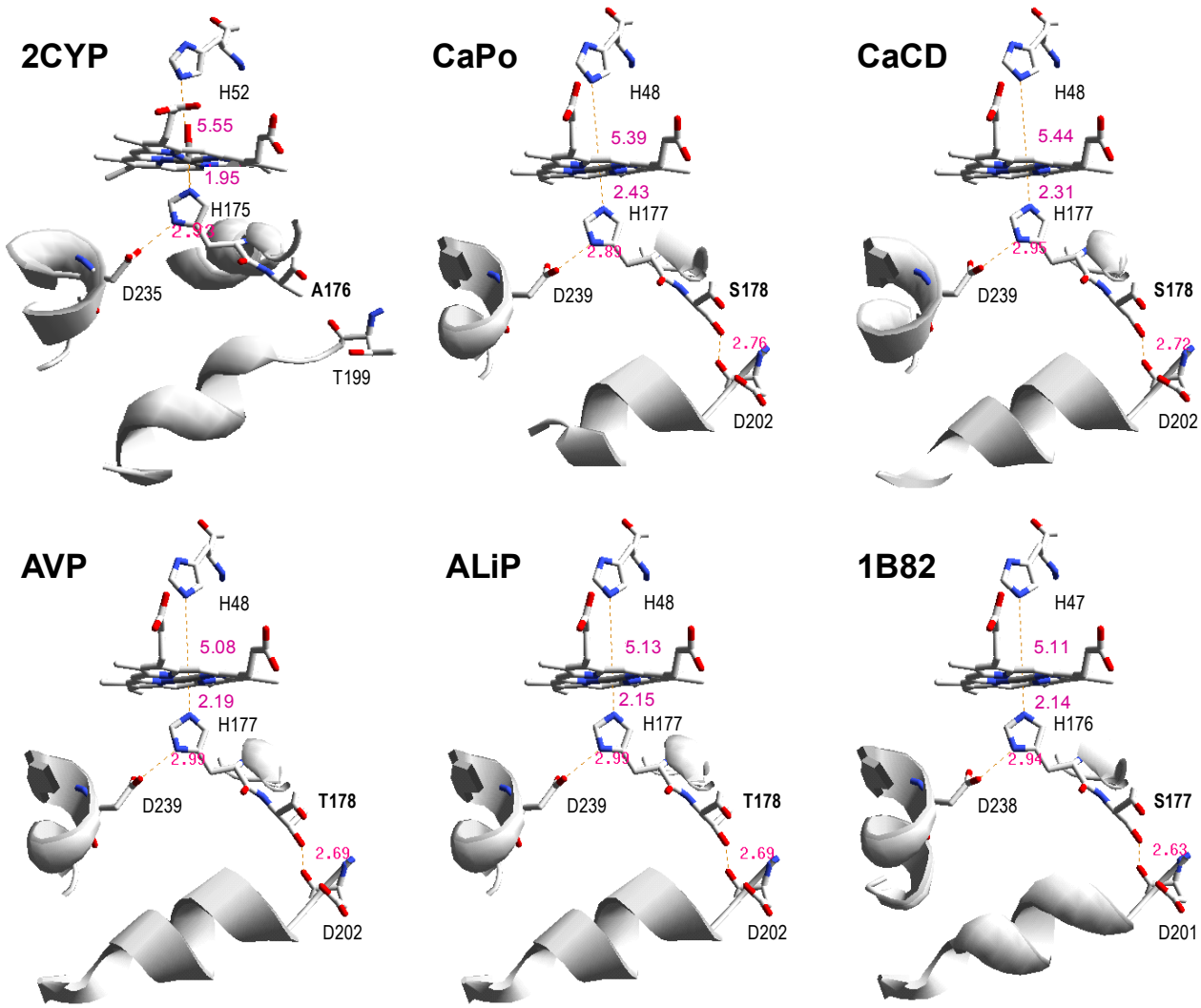

Figure S3. Proximal histidine, neighbor residues and distances (Å) in ancestral peroxidases (His177) and extant C*c*P (2CYP; His175) and *P. chrysosporium* LiPH8 (1B82; His176). His177-Asp239 and Ser/Thr178-Asp202 H-bonds would affect the position of the proximal histidine in the ancestral peroxidases, and consequently the heme iron electron-deficiency (homologous H-bonds are present in LiPH8, but the second one is absent from C*c*P). Distal histidine (His48 in the ancestors, His52 in CcP, and His47 in LiPH8) is also shown.

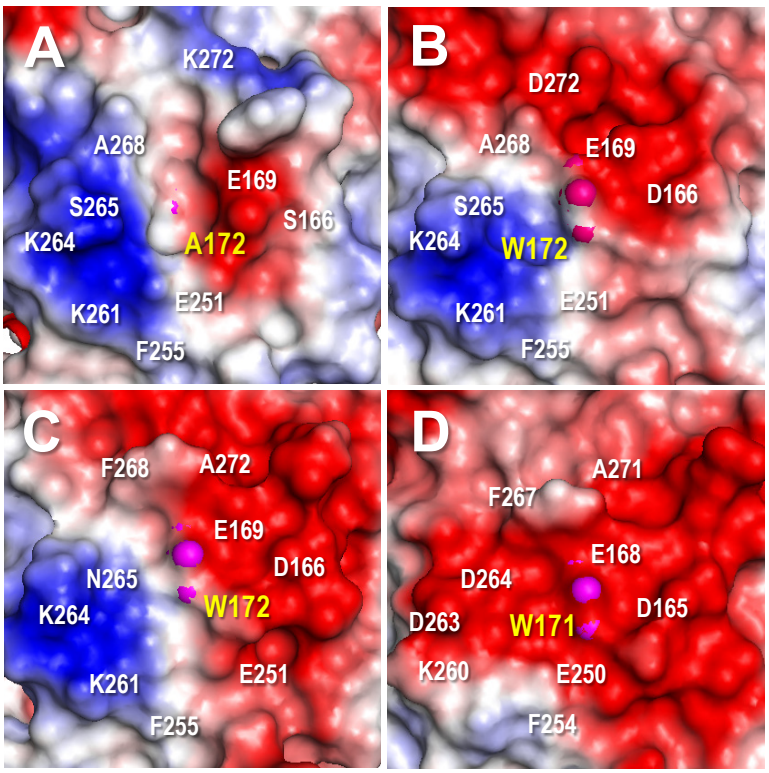

Figure S4. Surface environment of catalytic tryptophan. Electrostatic surfaces computed for ancestral CaCD (**A**), AVP (**B**) and ALiP (**C**) homology models, and extant LiPH8 (**D**) crystal structure (PDB 1B82) showing the environment of the exposed tryptophan (magenta spheres, yellow labels) in **B-D**, and the equivalent alanine residue in **A** (the positions of neighbor residues are also indicated). The presence of the catalytic tryptophan in AVP and ALiP and the absence of a net negative environment, as found in LiPH8 that is unable to oxidize RB5, contribute to the oxidation of this anionic dye by the two ancestral enzymes. The opposite effect is expected for VA, whose cation radical would be stabilized by the negative environment in LiPH8.