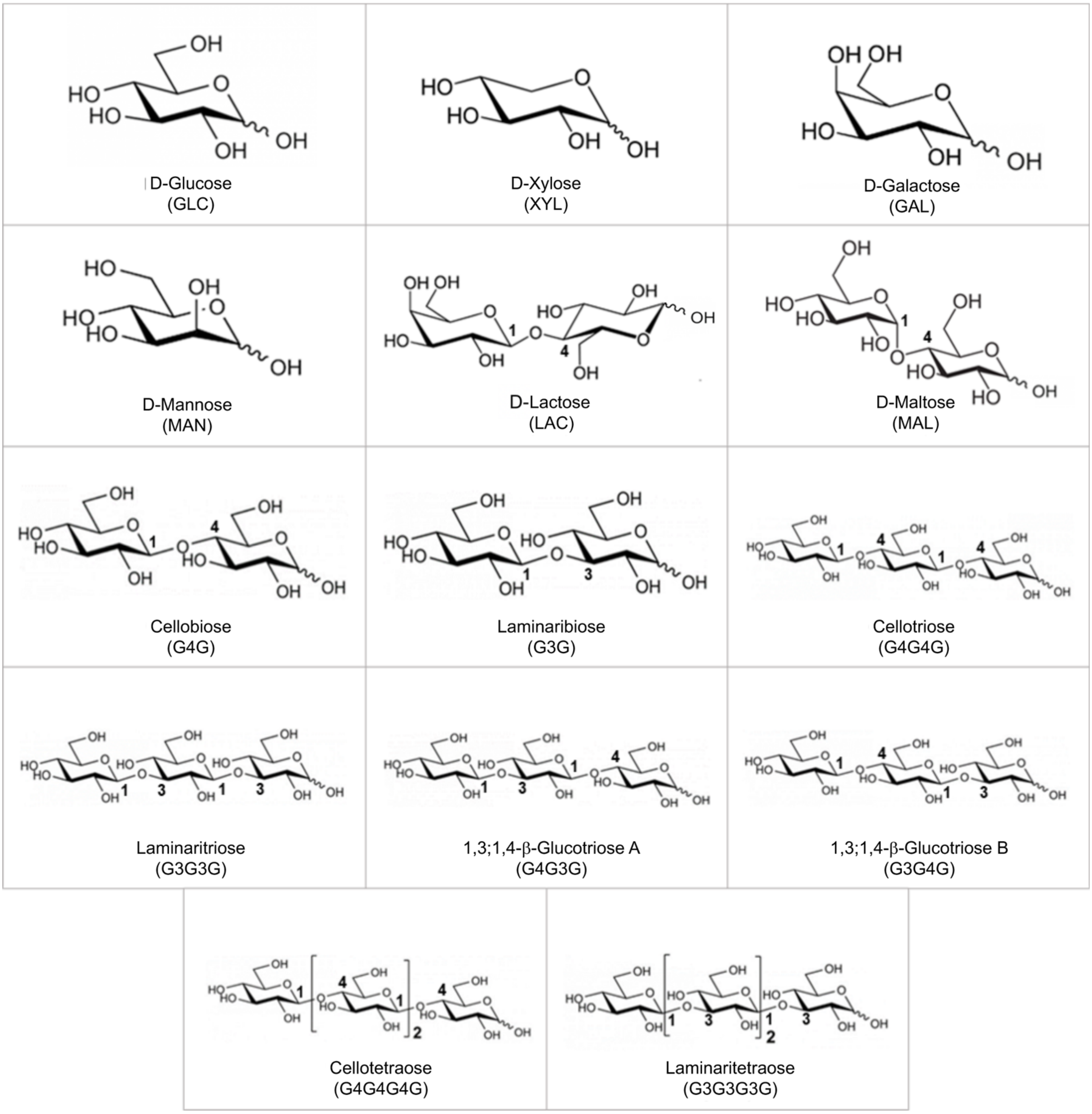
Additional File 1. Supplementary figures

Figure S1. Chemical structures of the monosaccharides and gluco-oligosaccharides tested for ODH activity. Carbon atoms involved in glycosidic bonds are numbered. D-galactose and D-mannose are epimers of D-glucose, respectively at C4 and C2. D-xylose differs from D-glucose for the absence of the group CH_2-OH at C5. D-lactose is a disaccharide composed by D-galactose linked to D-glucose by a β(1→4) glycosidic bond and D-maltose is a disaccharide made by two units of glucose joined with an α(1→4) bond.



D-Glucose (GLC)

D-Xylose (XYL)

D-Galactose (GAL)

D-Mannose (MAN)

D-Lactose (LAC)

D-Maltose (MAL)

Cellobiose (G4G)

Laminaribiose (G3G)

Cellotriose (G4G4G)

Laminaritriose (G3G3G)

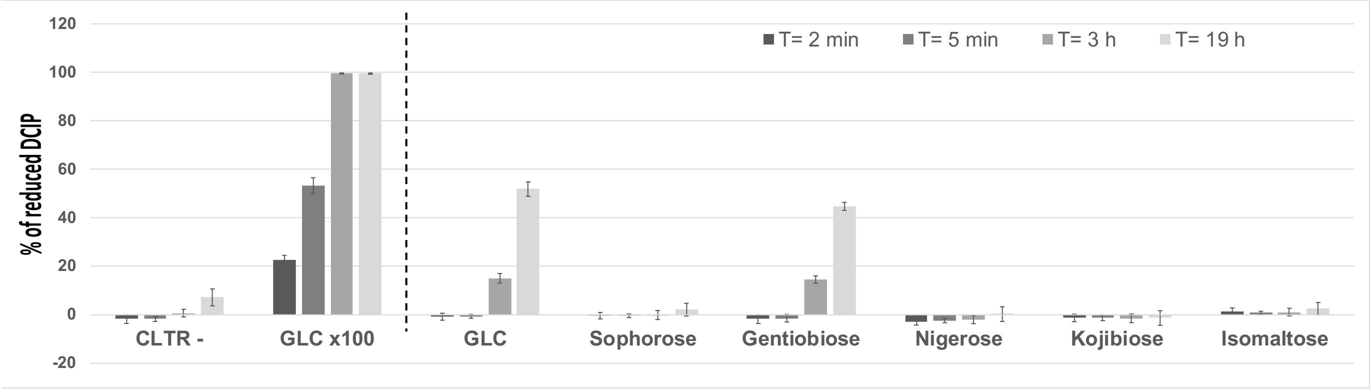
1,3;1,4-β-Glucotriose A (G4G3G)

1,3;1,4-β-Glucotriose B (G3G4G)

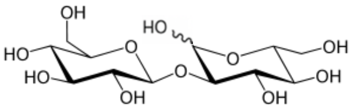
Cellotetraose (G4G4G4G)

Laminaritetraose (G3G3G3G)

Figure S2. Extended ODH substrate screening. To evaluate ODH activity towards all glucobiose isomers with a reducing end, substrate screening was further extended to sophorose, gentiobiose, nigerose, kojibiose and isomaltose, whose structures are represented within the squared boxes. The experiment was conducted as described for obtaining the data reported in Fig. 2 of the main text. Enzyme activity, comparable to the one observed on GLC, was only detected for gentiobiose. To exclude any possible artifacts, we verified by LC/MS that gentiobiose was not hydrolyzed (data not shown).

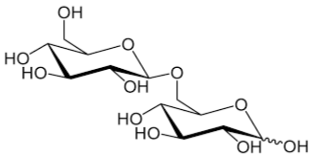


Sophorose (G2G)



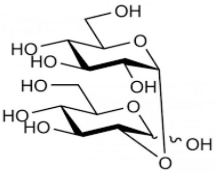
D-Glucose-**β(1→2)**-D-glucose

Gentiobiose (G6G)



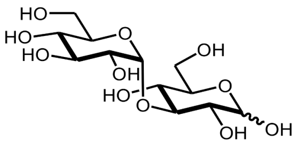
D-Glucose-**β(1→6)**-D-glucose

Kojibiose



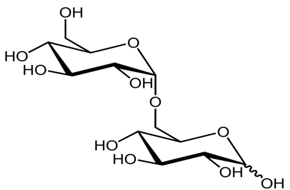
D-Glucose-**α(1→2)**-D-glucose

Nigerose



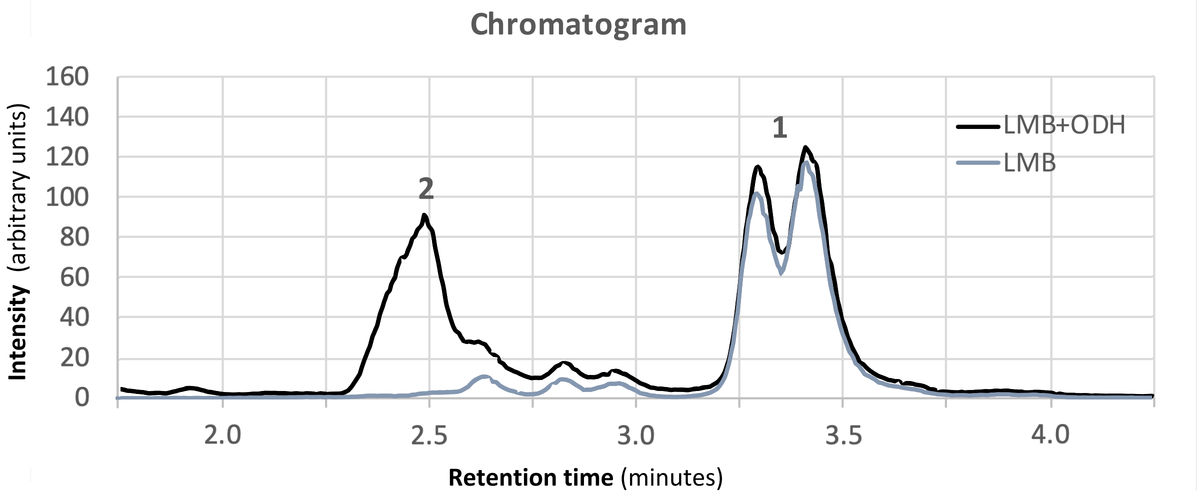
D-Glucose-**α(1→3)**-D-glucose

Isomaltose



D-Glucose-**α(1→6)**-D-glucose

Figure S3. LC-MS analysis of enzymatic products. Top panel: chromatografic separation of G3G (grey) and of an overnight G3G/DCIP/ODH reaction (black). G3G (MW=342 g/mol) appeared as a double chromatografic peak (peak 1) corresponding to the α- and β-anomers, as confirmed by the detection of ions having m/z values of 341 [M-H], 387 [M+Formate -H] and 683 [2M-H] (not shown). After enzyme oxidation only (peak 2), laminaribionic acid (MW=358 g/mol) was detected by ions having m/z values of 357 [M-H] and 715 [2M-H] (bottom panel), while no formate adduct was detected. DCIP (MW=268 g/mol, oxidized) was also detectected at an m/z value of 266 (and a minor peak at 268 for reduced DCIP). The chemical nature of laminaribionic acid confirms specific enzymatic oxidation of the C1 hydroxyl of G3G reducing end, as oxidation of other G3G hydroxyls would not result into hydrolysable enzyme products. The same enzymatic reaction (not shown) was assessed for GLC (MW=180 g/mol), detected by ions having an m/z value of 225 [M+Formate -H], and gluconic acid (MW=196 g/mol), detected at an m/z value of 195 [M-H] in GLC/DCIP/ODH overnight incubations, in agreement with what we previously reported [14].



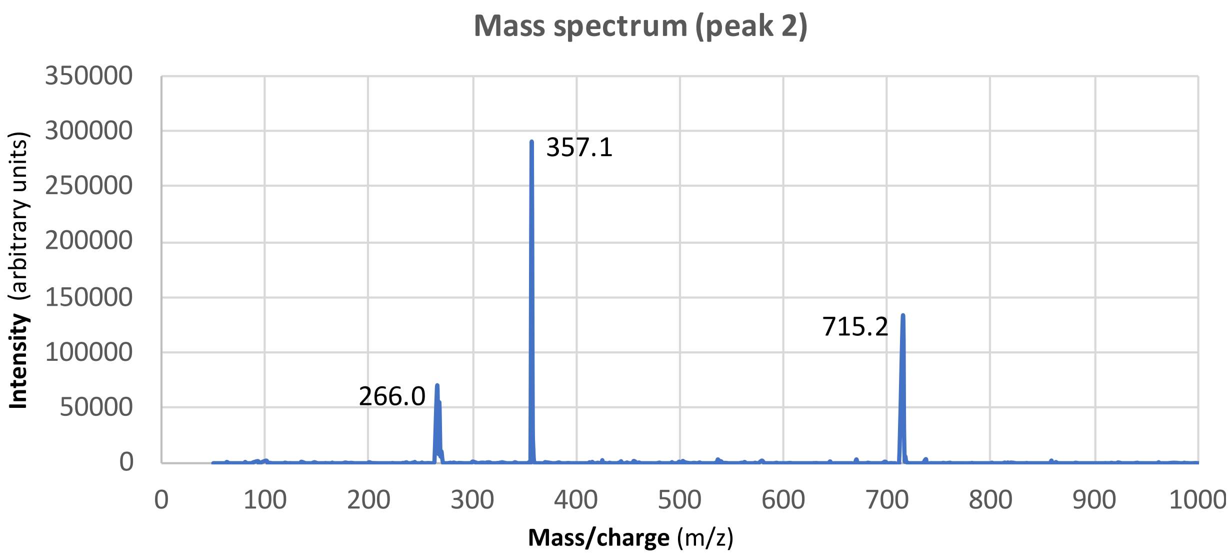


Figure S4. Full-range enzymatic kinetics of ODH on GLC. Initial rates of DCIP reduction as a function of GLC concentration, ranging between 0 and 3000 mM. The solid line represents a fit to the equation: $v_0 = (V'_{max} \times [S])/(K'_M + [S] + [S]^2/K_i)$, which takes substrate inhibition into account. In the equation v_0 is the initial velocity of the reaction, V'_{max} is the maximum velocity of the reaction corresponding to substrate saturation of the enzyme, K'_M is the Michaelis–Menten constant, [S] is the substrate analytical concentration and K_i corresponds to the dissociation constant for substrate binding to the enzyme. The fit is poor, suggesting that other inhibition models should be taken into account.

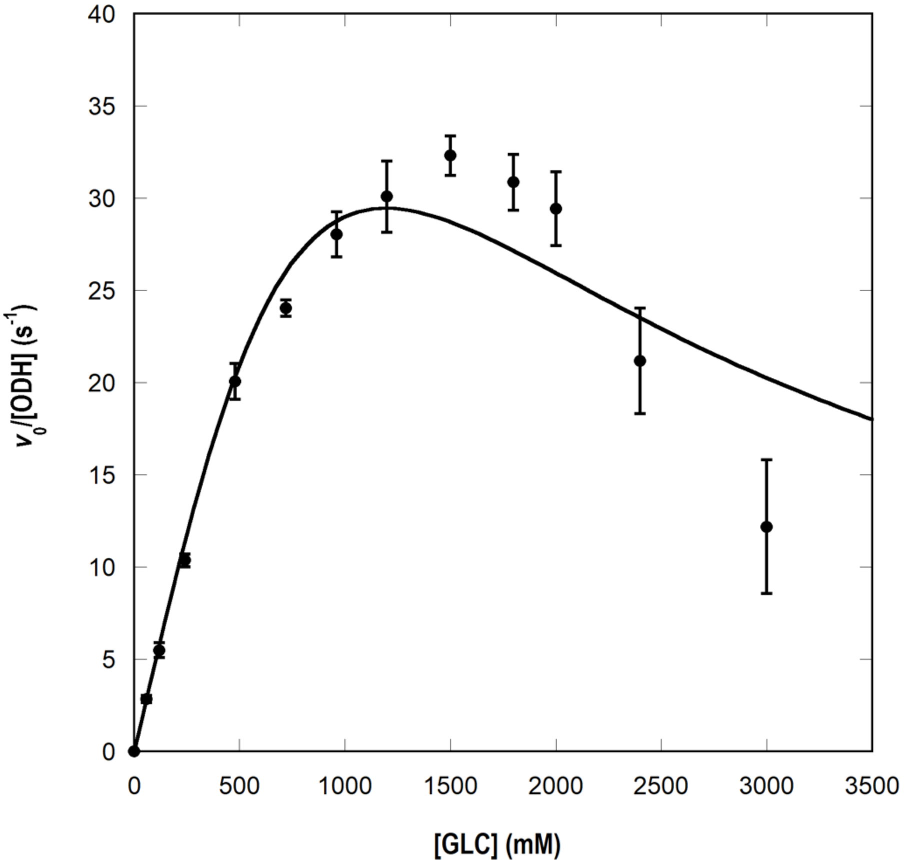


Figure S5. Comparison of ODH activity on different GLC anomers. Initial rates of DCIP reduction were measured on a 96-well plate as described in Methods (Spectrophotometric assays for enzymatic activity), with 25 mM of either freshly dissolved α-GLC (grey empty points) or preequilibrated GLC solutions (mixtures of α- and β-anomers, black solid points). Solid lines represent a fit to the Abs_{520} decrease (initial velocity), and the error bars the standard deviation of three independent replicas of the reaction mixtures. Measurements start 3-4 minutes after dissolving α-GLC in 30° C water, accounting for the time required to: dissolve α-GLC, add it to the reaction mixtures, dispense ODH to trigger the reaction and run data collection. Within the observed time window, ODH activity is twice faster on preequilibrated GLC (both anomers) than on freshly dissolved α-GLC.

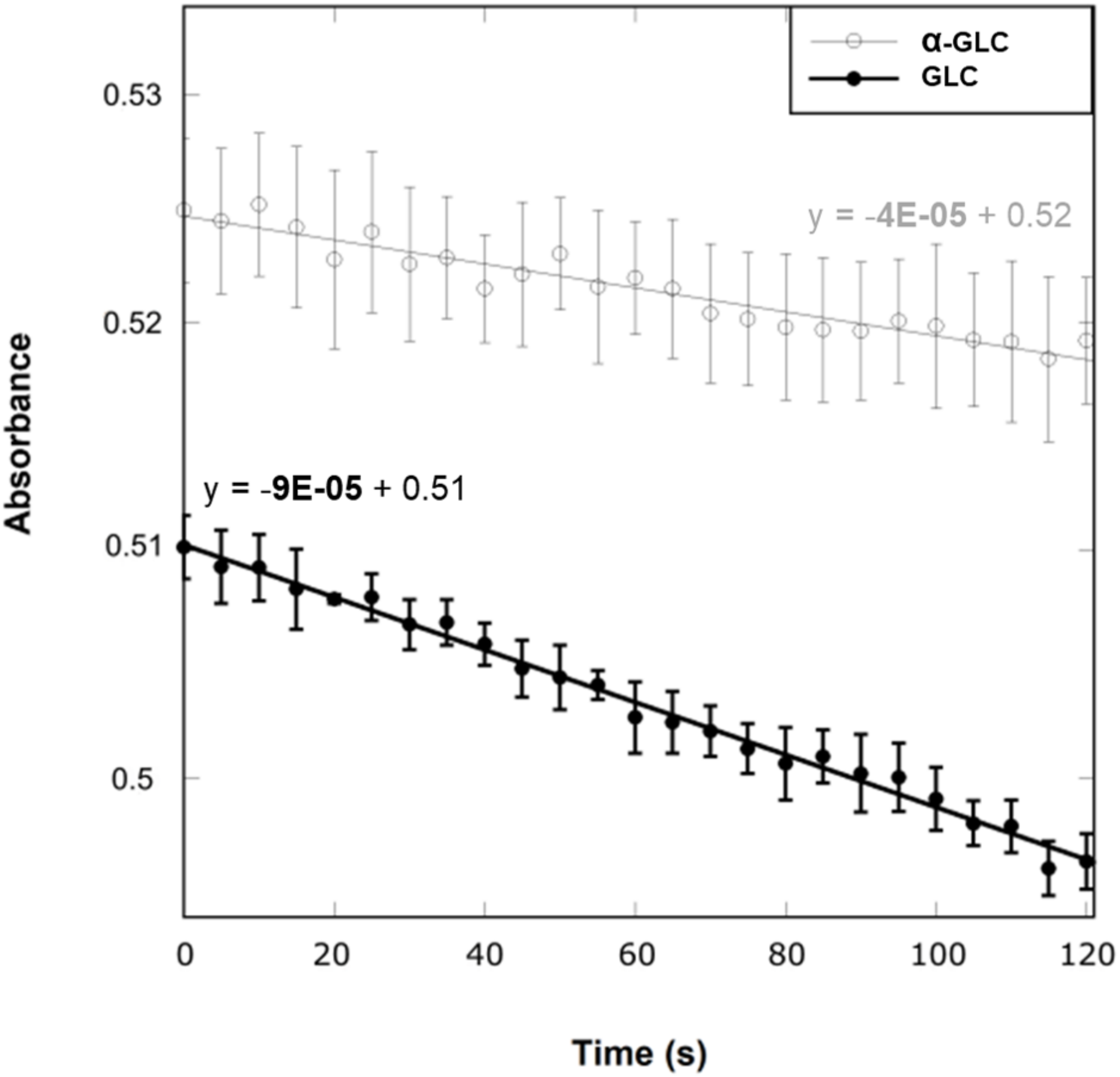
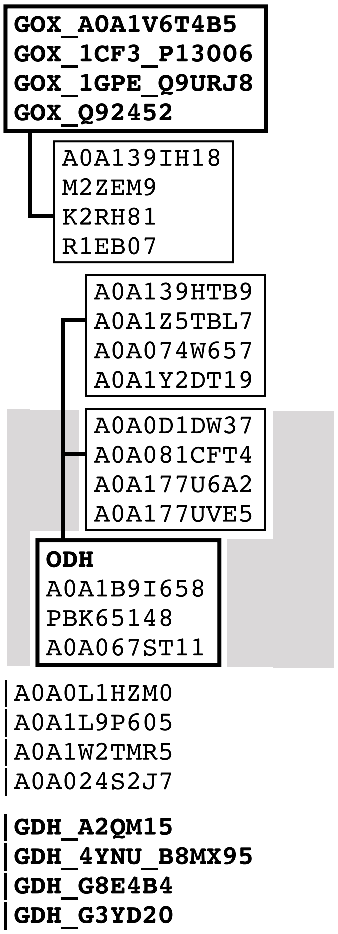


Figure S6. Multiple sequence alignment of ODH with enzymes from the GOX/GDH clade. Four equally distant genes per each previously defined subclade of the GOX/GDH clade [16] were used for Multiple Sequence Alignment (MSA). In the preliminary Table, subclade nomenclature, coloring scheme and order are as in [16], apart for the suggested renaming of the GDH III subclade into ODH. GOX-like and ODH-like subclades correspond to poorly defined groups that appear to be phylogenetically close to the GOX and the ODH subclades, respectively [16]. MSA analysis includes protein sequences from both Ascomycota (white background) and Basidiomycota (grey), corresponding to either characterized enzymes (bold) or to hypothetical genes from genomic data. Genes are named by their Uniprot identifier, preceded by a four letter PDB code if the three-dimensional structure is known, and by a GOX or GDH label for assigned functions. In the MSA, ODH amino acid sequence is in bold. The following coloring has been used to identify conserved residues involved in: substrate binding / catalysis / FAD ring distortion (blue); SBS1 (orange), SBS2 (yellow) and SBS3 (green); GLC-binding (H-bonds) in GOX/GDH enzymes (purple), and their loss in ODH (purple framed, golden box); G3G and GLC binding (H-bonds and CH-π interactions) in ODH (red); and substrate-binding loop and related residues (magenta).



Subclade	Phylum	Organism
GOX	**ASCO**	*Penicillium flavigenum* *Aspergillus niger* *Penicillium amagasakiense* *Talaromyces flavus*
GOX -like	**ASCO**	*Pseudocercospora musae* *Pseudocercospora fijiensis* *Macrophomina phaseolina* *Neofusicoccum parvum* UCRNP2
ODH -like	**ASCO**	*Mycosphaerella eumusae* *Hortaea werneckii* EXF-2000 *Aureobasidium namibiae* CBS 147.97 *Pseudomassariella vexata*
ODH -like	**BASI DIO**	*Ustilago maydis* 521 *Moesziomyces antarcticus* *Tilletia caries* *Tilletia caries*
ODH (GDH III)	**BASI DIO**	*Pycnoporus cinnabarinus* *Kwoniella pini* CBS 10737 *Armillaria solidipes* *Galerina marginata* CBS 339.88
GDH II	**ASCO**	*Stemphylium lycopersici* *Aspergillus versicolor* CBS 583.65 *Rosellinia necatrix* *Trichoderma reesei* RUT C-30
GDH I	**ASCO**	*Aspergillus niger* CBS 513.88 *Aspergillus flavus* NRRL3357 *Colletotrichum gloeosporoides* *Aspergillus niger* ATCC_1015

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|GOX_1GPE_Q9URJ8      SDPSKVAGKTYDYIIAGGGLTGLTVAAKLTENPKIKVLVIEKGFYESNDGAIIEDP----  88
|GOX_Q92452           SDPSKVAGKTYDYIIAGGGLTGLTVAAKLTENPKIKVLVIEKGFYESNDGAIIEDP----  88

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   |M2ZEM9            ---TAQLKKEYDYIVCGGGTAGLAVANRLSADPKNSVLVVEAGING--------------  60
   |K2RH81            ---SSQLLESYDYIICGGGTSGLTVANRLTELADATVLVIEAGPESIS------------  65
   |R1EB07            QPQGSDLLDSYDYIICGGGTSGLTVASRLSELADATVLVIEAGSDT--------------  71

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     |A0A074W657      RASNMSANNSFDYVVVGGGLTGLTVASRLSENPTIRVLVIEDGQDNHND-PRVSDV----  74
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|A0A1W2TMR5           AMAGAARAETFDYIIAGAGTCGLVLANRLSADPAVRVAVIEPGEDVRNN-ANVTDP----  76
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|GDH_G3YD20           -KSHADSPAHYDFVIVGGGTSGLVVANRLSELSDVTVAVIEAGESALNN-FNVSNV----  71
                                 .::: *.*  *   * :*:      : ::: *


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|GOX_1GPE_Q9URJ8      NAYGQI--FG---TTVDQNYLTVPLI--NNRTNNIKAGKGLGGSTLINGDSWTRPDKVQI  141
|GOX_Q92452           NAYGQI--FG---TTVDQNYLTVPLI--NNRTNNIKAGKGLGGSTLINGDSWTRPDKVQI  141

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   |R1EB07            -------------TTANWNYPSGPERFASGRSQTLPAGKALGGSSAINGRVYLRAEQSQI  118

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|GDH_G8E4B4           NGYGLA--FG---TDIDYAYQTTAQTYANNASTTLRAAKALGGTSTINGMAYTRAEASQI  130
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   |K2RH81              WP--------------------TEVAIETYLNQLEEA-YASIGMPRIQDPNGGEMRGLST  207
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  |A0A067ST11           YP--------------------DDMYGGPQQLAFVDTIVALTGISHFKDLNGGTPNCVSI  233

|A0A0L1HZM0             FT--------------------PYLVGQGFADLLKTT-SEAMGIPYNQDPNDGVMRGFNT  219
|A0A1L9P605             YQ--------------------YGLQNGSFASLAKET-WEAFGMPFNPDVNGGNIRGFSV  215
|A0A1W2TMR5             FP--------------------FSLVNGSLHDLAVDT-WAALGYELNEEVNAGSVRGFGA  224
|A0A024S2J7             FN--------------------PALLSGSFYGDVKAS-WAALGQTLNRDASSGDTAGFDV  222

|GDH_A2QM15             WP--------------------DI-PANNLTNTLNAT-FQGLGVPWTEDVNGGKMRGFNV  222
|GDH_4YNU_B8MX95        WS--------------------GSLASGNLSVALNRT-FQAAGVPWVEDVNGGKMRGFNI  220
|GDH_G8E4B4             YA--------------------YGSTNDSYPTSLNAT-YQALNVPWKEDIAGGSMVGFAS  224
|GDH_G3YD20             WP--------------------NSMTNSSVFPVLEQT-FEKLGVQYNPDSEGGKMVGFTV  219
                                                                  .     :   *
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|GOX_A0A1V6T4B5             IPNNL----HENQIRADAAREWLLPNY-QRDNLQILTGQKVGKVLFNQTA------SGPK  285
|GOX_1CF3_P13006            FPNTL----HEDQVRSDAAREWLLPNY-QRPNLQVLTGQYVGKVLLSQNG------TTPR  285
|GOX_1GPE_Q9URJ8            IMNNL----DENQVRVDAARAWLLPNY-QRSNLEILTGQMVGKVLFKQTA------SGPQ  285
|GOX_Q92452                 IMNNV----DENQVRVDAARAWLLPSY-QRPNLEILTGQMVGKVLFKQTA------SGPQ  285

   |A0A139IH18              YPGFFVINAQREQLRESAREAYYLPVV-GRPNLELLDETMCQRIDWENGS---G-GGDLK  255
   |M2ZEM9                  YPGFFVINAQREQRRESSREAYYLPIV-GRTNLELIDETMCQRIDWEKSS---D-GGDLK  255
   |K2RH81                  YPRTRQA-INDRDVRESAASAYYEPVR-DRTNLDVLLNTSVLRIVWAEND---DADGNAI  262
   |R1EB07                  YPRTQQN-VNDQDVREDANTAYYQQVA-DRTNLDVLTNTSCLRIVWAESD---DADGNAI  266

     |A0A139HTB9            IPNMVVP--EENQNRSSPFTAYAQNQVQERNNFLILTGHRVTEIVWQETK----ERAPLV  297
     |A0A1Z5TBL7            IPNMVVT--DGSQNRSSPFTAYAMHQVEQRDNFMILTGQRVTEIVWKE-------GDDMI  305
     |A0A074W657            IPNMVQP--GESQNRSSPYTAYILGEPEKRGNLVILTGHRAVEIRWRNNCTSLESDRILT  281
     |A0A1Y2DT19            IPNMVQP--DESETRASPYASWIKGTADTRPNLVILLGHRVVKLDWSSTE----EGADLV  289

     |A0A0D1DW37            TPNSI----GVNGQRTSAASAYYTPVQ-NRDNLTILTGTMAKNLLWDAAT----SSNLLR  302
     |A0A081CFT4            TPNSI----GTNARRTSSASAYYTPVQ-GRENLTVLTGTRARNLLWGAQS----ASG-LG  293
     |A0A177U6A2            TPNSMLP--GSGNLRSSSATAYLTPIEQKRSNLVVLTTWRGWKTTWSSTA----RT--PT  316
     |A0A177UVE5            FPNTFLP--GPGKLRSSSAIAYLSPIEFSRSNLVVLTNFRGWKLIWDPTK-----N--AT  305

  |ODH                      TPLSINW--HDDDHRSSSIEAYYTPVENNRQGWTLLIDHMATKVLFDG-T----NA-PLT  260
  |A0A1B9I658               HPNTISW--QDSDHRSSSATAYYSPVSE-RSNLAILLQHQATKIQFDGN----------K  275
  |PBK65148                 TPLTLNW--HDDDHRSSSVEAYLSPVEGIRTNWLTLAEHQVTKINWANAG----SI-PLT  283
  |A0A067ST11               TPLTINW--HDSDHRSSSAMAYLTPVESVRTNWLTLVTHQVTKINWNT-G----GI-PLT  285

|A0A0L1HZM0                 WPMTLNE---TGPTREDGARAFYYPVA-SRPNLHVFPNSMATRIIWDDSN---SAAAGVL  272
|A0A1L9P605                 WPQTLDR---EANVREHSARAYYYYPVR-DRSNLVIF-RGRVDRVSWAETN---ES--KVL  265
|A0A1W2TMR5                 WPQTLDR---DRNIRFDAARAYYYDIE-DRPNLTLI-KGTVKKLTWASCT---ASGQDAV  276
|A0A024S2J7                 WPQTLDP---VKNVRWDAATAFYWPVQ-DRPNLALL-NGTVSRILWKNDE---AD--VPE  272

|GDH_A2QM15                 YPSTIDY---TAYVREDAARAYYWPIA-SRPNLHLMLDTFVNRLVWKNGG---SQ-GNAT  274
|GDH_4YNU_B8MX95            YPSTLDV---DLNVREDAARAYYFPYD-DRKNLHLLENTTANRLFWKNGS---A--EEAI  271
|GDH_G8E4B4                 YPKTLNQ---DLNIRWDAARAYYFPYE-NRTNLKVVLNTTAKKLTWASAT---NG-TDAT  276
|GDH_G3YD20                 HPDTLDR---EMNVREDAARAYYWPYE-ARSNLKIISNTRANKVIWANTT---Q--GEAV  270
                                          *      :       * .   .      .


|GOX_A0A1V6T4B5             AVGVNFGTNK--AVNFNVYAKQEVLLAAGSAISPLILEYSGIGIKSVLDKAGVKQLLELP  343
|GOX_1CF3_P13006            AVGVEFGTHK--GNTHNVYAKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGIDTVVDLP  343
|GOX_1GPE_Q9URJ8            AVGVNFGTNK--AVNFDVFAKHEVLLAAGSAISPLILEYSGIGLKSVLDQANVTQLLDLP  343
|GOX_Q92452                 AVGVNFGTNK--AVNFDVFAKHEVLLAAGSAISPLILEYSGIGLKSVLDQANVTQLLDLP  343

   |A0A139IH18              ARGVEISNVD--NK-TVVRARHEVILSAGVYRTPGILEYSGIGNPKILKAHKIDTKIELP  312
   |M2ZEM9                  AHGVEISNVD--NK-TVIHARKEVILSAGVYRTPGILEYSGIGNPKILKSHKIDTKVDLP  312
   |K2RH81                  AAGVEVATAD--GTTAVLNATQEVILAAGAYRSASILEYSGVGNSQILQGLGIATKVYLP  320
   |R1EB07                  AAGVEVASAD--GSTATLNATAEVIVAAGAYRSASILEYSGVGNSQILQSLGIDTKVYLP  324

     |A0A139HTB9            ADGVHFQSS-KNSPVQFVKANREVLLAAGSLQSPQILELSGVGDSDVLGAAGVELKHELK  356
     |A0A1Z5TBL7            AERVHFQGC-RDCDISFVKADREVLLSAGSLQSPQILELSGVGDPDVLEAAGVPLKKAAF  364
     |A0A074W657            ADGVYYQAD-RHSERLFAKATREVLLAAGSMQSPQLLELSGVGDPAILDKAGVPVRKALM  340
     |A0A1Y2DT19            ASGIQFQQS-RSSPILTAHASRDVILAAGSMQSPQLLELSGVGDATVLANAGIPLVKSVP  348

     |A0A0D1DW37            SSGVVVQQG-RNGNQIRLVANKEVILAAGALNTPVLLQRSGVGAKTDLNSIGVDQRIELA  361
     |A0A081CFT4            SSGVVVQQS-HDGNLVTINAKREVVLAAGALNTPTLLQRSGVGAKADLDSIGVDQKLALD  352
     |A0A177U6A2            ATGVIIQQK-NGGPTYNVKASREIIVAAGAIRSPVFLEHSGIGDANILRNINVPVKVDLP  375
     |A0A177UVE5            ATGVVIQQS-PGGPTYNVSAKREVIVAAGSIKSPIFLEHSGVGNATVLSKLRVPLKVNLP  364

  |ODH                      AVGIEFGASDATGNRYKAFARKEVILAAGAIQTPALLQLSGIGDSDVLGPLGISTLSDLK  320
  |A0A1B9I658               ATGVEYGTSD--GQRYTVNAGKEVIISAGAIQTPALLQLSGVGDPALLNGLGINVVANVS  333
  |PBK65148                 ASGVEFAPTSGGSTRYTATANREVIIACGSIQSPALLQLSGVGDSDVLGPLGIDTHIDLK  343
  |A0A067ST11               ASGVEFAPASGGSTRYTASARREVILAAGAIQTPALLQLSGIGDSAILGPLGITTMIDLK  345

|A0A0L1HZM0                 ASGVEIVTAK--DVTETIHASKEVVVAAGAIRSPAFLEHSGVGNPAVLEPLGIETVNPLH  330
|A0A1L9P605                 AEGVEYTTED--GKAKILYAEKEVIVSAGAVRTPAILELSGVGNPELLKQLDIPVKVPLS  323
|A0A1W2TMR5                 ADGVEYVTPD--GGLIKVTAAKEVILSAGALRSPLILESSGVGNPSLLSKLGIKPKIDLP  334
|A0A024S2J7                 ASGVEYLTPD--GNIKTVNARREVILSAGALRTPLILELSGIGNPSILNGLGIETVVNSP  330

|GDH_A2QM15                 AAGVEITSSN--GTISVIGASQEVIISAGSLKSPGILELSGIGNRDILERYNISVRVDLP  332
|GDH_4YNU_B8MX95            ADGVEITSAD--GKVTRVHAKKEVIISAGALRSPLILELSGVGNPTILKKNNITPRVDLP  329
|GDH_G8E4B4                 ASGVEITAAD--GTTSVVTANKEVIISAGALVSPLLLELSGVGNPAVLSQYGIETVVELP  334
|GDH_G3YD20                 AVGIEVTNAY--GTET-IYADKEVILSAGALRSPAILELSGIGNPDVLNKHNIPVKVNIT  327
                            :  :               *  :::::.*   :  :*: **:*    *    :
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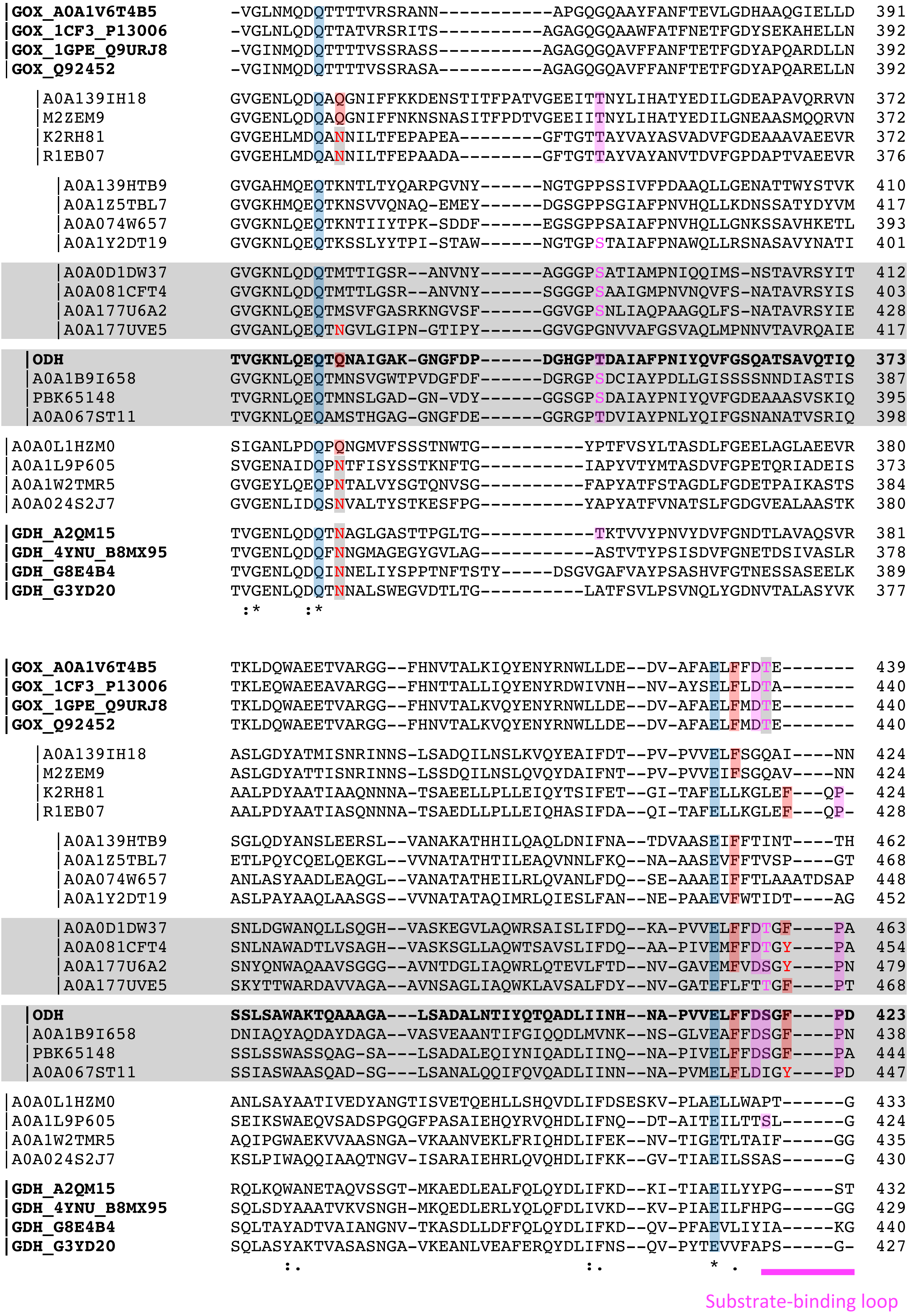

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GOX_1CF3_P13006    -VGLNLQDQTTATVRSRITS----------AGAGQGQAAWFATFNETFGDYSEKAHELLN  392
GOX_1GPE_Q9URJ8    -VGINMQDQTTTTVSSRASS----------AGAGQGQAVFFANFTETFGDYAPQARDLLN  392
GOX_Q92452         -VGINMQDQTTTTVSSRASA----------AGAGQGQAVFFANFTETFGDYAPQARELLN  392

A0A139IH18         GVGENLQDQAQGNIFFKKDENSTITFPATVGEEITTNYLIHATYEDILGDEAPAVQRRVN  372
M2ZEM9             GVGENLQDQAQGNIFFNKNSNASITFPDTVGEEIITNYLIHATYEDILGNEAASMQQRVN  372
K2RH81             GVGEHLMDQANNILTFEPAPEA--------GFTGTTAYVAYASVADVFGDEAAAVAEEVR  372
R1EB07             GVGEHLMDQANNILTFEPAADA--------GFTGTTAYVAYANVTDVFGPDAPTVAEEVR  376

A0A139HTB9         GVGAHMQEQTKNTLTYQARPGVNY------NGTGPPSSIVFPDAAQLLGENATTWYSTVK  410
A0A1Z5TBL7         GVGKHMQEQTKNSVVQNAQ-EMEY------DGSGPPSGIAFPNVHQLLKDNSSATYDYVM  417
A0A074W657         GVGKNLQEQTKNTIIYTPK-SDDF------EGSGPPSAIAFPNVHQLLGNKSSAVHKETL  393
A0A1Y2DT19         GVGKNLQEQTKSSLYYTPI-STAW------NGTGPSTAIAFPNAWQLLRSNASAVYNATI  401

A0A0D1DW37         GVGKNLQDQTMTTIGSR--ANVNY------AGGGPSATIAMPNIQQIMS-NSTAVRSYIT  412
A0A081CFT4         GVGKNLQDQTMTTLGSR--ANVNY------SGGGPSAAIGMPNVNQVFS-NATAVRSYIS  403
A0A177U6A2         GVGKNLQEQTMSVFGASRKNGVSF------GGVGPSNLIAQPAAGQLFS-NATAVRSYIE  428
A0A177UVE5         GVGANLQEQTNGVLGIPN-GTIPY------GGVGPGNVVAFGSVAQLMPNNVTAVRQAIE  417

ODH                TVGKNLQEQTQNAIGAK-GNGFDP------DGHGPTDAIAFPNIYQVFGSQATSAVQTIQ  373
A0A1B9I658         GVGKNLQEQTMNSVGWTPVDGFDF------DGRGPSDCIAYPDLLGISSSSNNDIASTIS  387
PBK65148           TVGRNLQEQTMNSLGAD-GN-VDY------GGSGPSDAIAYPNIYQVFGDEAAASVSKIQ  395
A0A067ST11         TVGKNLQEQAMSTHGAG-GNGFDE------GGRGPTDVIAYPNLYQIFGSNANATVSRIQ  398

A0A0L1HZM0         SIGANLPDQPQNGMVFSSSTNWTG----------YPTFVSYLTASDLFGEELAGLAEEVR  380
A0A1L9P605         SVGENAIDQPNTFISYSSTKNFTG----------IAPYVTYMTASDVFGPETQRIADEIS  373
A0A1W2TMR5         GVGEYLQEQPNTALVYSGTQNVSG----------FAPYATFSTAGDLFGDETPAIKASTS  384
A0A024S2J7         GVGENLIDQSNVALTYSTKESFPG----------YAPYATFVNATSLFGDGVEALAASTK  380

GDH_A2QM15         TVGENLQDQTNAGLGASTTPGLTG-----------TKTVVYPNVYDVFGNDTLAVAQSVR  381
GDH_4YNU_B8MX95    TVGENLQDQFNNGMAGEGYGVLAG-----------ASTVTYPSISDVFGNETDSIVASLR  378
GDH_G8E4B4         TVGENLQDQINNELIYSPPTNFTSTY-----DSGVGAFVAYPSASHVFGTNESSASEELK  389
GDH_G3YD20         TVGENLQDQTNNALSWEGVDTLTG----------LATFSVLPSVNQLYGDNVTALASYVK  377
                    :*    :*


GOX_A0A1V6T4B5     TKLDQWAEETVARGG--FHNVTALKIQYENYRNWLLDE--DV-AFAELFFDTE-------  439
GOX_1CF3_P13006    TKLEQWAEEAVARGG--FHNTTALLIQYENYRDWIVNH--NV-AYSELFLDTA-------  440
GOX_1GPE_Q9URJ8    TKLDQWAEETVARGG--FHNVTALKVQYENYRNWLLDE--DV-AFAELFMDTE-------  440
GOX_Q92452         TKLDQWAEETVARGG--FHNVTALKVQYENYRNWLLDE--DV-AFAELFMDTE-------  440

A0A139IH18         ASLGDYATMISNRINNS-LSADQILNSLKVQYEAIFDT--PV-PVVELFSGQAI----NN  424
M2ZEM9             ASLGDYATTISNRINNS-LSSDQILNSLQVQYDAIFNT--PV-PVVEIFSGQAV----NN  424
K2RH81             AALPDYAATIAAQNNNA-TSAEELLPLLEIQYTSIFET--GI-TAFELLKGLEF---QP-  424
R1EB07             AALPDYAATIASQNNNA-TSAEDLLPLLEIQHASIFDA--QI-TAFELLKGLEF---QP-  428

A0A139HTB9         SGLQDYANSLEERSL--VANAKATHHILQAQLDNIFNA--TDVAASEIFFTINT----TH  462
A0A1Z5TBL7         ETLPQYCQELQEKGL--VVNATATHTILEAQVNNLFKQ--NA-AASEVFFTVSP----GT  468
A0A074W657         ANLASYAADLEAQGL--VANATATHEILRLQVANLFDQ--SE-AAAEIFFTLAAATDSAP  448
A0A1Y2DT19         ASLPAYAAQLAASGS--VVNATATAQIMRLQIESLFAN--NE-PAAEVFWTIDT----AG  452

A0A0D1DW37         SNLDGWANQLLSQGH--VASKEGVLAQWRSAISLIFDQ--KA-PVVELFFDTGF----PA  463
A0A081CFT4         SNLNAWADTLVSAGH--VASKSGLLAQWTSAVSLIFDQ--AA-PIVEMFFDTGY----PA  454
A0A177U6A2         SNYQNWAQAAVSGGG--AVNTDGLIAQWRLQTEVLFTD--NV-GAVEMFVDSGY----PN  479
A0A177UVE5         SKYTTWARDAVVAGA--AVNSAGLIAQWKLAVSALFDY--NV-GATEFLFTTGF----PT  468

ODH                SSLSAWAKTQAAAGA---LSADALNTIYQTQADLIINH--NA-PVVELFFDSGF----PD  423
A0A1B9I658         DNIAQYAQDAYDAGA--VASVEAANTIFGIQQDLMVNK--NS-GLVEAFFDSGF----PN  438
PBK65148           SSLSSWASSQAG-SA---LSADALEQIYNIQADLIINQ--NA-PIVELFFDSGF----PA  444
A0A067ST11         SSIASWAASQAD-SG---LSANALQQIFQVQADLIINN--NA-PVMELFLDIGY----PD  447

A0A0L1HZM0         ANLSAYAATIVEDYANGTISVETQEHLLSHQVDLIFDSESKV-PLAELLWAPT------G  433
A0A1L9P605         SEIKSWAEQVSADSPGQGFPASAIEHQYRVQHDLIFNQ--DT-AITEILTTSL------G  424
A0A1W2TMR5         AQIPGWAEKVVAASNGA-VKAANVEKLFRIQHDLIFEK--NV-TIGETLTAIF-----GG  435
A0A024S2J7         KSLPIWAQQIAAQTNGV-ISARAIEHRLQVQHDLIFKK--GV-TIAEILSSAS------G  430

GDH_A2QM15         RQLKQWANETAQVSSGT-MKAEDLEALFQLQYDLIFKD--KI-TIAEILYYPG-----ST  432
GDH_4YNU_B8MX95    SQLSDYAAATVKVSNGH-MKQEDLERLYQLQFDLIVKD--KV-PIAEILFHPG-----GG  429
GDH_G8E4B4         SQLTAYADTVAIANGNV-TKASDLLDFFQLQYDLIFKD--QV-PFAEVLIYIA-----KG  440
GDH_G3YD20         SQLASYAKTVASASNGA-VKEANLVEAFERQYDLIFNS--QV-PYTEVVFAPS-----G-  427
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GOX_A0A1V6T4B5        GKINFDIWNLIPFTRGSVHILSSDPYLW----QYANDPKFFMNELDLLGQAAATKLGREL  495
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GOX_1GPE_Q9URJ8       GKINFDLWDLIPFTRGSVHILSSDPYLW----QFANDPKFFLNEFDLLGQAAASKLARDL  496
GOX_Q92452            GKINFDLWDLIPFTRGSVHILSSDPYLW----QFANDPKFFLNEFDLLGQAAASKLARDL  496

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   K2RH81             TRLECEVWSTLPFSRGNVHITTARPPTDGSTPALAINNNYFQLDYDNRAQIAAARFVRNL  484
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     A0A074W657       ATVGADLWNLIVLARGTIHITSNNSFDL-----PEIEPSYFGHPLDLQLQTLATQQAAQV  503
     A0A1Y2DT19       QTVGADIWNLIPLARGTVHVQSDNSWDQ-----PAIDAAYFGHELDTTLQVLATRQARDV  507

     A0A0D1DW37       NSYGIDIWTLLPFSRGSIRATSQNPYDG-----ARIDPNYFGLPIDMDMQVASLRASRRV  518
     A0A081CFT4       NSYGIDLWTLLPFSRGSVRATSGNPFDA-----PRVDPNYFGLPIDMDMQVQSLRAARRV  509
     A0A177U6A2       NGFGVEMWPLLPYSRGSVHTSSASTFAK-----TIVDPRYFSVPFDMDMQVAGCRGVRRV  534
     A0A177UVE5       NDYGIELWPLLPFSRGYVHAVSADAFSN-----ATVNPRYFSVPFDMDLQVASSRAARRI  523

  ODH                 -DVGIVMWPLLPFSRGNVTITSNNPFAK-----PSVNVNYFSVDFDLTMHIAGARLSRKL  477
  A0A1B9I658          GGLGIDLWQLLPFSRGTVKITSTDPFNY-----PSIDPRYFSADVDLKIQIAGLRMARKI  493
  PBK65148            -TLGIDQWQLLPFSRGTVQISTTDPFTQ-----PAVNVNYFSVDWDLDVQIASARLSRTI  498
  A0A067ST11          -TLGMLAWNLLPFSRGNVKITTTDPFTQ-----PQVTVNYYSVDFDLDVQVAGARLARKI  501

A0A0L1HZM0            NQIIAQLWNLLPLSRGSIHINSTDATLP-----PRIDPAFLQLPIDRYVQAAAAIRVRKY  488
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A0A1W2TMR5            EFLVSAMWLLLPFSWGSVHLTSVDAINA-----PAIDPKYFLVDFDLDVQIGLGRTAQKF  490
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GDH_G8E4B4            -SWGAEYWGLLPFSRGSIHISQANSTAG-----ALINPNYFMLDYDVELQVATAKFIRSV  494
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                             *  :  : *                       :     *


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   K2RH81             YALPPLNGSSVLDEIAPGLATV-------PPDA--TDDEWEVWLK-------GQFRSAWH  528
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     A0A074W657       YSTSPLAEMV-DKNILPATLP-----------V--TYEAWETFVK-------DTFTSVWH  542
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     A0A177U6A2       YQTSPVSDLFAAGEEIPGFDVSRGGIPDGPKHG--AFARWQKWIS-------EGYSSVAH  585
     A0A177UVE5       LQGDAFKNISSGPENKPGFKVVP---DDSVNHG--SYATWQAWIS-------KNFGSVAH  571

  ODH                 LGSPPLSSLL-VGETVPGFKTV----PNNGNGG--TDADWKKWIL--KPGNSAGFASVAH  528
  A0A1B9I658          FQTSPLRSIV-TEENAPGYNEV----PENNDGG--SDEDWSNWIL-------NGFSSVHH  539
  PBK65148            LSSPPLSDLS-TGEVIPGD-SV----PDNGERG--TEEDWTSWIT-------NGFAAVSH  543
  A0A067ST11          LTTPPLSSLS-TGETRPGS-AV----PDNASRG--TDAAWKSWIK-------STFDSVAH  546

A0A0L1HZM0            FATSPLADH-ITGELSPGFETV-------PQDAGWRDASWDAWIK-------QTLGGNSH  533
A0A1L9P605            WGTDPVRSL-AGQRLQPAVGDL-------PSNA--TDEALDKWIA-------SSFSANQH  522
A0A1W2TMR5            WNTEPVSDI-VGARVSPTDDDV-------PLNA--TDEQWAGYIS-------DSLTPNHH  533
A0A024S2J7            WTTAPISAV-IEARVDPNSTIL-------PDNA--TDAQWEDFTR-------SSLISNSH  528

GDH_A2QM15            FQSAPLANI-IAEETNPGFEAV-------AANG--SEEDWKAWLL-------TQYRSNFH  530
GDH_4YNU_B8MX95       LRSAPLNKL-IAKETKPGLSEI-------PATA--ADEKWVEWLK-------ANYRSNFH  527
GDH_G8E4B4            FGTGPFASV-AGTETTPGFDVI-------PADA--DEATWKSWAT-------KEYRSNFH  537
GDH_G3YD20            LGTAPLNSL-VGEEVSPGLDVL-------PASA--SSATWTKWVK-------ENYRTNYH  525
                           .       .  *                         :                *
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|GOX_A0A1V6T4B5       AVSTCSMMSRELGGVVDATAKVYGTQGLRVIDGSIPPTQVSSHVMTVFYGMALRIAESVL  597
|GOX_1CF3_P13006      GVGTCSMMPKEMGGVVDNAARVYGVQGLRVIDGSIPPTQMSSHVMTVFYAMALKISDAIL  598
|GOX_1GPE_Q9URJ8      AVSSCSMMSRELGGVVDATAKVYGTQGLRVIDGSIPPTQVSSHVMTIFYGMALKVADAIL  598
|GOX_Q92452           AVSSCSMMSRELGGVVDATAKVYGTQGLRVIDGSIPPTQVSSHVMTIFYGMALKVADAIL  598

   |A0A139IH18        GVGTAAMLPRKWGGVVNGDLKVYGTSNVRVVDASAVPFNMGGHPTSTMYALAERAAHLIL  585
   |M2ZEM9            GIGTAAMLPRKWGGVVDGNLKVYGTSNVRVCDASAVPFNMGGHPTSTMYALAERAAHLIL  585
   |K2RH81            PVGTTSMLPRQNGGVVDAELKVYETANVRIVDAGVFPFQINGHPSATVYAVAERAADIIK  588
   |R1EB07            PVGTTSMLPRAAGGVVDPQLRVYETANVRVVDAGVMPFQVNGHPSATVYAVAERAADMIK  592

     |A0A139HTB9      YVATCAMMKEEFGGVVDSKLKVYGIENVRVVDASVLPIQLSAHLSSSLYGIAEKGAQMVR  620
     |A0A1Z5TBL7      YIATLAMMKEEYGGVVDNRLKIYGIENVRAIDASVLPIQLSAHLSSSLYGIAEKAAAMIK  625
     |A0A074W657      PIATLSMMKEEFGGVVDNKLKVYGLSNVRVVDASVLPVQLSAHLSSSLYGIAEKAAVMIR  602
     |A0A1Y2DT19      PVATLSRMAEQDGGVVDESFRVYGVQNVRVVDASVLPVQLSAHLSSSLYGIALKAAGIIR  610

     |A0A0D1DW37      QLGTAAMGSRSLGAVVDAKFKVYGTSNVRVVDASVLPVQISAHLSSTLYGVAEKAADTIL  632
     |A0A081CFT4      QLGTAAMGSKQNGGVVDGNFKVYGTTNVRVVDASVVPVQLSAHLSASLYGVAEKAADAIL  623
     |A0A177U6A2      PVGTCALLPRQMGGVVDASFKVYGTANVRVVDASTLPQLISAHLSSTLYGIAERAADQIS  645
     |A0A177UVE5      PIATCSLAPRAMGGVVDPNFLVYGTTNVRVVDASTLPMQISAHLSSTLYGIAERAADSIK  631

  |ODH                PIGTAAMMKRSLGGVVDAQLKVYDTTNLRVVDASMMPLQISAHLSSTLYGVAEKAADLIK  588
  |A0A1B9I658         PIATCSMMSEELGGVVGSDLKVYNTENLRIVDASVLPIQFSAHLSATLYGLAENAADM--  597
  |PBK65148           PIGTLAMMKRSLGGVVDSQLKVYDTSNVRVVDASIVPIHISAHLSAGLYGVAEKAADLIK  603
  |A0A067ST11         PIGTAAMMKRSLGGVVDSQLKVYGTTNVRVVDASIMPLQISAHLSSTLYGVAEKAADLIK  606

|A0A0L1HZM0           PLSTCAMMQKDLGGVVDTEGKVYGTQNVRVVDASIFPTQISGHLSASVYAVAGRIAAVMM  593
|A0A1L9P605           LVGTASMLPMEFGGVVDTNLVVYGTENVRVVDASVLPGQNSGHLTSLIYAVAERAADIIK  582
|A0A1W2TMR5           PLGTASMMSRELGGVVDAELKVYGTGNVRVVDASVLPIQFSGHLTATLYAVAERAAEIIA  593
|A0A024S2J7           SLGTAAMMKREWGGVVDSQMRVYGTGNVRVVDASVLPMQVSGHLTATLYAVAERAGEMIV  588

|GDH_A2QM15           PVGTAAMMPQDKGGVVNDRLTVYGTSNVRVVDASVLPFQVCGHLVSTLYAVAERASDLIK  590
|GDH_4YNU_B8MX95      PVGTAAMMPRSIGGVVDNRLRVYGTSNVRVVDASVLPFQVCGHLVSTLYAVAERASDLIK  587
|GDH_G8E4B4           PVATAAMLPKEKGGVVDAQLKVYGTTNVRVVDASVLPFQVCGHLVSTLYAVAEKASDLIK  597
|GDH_G3YD20           PVGTTSMLPREKGGVVSPELKVYGTKNVRVVDASVLPFQLCGHLTSTLYAVAERASDLIK  585
                       :.: :      *.**.    :*   .:*  *..  *    .*  : .*.:* . .


|GOX_A0A1V6T4B5       EDYAKKA-----------------------------------------  604
|GOX_1CF3_P13006      EDYASMQ-----------------------------------------  605
|GOX_1GPE_Q9URJ8      DDYAKSA-----------------------------------------  605
|GOX_Q92452           DDYAKSA-----------------------------------------  605

   |A0A139IH18        TGEKG-------------------------------------------  590
   |M2ZEM9            TGEKG-------------------------------------------  590
   |K2RH81            QSFSAEIEIFNWHLTISTQNHQPLKVKPPVDISKPRNEELFSQTCLAT  636
   |R1EB07            ESFAGGGVGASGGTEGSG-TGTPGA-AARMGRVKGRVVRL--------  630

     |A0A139HTB9      LDWGGE------------------------------------------  626
     |A0A1Z5TBL7      EDQKGFREQPWAHQH---------------------------------  640
     |A0A074W657      EDQI--------------------------------------------  606
     |A0A1Y2DT19      EDQ---------------------------------------------  613

     |A0A0D1DW37      AR----------------------------------------------  634
     |A0A081CFT4      AK----------------------------------------------  625
     |A0A177U6A2      ASQ---------------------------------------------  648
     |A0A177UVE5      AAQT--------------------------------------------  635

  |ODH                AAQ---------------------------------------------  591
  |A0A1B9I658         ------------------------------------------------  597
  |PBK65148           ASY---------------------------------------------  606
  |A0A067ST11         ASY---------------------------------------------  609

|A0A0L1HZM0           KAVTMK------------------------------------------  599
|A0A1L9P605           ETL---------------------------------------------  585
|A0A1W2TMR5           GAV---------------------------------------------  596
|A0A024S2J7           GDRSGL------------------------------------------  594

|GDH_A2QM15           ADSALF------------------------------------------  596
|GDH_4YNU_B8MX95      EDAKSA------------------------------------------  593
|GDH_G8E4B4           AAA---------------------------------------------  600
|GDH_G3YD20           ESY---------------------------------------------  588
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Figure S7. Close-up view of the active site flavin. Protein and FAD carbon atoms are shown in wheat and yellow respectively, O atoms in red, N atoms in blue. **a** Interactions between the isoalloxazine ring and the protein. The isoalloxazine ring is rigidly fixed through a dense network of hydrogen bonds. Most contacts involve the protein backbone except for Ser573, which stabilizes the cofactor also with its side chain. Ser573 is modelled in double conformation: one conformer interacts with the flavin system, the other interacts with Asn97 side chain. Asn97 is located on the *si*-face of the isoalloxazine ring, while the two catalytic histidines, His528 and His571, lie on the *re*-face: the orientation of their imidazole ring is due to hydrogen bonding with Gln329 and Glu414 respectively. **b** Residues facing the active site. Except for Gln331, most of the residues directly facing the active site possess aromatic side chains: Tyr64, Phe416 and Trp430. Water molecules are depicted as red spheres. Hydrogen bonds (distance < 3.2 Å) are represented with a dotted line.

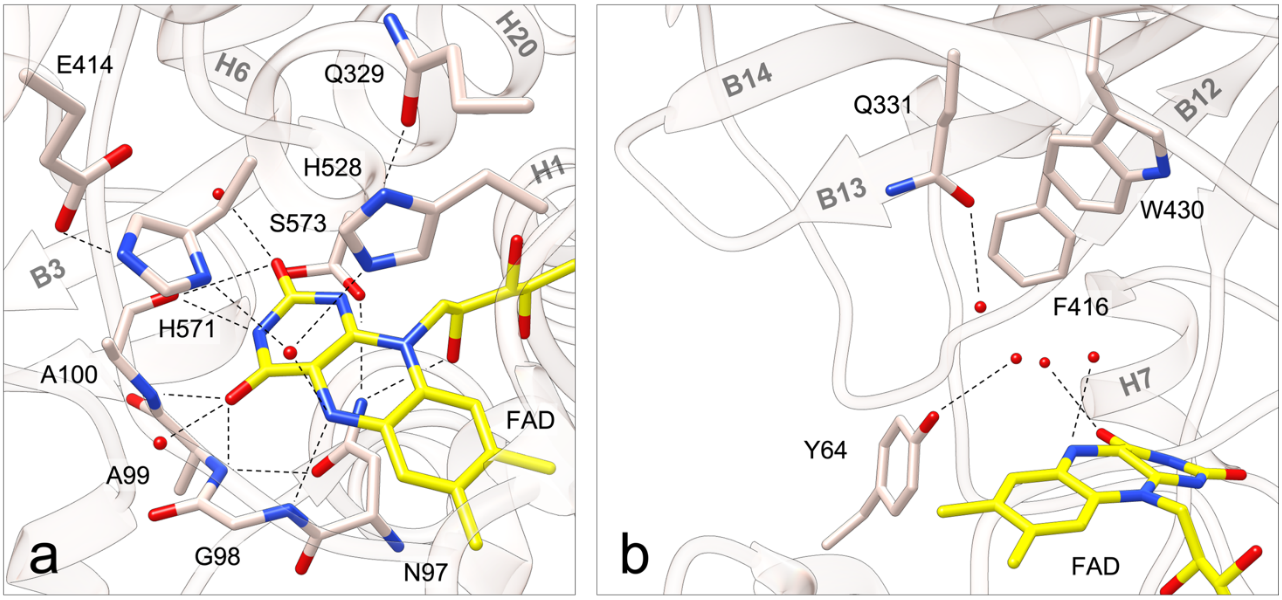


Figure S8. Details of the two anomeric forms of glucose (GLC) and laminaribiose (G3G) bound to ODH active site, **a** Top view (perpendicular to the isoalloxazine plane) and **b** side view of β-G3G in ODH-G3G; **c** top view and **d** side view of α-G3G in ODH-G3G; the protein is shown in green, the FAD cofactor in yellow, G3G in pink (β anomer) or magenta (α anomer). **e** Top view and **f** side view of β-GLC in ODH-GLC; **g** top view and **h** side view of β-GLC in ODH-GLC; the protein is shown in light blue, the FAD cofactor in yellow, GLC in orange (β anomer) or gold (α anomer); O atoms are in red, N atoms in blue. Water molecules are depicted as red spheres. Hydrogen bonds (distance < 3.2 Å) are represented with dotted lines.

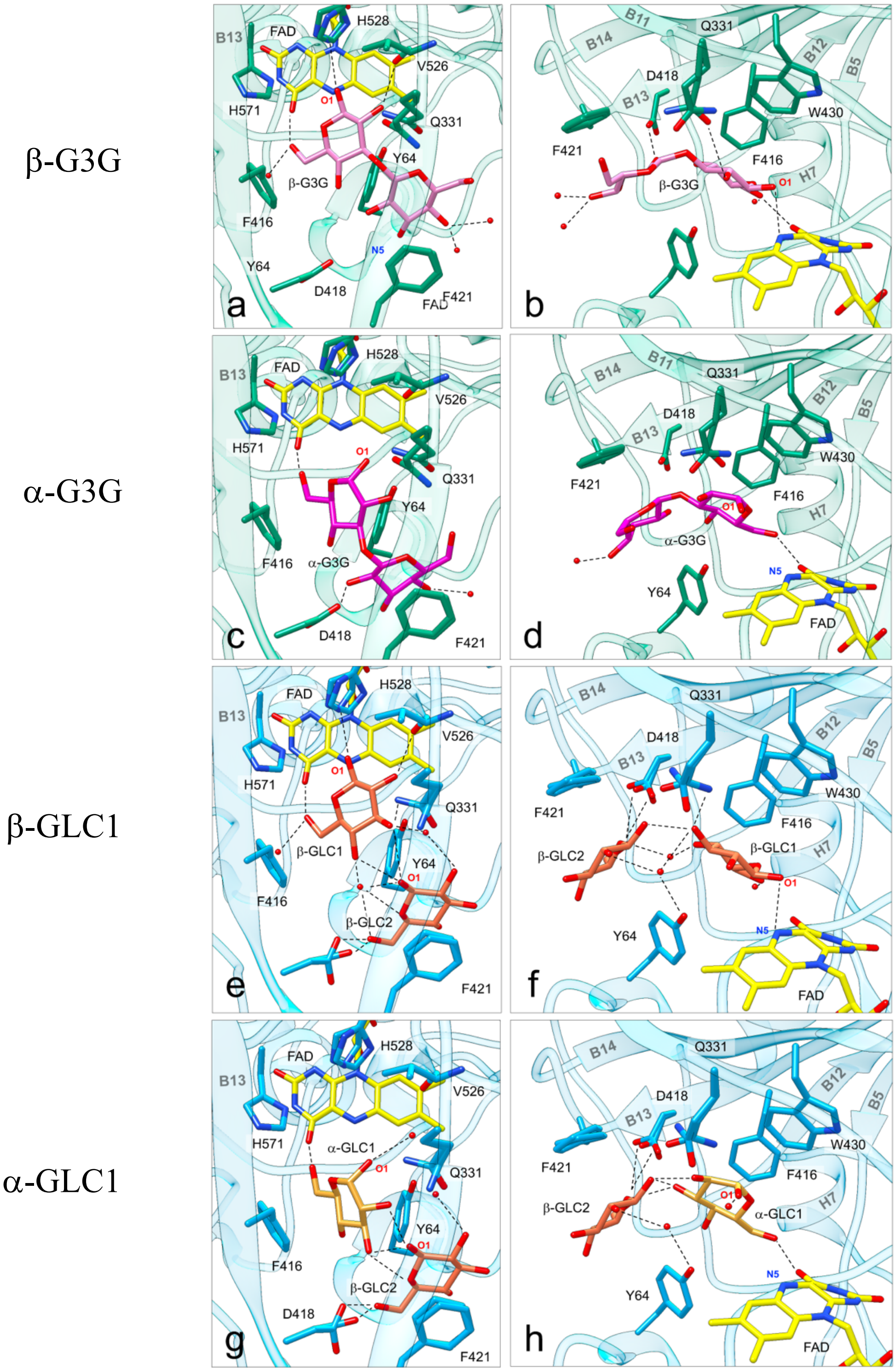
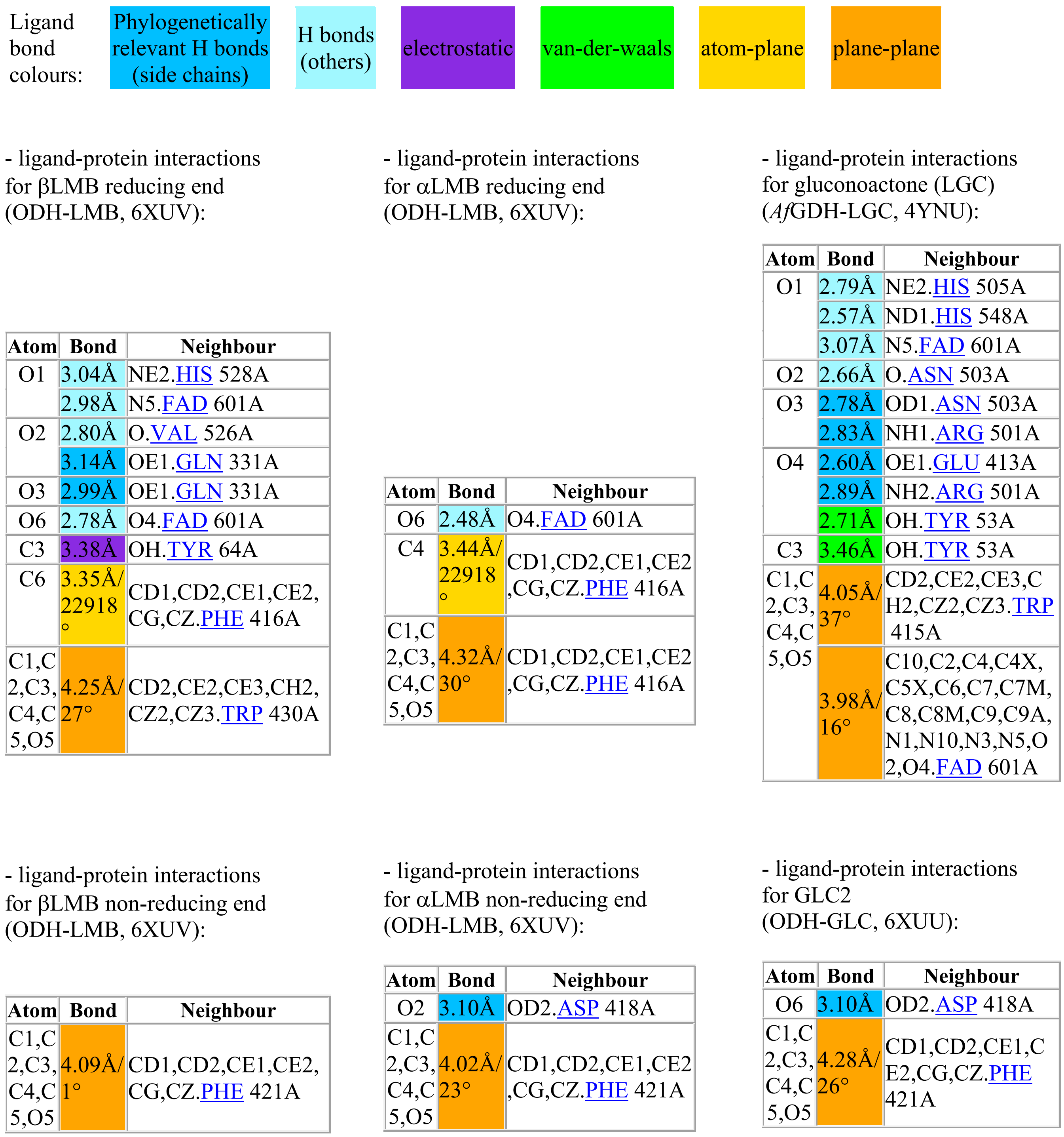


Figure S9. PDBe Motif analysis of protein-ligand interactions in ODH-G3G, ODH-GLC and *Af*GDH-LGC. Comparison of the simplified PDBe outputs for: i) (upper panel) β- and α-LMB reducing end pyranose in ODH-G3G (pdb: 6XUV) and gluconoactone (LGC) in *Af*GDH-LGC (4YNU); ii) (lower panel) β- and α-LMB non-reducing end pyranose in ODH-G3G (pdb: 6XUV) and GLC2 in ODH-GLC (pdb: 6XUU). As for the reducing end of β- and α-G3G, similar results were obtained for α- and β-GLC1 in ODH-GLC (not shown). Colors represent different bond types. Atom pairs, distances and eventually angles are reported. No van-der-waals interactions have been shown, apart from two crucial ones between Tyr53 hydroxyl and LGC in *Af*GDH-LGC (for more details see Additional file 2, Section 4).



Ligand bond colours: Phylogenetically relevant H bonds (side chains) | H bonds (others) | electrostatic | van-der-waals | atom-plane | plane-plane

- ligand-protein interactions for βLMB reducing end (ODH-LMB, 6XUV):

Atom	Bond	Neighbour
O1	3.04Å	NE2.HIS 528A
	2.98Å	N5.FAD 601A
O2	2.80Å	O.VAL 526A
	3.14Å	OE1.GLN 331A
O3	2.99Å	OE1.GLN 331A
O6	2.78Å	O4.FAD 601A
C3	3.38Å	OH.TYR 64A
C6	3.35Å/22918°	CD1,CD2,CE1,CE2,CG,CZ.PHE 416A
C1,C2,C3,C4,C5,O5	4.25Å/27°	CD2,CE2,CE3,CH2,CZ2,CZ3.TRP 430A

- ligand-protein interactions for αLMB reducing end (ODH-LMB, 6XUV):

Atom	Bond	Neighbour
O6	2.48Å	O4.FAD 601A
C4	3.44Å/22918°	CD1,CD2,CE1,CE2,CG,CZ.PHE 416A
C1,C2,C3,C4,C5,O5	4.32Å/30°	CD1,CD2,CE1,CE2,CG,CZ.PHE 416A

- ligand-protein interactions for gluconoactone (LGC) (*Af*GDH-LGC, 4YNU):

Atom	Bond	Neighbour
O1	2.79Å	NE2.HIS 505A
	2.57Å	ND1.HIS 548A
	3.07Å	N5.FAD 601A
O2	2.66Å	O.ASN 503A
O3	2.78Å	OD1.ASN 503A
	2.83Å	NH1.ARG 501A
O4	2.60Å	OE1.GLU 413A
	2.89Å	NH2.ARG 501A
	2.71Å	OH.TYR 53A
C3	3.46Å	OH.TYR 53A
C1,C2,C3,C4,C5,O5	4.05Å/37°	CD2,CE2,CE3,CH2,CZ2,CZ3.TRP 415A
	3.98Å/16°	C10,C2,C4,C4X,C5X,C6,C7,C7M,C8,C8M,C9,C9A,N1,N10,N3,N5,O2,O4.FAD 601A

- ligand-protein interactions for βLMB non-reducing end (ODH-LMB, 6XUV):

Atom	Bond	Neighbour
C1,C2,C3,C4,C5,O5	4.09Å/1°	CD1,CD2,CE1,CE2,CG,CZ.PHE 421A

- ligand-protein interactions for αLMB non-reducing end (ODH-LMB, 6XUV):

Atom	Bond	Neighbour
O2	3.10Å	OD2.ASP 418A
C1,C2,C3,C4,C5,O5	4.02Å/23°	CD1,CD2,CE1,CE2,CG,CZ.PHE 421A

- ligand-protein interactions for GLC2 (ODH-GLC, 6XUU):

Atom	Bond	Neighbour
O6	3.10Å	OD2.ASP 418A
C1,C2,C3,C4,C5,O5	4.28Å/26°	CD1,CD2,CE1,CE2,CG,CZ.PHE 421A

Figure S10. Surface representation of ODH substrate-binding cavity in ODH-GLC (6XUU). Four GLC molecules bind to this region, numbered according to their proximity to the active site FAD. They seem to be caught in the diffusion process from the bulk to the flavin system, with GLC4 and 3 binding to the funnel-shaped opening to the solvent of ODH active site, while GLC2 and 1 bind close to the FAD cofactor within a V-shaped tunnel. ODH surface is shown in light blue, GLC molecules in orange (C atoms) and red (O atoms) sticks.

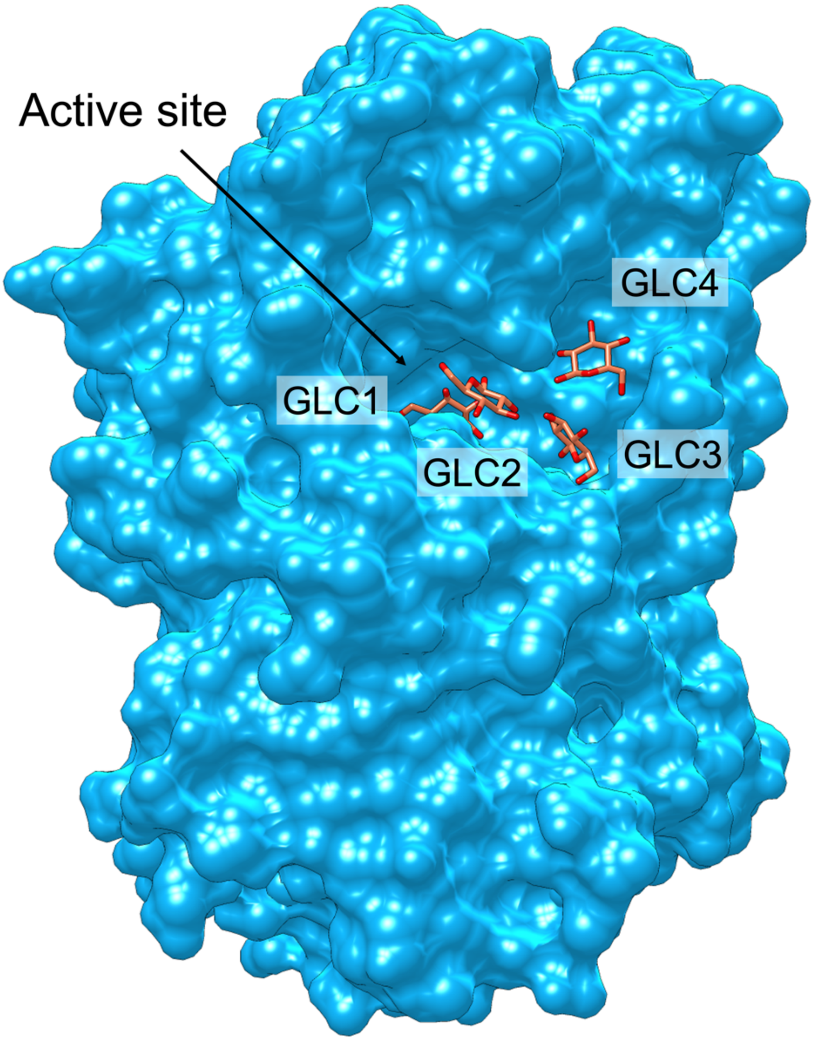


Figure S11. External sugar-binding sites (SBSs) in ODH-GLC (6XUU). All three sites are located on the convex face of ODH, whose surface is represented in the center and colored in light blue. SBSs are numbered according to their proximity to the active site entrance, located on the concave face of the protein (not visible). The three insets show the detail of ODH-ligand interactions. GLC molecules and the surrounding protein atoms are represented as orange and light blue sticks respectively (C atoms); O atoms are in red, N atoms in blue and water molecules are depicted as red spheres. Hydrogen bonds (distance <3 .2 Å) are represented with dotted lines. The $2F_o$-F_c electron density map is also depicted for the GLC molecules and contoured at 1σ.