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A novel oxidoreductase family sharing a conserved FAD-binding domain

Flavoproteins play a key role in many biological processes, ranging from redox catalysis to DNA repair. Of the hundreds of flavoproteins isolated to date, most contain a dissociable flavin cofactor. However, in about 30 flavoenzymes, the isoalloxazine ring of the flavin group is covalently linked to a histidine, cysteine or tyrosine residue in the polypeptide chain^{1,2}. The exact mechanism of covalent flavinylation is still obscure – as is the reason for covalent binding^{3–5}. We report here on a newly discovered family of oxidoreductases that share a conserved FAD-binding domain. The homologous enzymes are found in both eukarya and eubacteria, and are involved in a diverse range of redox reactions.

Recently, we solved the first crystal structure of an α -(*N*³-histidyl)-FAD-containing flavoprotein: vanillyl-alcohol oxidase (VAO)⁶. This fungal enzyme catalyses the oxidation of a variety of lignin-derived phenolic compounds^{7,8}. We performed sequence comparisons with the recently determined primary structure of VAO (Ref. 9), using the gapped-BLASTP program¹⁰. We found that VAO shows sequence similarity to several other flavoproteins of similar length, including *p*-cresol methylhydroxylase¹¹ (bits = 314, E = 6×10^{-85}), D-lactate dehydrogenase¹² (bits = 57, E = 2×10^{-7}), glycolate oxidase subunit D (Ref. 13; bits = 41, E = 0.016) and L-gulono- γ -lactone oxidase¹⁴ (bits = 36, E = 0.40). L-gulono- γ -lactone oxidase shows significant sequence similarity to some other flavoenzymes and uncharacterized open reading frame products^{12,15-18}. The N- and C-termini of these proteins show most sequence conservation and were used for a more extensive search: a group

of 54 homologous protein sequences, with representatives in all kingdoms, was found. Figure 1 shows an alignment of the sequences for which gene products have been identified. Except for alkylldihydroxyacetonephosphate synthase¹⁷ and L-galactono- γ -lactone dehydrogenase¹⁸, all the enzymes listed are known to contain FAD and catalyse redox reactions in a wide variety of metabolic pathways.

Compelling evidence that the homologous sequences represent a structurally related family comes from inspection of the three-dimensional structure of VAO. VAO consists of two domains; the active site of the enzyme is formed at their interface (Fig. 2 and Ref. 6). The FAD-binding domain (residues 1–270 and 500–560) provides the cofactor-binding site, whereas the cap domain (residues 271–499) forms the substrate-binding site. From this it is evident that the regions conserved in members of the novel oxidoreductase

HDNO	b	(37)	PSLIARCLSGAGVDAKSVRYACDNGLE--ISVRSGGHNP-NGYATN-DGG---IVLDDL-RLMNSIH-IDTA--GSRARIGGGVISGDLVKKEAAGFGL--AAVTGMHP--KV
GLCD	b	(55)	PLLVLVPKQMGHRLTAIVLAVCHLRVP--VTVTRAGTGL-SGQALPLEKG---VLLVMAR-FKEILDINPV--GRRARVQPGVRNLAISQVAPPHNL--YYAPDPSSQIV
MMCO	b	(30)	PAYVVEAADBEQVEAAAVRLAAEQKRP--VGVNATGHG--PSVSA--DDA--VLVNTR-RRMEGVS-VDAA--RATAWIEAGARWRKSVLEHTAPHGL--APLNGSSP--NV
FASC	b	(26)	PVVVVVPRTVADVQEAALRYTAARNLS--LAVRSGSHST-YG-QCQADGG---VVLDM-KRFNTVHDVRS---QQTATIDAGARWSDVVAATLSRQQ---TPPVLTDTYLT
PCMH	b	(56)	PSAAVATTVQVQGVATKIP--WTITSTGRNFGYSGAAPVQRG--QVILLD-KKMKNIKIDPE--MCYALVPEPGVTFGQMVDYIQENNLVPMVLSFAPS-AIA
VAO	f	(70)	ASAIIVAPRNADVQSIIVGLANKFSFP--LWPISIGRNSGYGGAAPRVSG--SVVLDMGKNMNRVLEVNVE--GAYCVVEPGVITYHDHNYLEANNLRDKLWLDVDPDLGGG
DALO	f	(22)	PERYVQSSIDEVELVKSARLAEKS--LVTVGSGHS--PSNMCVTDEW--LVNLDR-LDKYQKFVEYPELHYADVTVDAGMRLYQLNEFLGAKGYS-IQNLGSGIS--EQ
DLD	f	(143)	PEIVLYPRNTDEVSLLKIKICHKYSIP--VIPPSSGTSL-EGHFLPTRPG-SCVVLDISKYLNKIIQLNKE--DLDVVQGGVPWEELNEDYLNHDHGL--LFGCDPGP--GA
GLD	p	(21)	TRNFNPQETLADLEAEVKAHEKKNP--IRVPSGLSP-NGIGLS-RSG--MVNLAL-MDKYLEVDDEKKE---RVRVQAGIRVQQLVDAIQEYGLT-LQNFASIR--EQ
BBE	p	(70)	PSAIITLPGSKEELSTNTRCIRKGSWT--IRLRSGGHSY-BGLSYTSDTP--FILIDL-MNLNRVS-IDLE--SETAWVESGSTLGEIYYAITSSS--KLGFTAGWCPTV
HOX	p	(43)	IDFVYVVYTPQGACTALDRAMKECSPGTVRIVSGGHCVYEDFVDECVKA---IINV-TGLVESG-YDDD--RGYFVSSGDTNWGSPFKTLFRDHGR--VLPGGSCSY--SV
GLO	a	(20)	PEVYIQTSTVEEVREVLLALAREQKKK--VKVVGGSHS--PS-DIACDQ--FIIMHGK-MNRVLQYDQKE--KKQITVVEAGILLADLHPQLDEHGLA-MSNLGNFS--DV
ADPS	a	(205)	PDIVLWPTCHDDVVKIVNLACKYNLC--IIPIGGGTSVSYGLMCPADETRTIIISLDTSQ-MNRILWVDEN--NLTAHVEAGITGQELERQLKESGY--CTGHEPDSLEFS
MURB	b	(16)	AQHITVCAEDBQQLLNAWQYATAEQGP--VLILGEGSNV-LFLEDY-RGT--VIIN--RIKIGIBIHD-PAWAYLHVGAAGENWHRLVKYTTLQEGM--PGLLENIAL--IP
Consensus			P..hh.P.s.--h..hh+hs....P..h.hhssGHS...s.s...-s...hhh+.+.n.hh.h.-.-....s.h.ssh...-h...h.-.sh.....ss.....
Structure			Pyrophosphate
HDNO	P08159		GFCGLALNNGGVFLTPKYGLASDNLILGATLVTTAT--GDVIYCS-----DDEERPELFWAVRGAGP-NFGVVTEVEVQLYE(223) LAAVKREYDPENFRH-NYNI(5)
GLCD	P52075		SIGGNVAENAGGVHCLKRYGLTVHNLLKIEVQTLAD--GEALTLG--SDALDSPGFDLLALFTGSGE-MLGVTTEVTVKLLP(223) FHAVKAAFDPDGLLNP-GKNI(27)
MMCO	P43485		GAVGYLVGGAGGLHRRFYGAADHVRRLRLQLD--GRLRDVT-----AGTDPLDFWAVRGAGP-NFLGVLMVEVDFLFP(227) LRAVKAGYDPDNMFRV-NFNI(27)
FASC	Z29635		TVGGTSLVSGGFGSSHGFLQTDNVDSLAVVTGS--GDFRECS-----AVSNSLELFDVAVRGGLG-QFGVIVNATIRLTA(223) IARAKRRFPDPRILAP-GYRM(3)
PCMH	L14748		GPVGNMTDRGCVGYTP--YGEHFMMQCGMEVVLAN--GDEVYRTG-MGGVPGSNTWQIFKWGYP(9) NYGICTKMGFWMLP(259) VRWTRTSSSLRAARASTITI(10)
VAO	Y15627		SVLGNNAVERRGVGYTP--YGDHMMHSGMEVVLAN--GDLRLTG(19) QPWSKIAHLFVPGYGP(9) NMNGIVTIRGIWMLP(256) NEVLKNNAVDPNGIAPGSGV(12)
DALO	P54783		SVAGIISTGSHGSSP-YHGLISSQYVNLTVNGK--GELKFTD-----AENDPEVFKAALLSVG-KIGIIVSATIRVVP(300) FRKIRKEQDPDNVFLANKQWA(13)
DLD	X71628		QIAGCIANSAGHTNAYRYGTMENNVNITMCMD--GTIVYKTKR-PRPKSSAGYNLNGLIIGSEG-TLGIVTEATIKCHV(228) MRKLLKALDPKRIILNP-DKIF(12)
GLD	Z97060		QIGGI IQVGAHGTGA-RLPPIDEQVIGMKLVTIP--KGTIELS-----KDNDEPFLHARCLGQ-GLGVVAEVTLCQVE(228) YNKARRELDPNRILSNMNVK(8)
BBE	P30986		QTGGHISGGGFGMMSRYGLAANDVVDAILIDAN--GAILDRQ-----AMGEDVFWAIRGGGVGGAIAYNWKILKLP(256) LIRAKTLIDPNNVFNH-PQSI(22)
HOX	U89770		GLGGHIVGGGDGILARLHGLPVDWLSGVEVVVKPVLTEDSVLKVYHKDSEGNDELFWAHTGGGCGNFGIITKYYPKDLF(293) LIKAKWLWDPNEIFTN-KQSI(12)
GLO	P10867		TVAGVIG-SGTNGTGIKHGILATQVVALTLMTAD--GEVLECS-----ESRNADVQFQAARVHLG-CLGILITVTTLQKVP(230) FCDIREKLDPMTGMLN--SYL(5)
ADPS	Y08826		TVGGWISTRASGMKKNIYGNIEDLVHMKVVTIPR--GVIEKSCQ--GPRMSTGPDIIHFMGSEG-TLGVITEATIKQIR(255) LKSVKYDYVDPNTNFG--NRNL(1)
MURB	P08373		CGVCGSSPIQNI GAYVELGRVCAYVDSVELATGK--QVRLTAKE-----CRFGYRDSIFKHEYQD-RFAIVA-VGLRLPK(200)
Consensus			shsG.hs.ss.s.s.+Ysh..-nhhsh.hhss...s-hh..s.s...-s...-hP.s..ss.s.s..shhs..sh+h.P h..h+...-P..hF.....h
Structure			ADP Isoalloxazine Adenine

Figure 1

Multiple alignment of 13 homologous sequences for which gene products have been identified. The gene products of all but fasciation enzyme (FASC) and mitomycin C oxidase (MMCO) have been characterized. All (other) homologous sequences can be viewed at <http://gcg.tran.wau.nl/local/Biochem>. The origin of the sequences is shown in the upper panel (b, bacterial; f, fungal; p, plant; a, animal), while the SWISS-PROT accession numbers are listed in the lower panel. Sequences were aligned using the ClustalW²⁵ program and manual alignment. The number of residues present in gaps in the sequence and at the termini are indicated in parentheses. Conserved ($\geq 50\%$) residues are shown in green. A consensus sequence is shown beneath the alignment [s, small (G,A,S,T); -, negatively charged (E,D); n, neutral (N,Q); +, positively charged (R,K); h, hydrophobic (I,L,V); *, any residue]. All homologous sequences were retrieved using the position-specific iterative BLAST (PSI-BLAST) method. By screening the NCBI non-redundant protein sequence database with the homologous N-terminal part of 6-hydroxy-D-nicotine oxidase (HDNO), all 54 homologous sequences were identified within four iterations ($E < 0.01$). The bacterial flavoenzyme UDP-N-acetylenolpyruvylglycosamine reductase (MURB) was also detected, which shows, despite a very low sequence similarity with the N-terminus of vanillyl-alcohol oxidase (VAO)⁶, a similar architecture to its FAD-binding domain²⁶. The conserved histidine residue to which FAD is bound in HDNO and BBE is shown in red. The points of interaction between the FAD cofactor and specific conserved residues (predicted from the structure of VAO) are indicated in blue. ADPS, alkylidihydroxyacetonephosphate synthase; BBE, berberine bridge enzyme; DALO, D-arabino-1,4-lactone oxidase; DLD, D-lactate dehydrogenase; GLCD, glycolate oxidase subunit D; GLD, L-galactono-γ-lactone dehydrogenase; GLO, L-gulonono-γ-lactone oxidase; HOX, hexose oxidase; PCMH, p-cresol methylhydroxylase.

Figure 2

Ribbon diagram of a vanillyl-alcohol oxidase monomer from *Penicillium simplicissimum*. The covalently bound FAD is represented as a ball-and-stick model. The FAD-binding domain is shown in green and the cap domain is shown in red. The conserved regions at the N- and C-termini of the protein are shown as full-coloured ribbons. The figure was rendered using MOLSCRIPT²⁷.

family (shown in green in Fig. 2) represent a conservation of this FAD-binding domain¹⁹. It is apparent from the VAO structure that several conserved residues are involved in binding the FAD cofactor (Fig. 1). The intervening non-conserved region of about 240 residues exactly corresponds to the cap domain of VAO; the topology of this domain might therefore differ among the members of the family.

Interestingly, several of the VAO-related enzymes described above harbour a covalently bound FAD. For 6-hydroxy-D-nicotine oxidase and berberine bridge enzyme, the FAD-linking residue (shown in red in Fig. 1) is known^{20,21}. Remarkably, this histidine residue is conserved in 17 members of the family, which suggests that they are also covalently flavinylated.

Indeed, L-gulonon- γ -lactone oxidase¹², D-arabino-1,4-lactone oxidase²² and hexose oxidase²³ have been reported to contain a covalently bound flavin. Furthermore, the position of the flavinylated histidine residue in 6-hydroxy-D-nicotine oxidase is homologous to Arg104 of VAO. In VAO, Arg104 lies close to the 8 α -methyl group of the flavin ring, although this group is covalently linked to His422 (Ref. 6). Thus, if all sequences containing the conserved histidine residue truly represent covalent flavoproteins, then the percentage of covalent flavoproteins within this novel oxidoreductase family is relatively high. Moreover, in VAO and *p*-cresol methylhydroxylase, the flavin group is covalently linked to a residue in the cap domain^{3,6}. This indicates that, unlike the

well-known Rossmann fold²⁴, this newly discovered, conserved FAD-binding fold favours covalent flavinylation.

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