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Evolution of Enzymatic Activities of Testis-Specific Short-Chain Dehydrogenase/Reductase in *Drosophila*

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Abstract The testis-specific gene Jingwei (*jgw*) is a newly evolved short-chain dehydrogenase/reductase in *Drosophila*. Preliminary substrate screening indicated that JGW prefers long-chain primary alcohols as substrates, including several exotic alcohols such as farnesol and geraniol. Using steady-state kinetics analyses and molecular docking, we not only confirmed JGW's substrate specificity, but also demonstrated that the new enzymatic activities of JGW evolved extensively after exon-shuffling from a preexisting enzyme. Analysis of JGW orthologs in sister species shows that subsequent evolutionary changes following the birth of JGW altered substrate specificities and enzyme stabilities. Our results lend support to a general mechanism for the evolution of a new enzyme, in which catalytic chemistry evolves first followed by diversification of substrate utilization.

Keywords Enzyme evolution · Short-chain dehydrogenase/reductase · *Drosophila* alcohol dehydrogenase · Exon-shuffling · *Drosophila*

Abbreviations

JGW	Jingwei
SDR	Short-chain dehydrogenase/reductase
ADH	Alcohol dehydrogenase

Introduction

Drosophila alcohol dehydrogenase (ADH) is an NAD(P)-dependent short-chain dehydrogenase/reductase (SDR), a family of enzymes that are structurally and biochemically distinct from other ADHs (Benach et al. 2001). Diverse in sequence and function (Oppermann et al. 2003), SDRs share a common protein fold consisting of a central β-sheet surrounded by α-helices (β-α-β-α-β) with an NAD(P)-coenzyme binding site in the N-terminal domain containing a typical Gly-Xaa-Gly-Xaa-Xaa-Gly motif (Benach et al. 2001; Jornvall et al. 1995). The active site contains a Ser-Tyr-Lys catalytic triad and in *D. melanogaster* ADH, Asp38 confers its specificity towards NAD. Although it has been suggested that some SDRs arose through gene fusion of a common ancestral coenzyme binding domain with various substrate-specific domains (Benach et al. 2001), little is known about the evolution of the biochemical characteristics of SDRs, despite their biological importance. *Jingwei*

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(*jgw*) is a recently evolved gene that provides an opportunity to explore the evolution of new enzyme functions (Kaessmann et al. 2009; Ladenstein et al. 2008).

Jingwei is a newly evolved member of SDR found in two closely related African *Drosophila* species, *D. teissieri*, and *D. yakuba*, but not in *D. melanogaster*. *Jgw* was created when a processed alcohol dehydrogenase messenger RNA was reverse-transcribed and inserted into the third intron of a gene called *Yellow emperor* (*Ymp*) (Long et al. 1999; Wang et al. 2000). Unlike ancestral ADH, which is expressed mainly in the fat body and digestive tract, *D. teissieri* JGW is expressed in testes, while *D. yakuba* JGW shows a divergent pattern of expression (Long and Langley 1993). Whereas the ancestral ADH predominantly uses ethanol as substrate, JGW prefers long-chain primary alcohols as substrates, including several exotic alcohols such as farnesol and geraniol (Zhang et al. 2004). These observations suggest that JWG has evolved a novel function, possibly in hormone and pheromone biosynthesis/degradation (Zhang et al. 2004). Here, we use enzymatic kinetics analysis and molecular docking to further characterize the structural and functional consequences of the evolutionary changes accumulated in the *Jingwei* lineages. The results show that *jingwei* has evolved new functions in substrate specificity.

Materials and Methods

Structural Analysis

Molecular modeling was performed on a Dell E1505. PyMOL (http://www.pymol.org/) and CCP4 (http://www.ccp4.ac.uk/index.php) were used to generate a primary model of JGW and to verify all model structures that were derived from this primary model. Molecular graphics were created with PyMOL software. The 3D structures of *D. teissieri* and *D. yakuba* were constructed with MacroModel™ (Schrödinger), using crystal structures of *D. melanogaster* [PDB 1mg5 (Benach et al. 2005)] and *D. lebanonensis* (PDB 1a4u, Benach, et al. 1999) ADHs as templates. When needed, NAD^+ was modeled using coordinates from the crystal structures of the binary and ternary enzyme complexes (PDB ID 1mg5 and 1b14). Amino acid numbering follows that for *D. melanogaster* ADH which contains 255 amino acids, as do the JGWs of *D. teissieri* and *D. yakuba*. *D. lebanonensis* ADH is one amino acid shorter at the N terminus (Benach et al. 1998; Chambers 1991). For mutational and dynamics simulations, models were refined using Polak-Ribiere conjugate gradient minimization and stochastic dynamics routines in MacroModel™ (Schrödinger). Ten thousand steps of steepest decent energy minimization were carried out using the OPLS_2005 force field, followed by a series of alternating short (1 ps) stochastic simulations and additional energy minimization steps to equilibrate the system. The refined structures underwent protein preparation and grid refinement before being docked with ligand libraries using Glide™ (Schrödinger). Two libraries were used, one containing 1-butanol, 2-butanol, isopropanol, and ethanol, and the other containing geraniol, farnesol, 2-heptanol, 2-pentanol, 3-pentanol, and 2-butanol.

Jgw Cloning and Protein Expression

We obtain recombinant JGWs using the *pGEX* protein expression and purification system (Amersham Pharmacia). The *gst-jgw* fusion genes were sequenced to ensure that they were in-frame. The *gst-jgw* expression plasmids were transformed into host bacteria BL21 or BL21-CodonPlus-RIL (Stratagene), taking into account of the special codon usage in *jgw* (Zhang et al. 2005). The resulting transformants were grown overnight at 25°C in 2YT-G medium supplemented with 100 mg/ml of ampicillin (additional 34 μg/ml chloramphenicol for BL21-CodonPlus-RIL). The overnight culture was diluted 10 times in fresh medium and grown for additional 3 h before adding IPTG to a final concentration of 0.1 mM. After 5 h induction, cells were harvested by 10 min centrifugation at $5{,}000\times g$. The cell pellet was washed, re-suspended in extraction buffer, and lysed by sonication. After centrifugation at $20{,}000\times g$ for 15 min at 4°C to remove cell debris, the supernatant was incubated with Glutathione Sepharose 4B (Amersham Pharmacia manual). The GST-JGWs were then purified according to the manufacture's instructions.

Large-scale production of JGW protein was conducted in a 20-l bacteria fermenter. Briefly, JGW expression was induced by adding IPTG to a final concentration of 0.5 mM at $OD_{600} = 1.8$. After further incubation for 12–15 h at 25°C, cells were harvested by centrifugation at $6{,}500\times g$ for 15 min at 4°C. Cell pellets were resuspended in 4°C extraction buffer (1 g/ml) and lysed using a French press operated at 16,000–18,000 lb/in^2 with the cell suspension chilled to 4°C after each pass. After centrifugation, Triton X-100 (1%, final concentration), and dithiothreitol (0.5 mM DTT, final concentration) were added to the supernatant to solubilize the protein. Protein purification then followed the small-scale protocol described above.

Protein preparations were judged pure as determined by SDS-PAGE, and no bands other than the desired GST-JGW or GST-ADH were observed. Protein concentrations were determined using the Bio-Rad protein assay kit, based on the method of Bradford (Bio-Rad). Enzyme activities were retained over several freeze–thaw cycles demonstrating that preparations were stable.

Kinetic Measurements

Kinetic measurements of ADH activity were performed at 25°C in 1 ml of reaction buffer (0.025 M MOPS pH7.4 or 0.025 M CHES pH 9.4 and 0.1 M NaCl), various concentrations of alcohols, and NAD^+ at a fixed concentration of 500 μM (Winberg et al. 1986). Reactions were started by adding enzyme and the initial rate of NADH formation was measured at 340 nm with a Cary 300 UV–Vis spectrophotometer (Varian). All the experiments were performed in duplicate. Deviations from averages were less than 5%. K_m and V_{max} were calculated by Lineweaver–Burk plot as Winberg et al. (1982). The substrate specificity of each alcohol dehydrogenase was determined by comparing the rate of reaction with 1 mM of a given alcohol to that with 1 mM ethanol.

The enzyme active-site concentrations (e) were obtained in the titration experiments according to Winberg et al. (1986). The rate assay was carried out at 25°C, with a varied concentration of enzyme and a fixed concentration of 0.5 mM NAD and 100 mM alcohol in a total volume of 1 ml of 0.025 M MOPS pH 7.4 and 0.1 M NaCl. The velocity v (ΔA 340 nm/min) is a linear function of the active-site concentration in the reaction. As $v = k{\cdot}e$, the rate constant k (ΔA 340 nm/min/μM enzyme active sites) was obtained from a plot of v against e.

Inactivation of JGW by Guanidine-HCl or Tween-20 was determined by measuring its remaining activity after incubating JGW with various concentrations of each detergent for 15 min at 25°C using ethanol as substrate. The reaction buffer was the same as for kinetic measurements. In all the assays, the reaction rate was linear for at least 5 min.

Results

Changes During JGW Evolution

To explore the effects of amino acid replacements accumulated during the evolution of JGW, we modeled the structure of JGWs based on orthologous ADHs from *D. melanogater* (PDB ID 1mg5, Benach et al. 2005) and *D. lebanonensis* (PDB ID 1a4u, Benach et al. 1999). JGW evolution proceeded through three stages: *jgw*I (when *adh* first inserted into *Ymp*), *jgw*II (just before the split between *D. teissieri* and *D. yakuba*), and current *jgw* (Long and Langley 1993). Distinct amino acid substitution patterns were observed for each of the three evolutionary stages (see Table 1 for evolutionary amino acid changes, Fig. 1 for 3-D representation, and Supplemental Figure 1 for secondary structure and amino acid sequence alignment). Three changes are worth noting on the *jgw*I to *jgw*II lineage: Glu41Lys, His191Gln, and Glu205Lys. Glu41Lys replaces a negatively charged carboxyl with a positively charged amine at a position close to Asp38, which is involved in the coenzyme (NAD) binding (Benach et al. 1998, 2001). Glu41Lys might therefore alter coenzyme affinity. Replacements His191Gln and Glu205Lys are in the loop formed from residues 187–211 that control substrate access to the catalytic center (Benach et al. 1998, 2001). In particular, the Glu205Lys replacement causes changes in charge, ion pairing, and hydrogen bonding. These changes may influence substrate specificity. The remaining amino acid replacements in the *jgwI–jgwII* lineage are typical of many proteins, most of which are either distant from the active site (the Ser-Tyr-Lys catalytic triad) or lie on the protein surface where they are less likely to impact folding and function (Bustamante et al. 2000; Dean et al. 2002). One change in the hydrophobic core, Leu120Met, is structurally conservative.

The distributions of replacements differ in the three lineages, *jgwI* to *jgwII*, *jgwII* to *D. teissieri,* and *jgwII* to *D. yakuba*. Five of nine changes on the *jgwI–jgwII* lineage lie within 20 Å of the catalytic hydroxyl of Ser138, whereas only two of 19 changes occurred within this range on the subsequent *jgwII–D. teissieri* and *jgwII–D. yakuba* lineages ($P < 0.025$, Fisher's exact test). Moreover, most of the 19 replacements involve surface residues far from the catalytic triad (Ser138-Tyr152-Lys155) and are physiochemically conservative (polar for polar, hydrophobic for hydrophobic, etc.,). One notable change in the *D. teissieri* lineage is Arg103Ala, a potential contact site with NAD^+. This replacement may influence NAD^+ binding. The nature and distributions of amino acid replacements in the three evolutionary lineages of JGW are consistent with the notion that substrate specificity evolved early in JGW evolution followed by further functional refinements in the subsequent lineages leading to the modern JGW enzymes.

Enzyme Function

To further probe the substrate-binding cavity of JGW, we performed enzyme assays to examine the catalytic behavior of JGW. The ratio of k_{cat}/K_M is often taken as measure of catalytic efficiency (Albery and Knowles 1976). The k_{cat}/K_M values of TJGW (*D. teissieri*) and YJGW (*D. yakuba*) reveal that JGWs are similar in many respects to ADH: all have a strong preference for long-chain secondary alcohols over short-chain primary alcohols (Fig. 2; e.g., 2-butanol is a better substrate than 1-butanol), none use methanol as a substrate (interactions with substrate alkyl groups are important for catalysis), and none use 3-hydroxybutyrate as a substrate (although substituting the carboxyl group with an amine restores catalytic activity—e.g., all oxidize 2-hexanolamine). Unlike ADH, however, both JGWs

Table 1 Amino acid substitutions between current ADH derived domain of JGW and their corresponding ancestral stage I and II

Evolutionary stage	Sites	Replacement			Position	Secondary structure	Ion pair of DM-ADH	Ion pair of DT-JGW	Ion pair of DY-JGW	H-bond of DM-ADH	H-bond of DT-JGW	H-bond of DY-JGW
Ancestral JGW I												
Current JGW	41	E	→	K	Surf	–	0	0	0	1	2	2
	120	L	→	M	Core	α5	0	0	0	2	1	0
	121	D	→	E	Surf	α5	1	1	1	0	2	1
	128	G	→	C	Surf	–	0	0	0	5	0	0
	191	H	→	Q	Surf	–	0	0	0	1	1	1
	205	E	→	K	Surf	α7	2	1	1	1	2	2
	215	S	→	P	Surf	α8	0	0	0	0	0	0
	234	I	→	L	Surf	β9	0	0	0	0	1	0
	246	Q	→	K	Surf	–	0	0	0	1	2	2
Ancestral JGW II												
Current DT-JGW	1	S	→	A	Surf	–	0	0		0	2	
	5	T	→	S	Surf	T	0	0		2	0	
	10	L	→	I	Core	β1	0	0		1	0	
	52	I	→	L	Surf	α2	0	0		2	1	
	57	T	→	C	Surf	β3	0	0		3	0	
	86	V	→	I	Core	–	0	0		1	1	
	248	S	→	C	Surf	–	0	0		1	0	
Current DY-JGW	1	S	→	A	Surf	–	0		0	0		3
	3	T	→	S	Surf	–	0		0	1		1
	5	T	→	S	Surf	T	0		0	2		0
	27	L	→	V	Core	–	0		0	2		0
	57	T	→	A	Surf	β3	0		0	3		0
	58	V	→	I	Core	β3	0		0	0		2
	74	K	→	E	Surf	α3	0		0	1		1
	78	T	→	C	Surf	α3	0		0	0		0
	81	A	→	S	Surf	α3	0		0	2		1
	82	Q	→	R	Surf	α3	0		3	2		1
	88	V	→	I	Core	β4	0		0	0		0
	103	R	→	A	Surf	α4	3		0	2		0

JGW is short for *Jingwei* protein

DM D. melanogaster, DT D. teissieri, DY D. yakuba

exhibit a higher specificity for diols. We conclude that JGW is a new member of SDR family with an evolved substrate specificity distinct from the parental ADH, and that these changes in substrate specificity arose through amino acid replacements outside the active site.

It has been reported that *Drosophila* alcohol dehydrogenases also oxidize aldehydes in certain buffer systems, though the kinetic values of aldehyde oxidation are not comparable to those for alcohol dehydrogenation (Heinstra et al. 1989; Henehan et al. 1995; Winberg and McKinley-McKee 1998). Four aldehydes: formaldehyde, formamide hexanal, benzaldehyde, and 4-methoxyl were tested for possible aldehyde dehydrogenase activity. No increase in A_{340} was observed at neutral pH 7.4 (Data not shown), a result consistent with a previous study that detected aldehyde dehydrogense activity only at high pH 9–10 (Winberg and McKinley-McKee 1998).

pH Effects and the JGW Active Site

The pH profiles of enzymes provide insights on the amino acid residues likely involved in catalysis. The optimal pH for JGW oxidizing ethanol and isopropanol lies above 9.5 (Fig. 3), consistent with the notion that an ionized Tyr152 (pKa = 10) is involved in catalysis (Benach et al. 1999, 2005). In ADH, both coenzyme and alcohol binding are strongly influenced by pH in the range 6–10 as a result of Tyr152 ionization (Winberg and McKinley-McKee 1988). Cysteine has a pK_a which is similar to that of Tyr. Site-directed mutagenesis in Drosophila ADH shows that

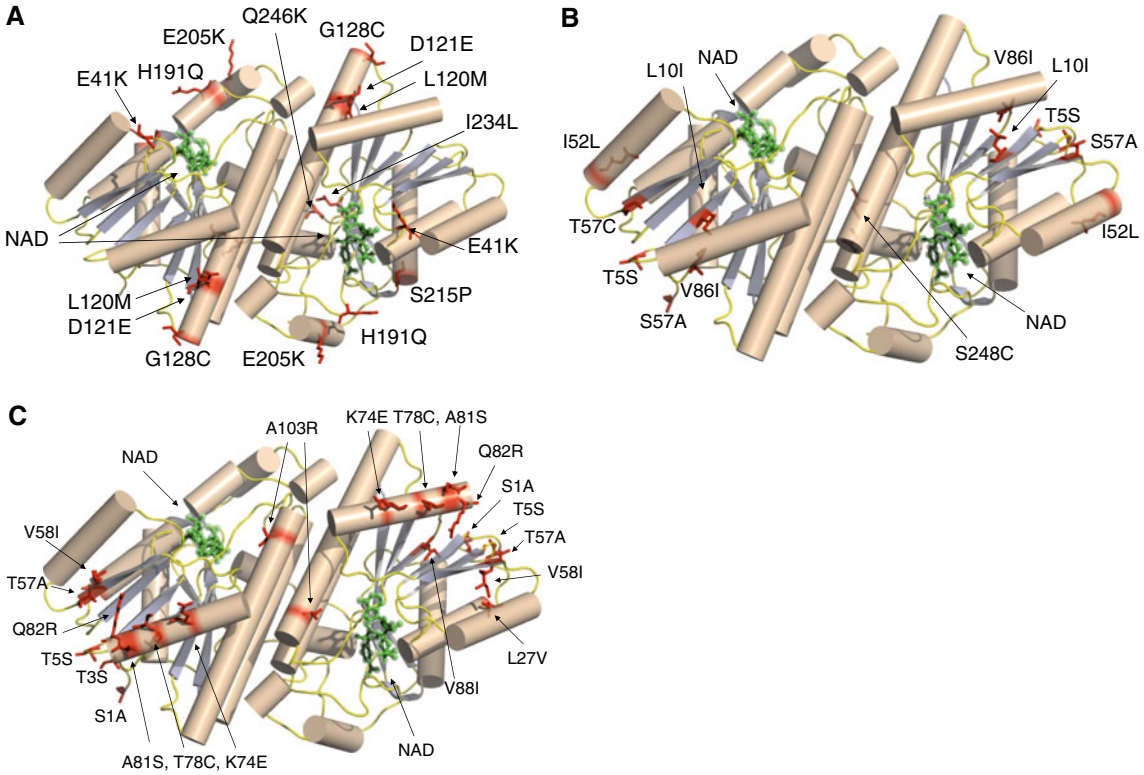


Fig. 1 Three-dimensional representation of the evolutionary amino acid replacements in the ADH derived domain of JGW. **a** Changes happened from JGW I to JGW II, **b** for JGWII to DT-JGW (*D. tessieri JGW*), and **c** for JGWII to DY-JGW (*D. yakuba JGW*)

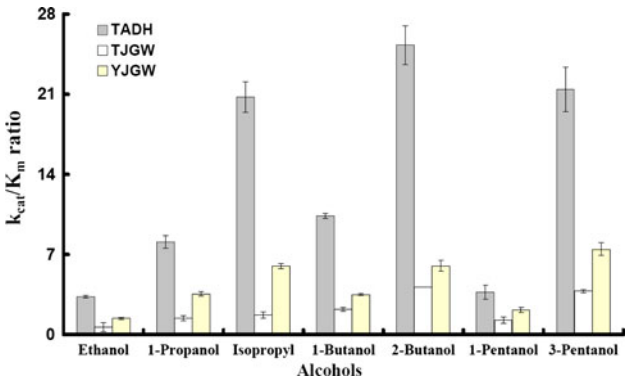
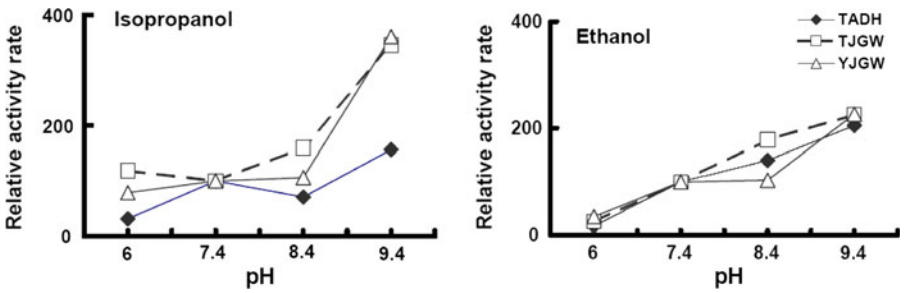


Fig. 2 The catalytic efficiency of JGWs to convert short-chain primary and secondary alcohols. *TADH D. teissieri* Alcohol dehydrogenase, *TJGW D. teissieri* JGW protein, and *YJGW D. yakuba* JGW protein

neither Cys135 nor Cys218, both of which are buried in the hydrophobic core, is directly involved in catalysis (Chen et al. 1990). The pH optimum is similar to that of ADH (Juan and Gonzalez-Duarte 1981; Winberg and McKinley-McKee 1992), and we infer that the catalytic properties JGW are broadly similar to those of ADH.

Nevertheless, higher pHs affect JGW activities far more than ADH activities, when isopropanol is used as a substrate (Fig. 3). The fact that the *D. teissieri* and *D. yakuba* JGWs exhibit a similar profile in response to changes in pH suggests that the amino acid substitutions responsible occurred on the *jgwI–jgwII* lineage before the two species diverged. Thus, when compared to ADH, the active sites of both JGWs are conserved but the molecular environment and electrostatic properties surrounding the catalytic residues differ subtly.

Fig. 3 The pH effects on the enzyme activities of ADH and JGWs. The enzyme activity at pH 7.4 was set to 100% as the control



Stability Analysis

To form a stable dimer is important for Drosophila ADH to optimize its catalytic function. It has been noticed that mutation Alal59Thr disrupts ADH function by interfering the dimer stability (Chenevert et al. 1995). To investigate the functional impact of amino acid changes outside of the active site, we monitored JGW activity in the presence of guanidine-HCl and Tween 20. The ionic detergent guanidine-HCl denatures protein structures (Pace 1990). The nonionic surfactant Tween 20 forms micelles in solution, helping protein folding at low concentrations but destabilizing protein structures at high concentrations (Scopes 1996). As shown in Fig. 4, we found that increasing concentrations of guanidine-HCL and Tween20 reduced JGW activity, with *D. teissieri* JGW more sensitive than *D. yakuba* JGW and *D. teissieri* ADH.

Homology Modeling of JGWs

To explore the structural basis of the evolved preference for long-chain primary alcohols as substrates, including several exotic alcohols such as farnesol and geraniol (Zhang et al. 2004), we constructed a 3D structural model of JGW. The ADH derived domains of JGWs at the current and the two ancestral stages were homology-modeled using multiple sequence and structural alignments of ADHs of *D. teissieri, D. yakuba, D. melanogaster,* and *D. lenbanonesis* (Supplementary Figure 1). The *D. melanogaster* ADH–NADH–acetate complex was chosen as the template for modeling the Michaelis complexes.

Homology-modeling and energy minimization of the ADH domain of JGW generates a structure whose Ramachandran map shows 1.1% residues in generous and 0.4% residues in outside regions of the (φ, ψ) map (including Gly and Pro) out of a total of 254 residues. About 97% of the residues are in the allowed and core regions (Supplement Figures 2 and 3) based on an analysis of 118 structures of at least 2.0 Angstroms resolution with a free R-factor no greater than 20%. No severe clashes between side chains were observed in the model after the last round of model building. Our current JGW model is similar in quality to previously published models (PDB ID 1mg5) (Benach et al. 2005).

The hydrophobic cavity of Drosophila ADH is formed with an active-site loop composed of residues 182–215 (connecting βF and αG) that is disordered in the apo form but forms a short helix in the ternary form (from 187 to 192), a small helix (α1), and Trp251 from the C-terminal region of the other subunit. The catalytic triad (Ser138, Tyr152, and Lys156) is conserved in all SDRs (Jornvall et al. 1995; Winberg et al. 1999). Amino acid replacements in *jgw* lineages do not change the apolar nature of the active-site cavity which discriminates against multisubstituted alcohols, such as sugars or polyols (Data not shown).

Simulated Substrate Docking

We next used molecular modeling of two JGWs to investigate substrate binding. Alcohols were docked directly in the active sites of *D. teissieri* JGW and *D. yakuba* JGW next to NAD^+. Two libraries were used: the first one contained 1-butanol, 2-butanol, isopropanol, and ethanol; and the second one contained geraniol, farnesol, 2-heptanol, 2-pentanol, 3-pentanol, and 2-butanol. The top hit in the first library was 2-butanol with docking scores of −4.86 and −5.86 kJ/mol for TJGW and YJGW, respectively (Table 2). The docking score is the predicted energy associated with binding. The lower the value of the score, the better the ligand binds to the protein. Simulations show that alcohols form H-bonds with Tyr152 (always seen) and Ser139 or Lys155 (sometimes seen) of the catalytic triad. As shown in Table 2, geraniol (a branched unsaturated C9 alcohol) had the best docking scores of −7.79 and −7.66 kJ/mol for TJGW and YJGW, respectively, followed by farnesol (a branched unsaturated C15 alcohol) with compatible scores of −7.83 and −6.78 kJ/mol. In general, the results from simulated substrate docking confirmed our experimental observation that longer-chain alcohols bound both TJGW and YJGW more tightly than short-chain alcohols (Table 2).

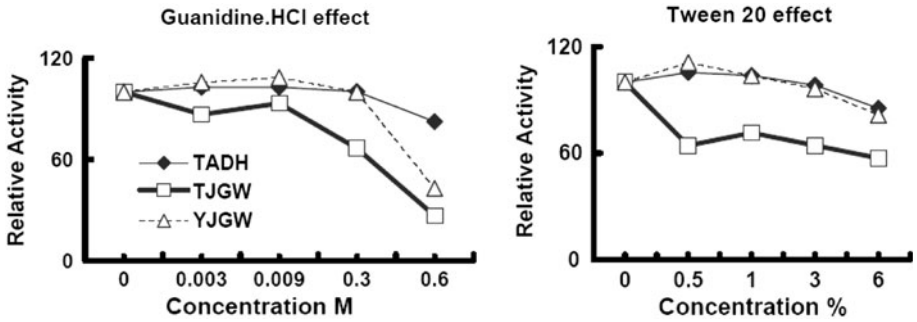


Fig. 4 The detergent effects on the enzyme activities of ADH and JGWs. The enzyme activity assayed in the absence of a detergent was set to 100% as the control

Table 2 Docking scores of *D. teissieri Jingwei* (DT-JGW) and *D. yakuba Jingwei* (DY-JGW) using different alcohols as substrates

	Primary alcohol				Secondary alcohol				
	Ethanol	1-Butanol	Farnesol	Geraniol	2-Propanol	2-Butanol	2-Pentanol	3-Pentanol	2-Heptanol
DT-JGW	−0.65	−3.35	−7.83	−7.79	−2.67	−4.86	−5.6	−5.05	−6.58
DY-JGW	−0.97	−3.7	−6.78	−7.66	−2.82	−5.86	−5.21	−5.01	−5.86

GlideScore (GScore) is given by: *GScore* a * vdW + b * Coul + Lipo + Hbond + Metal + BuryP + RotB + Site, where *vdW* van der Waals energy, *Coul* coulomb energy, *Lipo* lipophilic contact term, *HBond* hydrogen-bonding term, *Metal* metal-binding term, *BuryP* penalty for buried polar groups, *RotB* penalty for freezing rotatable bonds, *Site* polar interactions in the active site, and the coefficients of vdW and Coul are: $a = 0.065$, $b = 0.130$ for standard precision (SP) Glides

Discussion

A long enduring paradigm in enzymology is the central role of catalytic chemistry and substrate binding during evolution of new functions (Babbitt and Gerlt 1997; Horowitz 1945). Comparisons of various enzyme structures and catalytic mechanisms suggest that the evolution of new functionalities can be achieved in two ways: first, incorporating new amino acids within an active site, while retaining those essential to catalyze a partial reaction, can produce a new catalytic mechanism; and second, changing amino acids responsible for substrate binding, while retaining those essential for the complete reaction (Petsko et al. 1993). A classic example is the enolase superfamily which catalyzes a variety of isomerizations, racemizations, cyclizations, dehydrations, and ammonia lyase reactions, all of which proceed through a common divalent metal cation stabilized enediolate transition state produced by abstraction of a proton α to a carboxylate (Babbitt et al. 1995; Perona and Craik 1997). These observations suggest that catalytic chemistry evolves first followed by diversification of substrate utilization. Unfortunately, the long divergence times among members of such ancient enzyme families obscure phylogenetic relationships. JGW, as a recently evolved young gene with a clear phylogenetic history, provides us an opportunity to probe the generality of this thesis.

According to the chemistry first theory (Perona and Craik 1997), amino acid residues directly involved in catalytic functions are evolutionarily conserved and appear invariant in their spatial positions. The evolution of JGW is in agreement with this theory. Our analyses show that like all SDRs, JGW, and its parental *Drosophila* ADH share a common protein structure with a typical Rossmann fold containing a canonical GlyXaaXaaGlyXaaGly NAD^+/$NADP^+$ binding motif, a conserved aspartate residue (Asp38) that regulates coenzyme binding, and a Ser-Tyr-Lys catalytic triad (Benach et al. 1998, 1999). The observed enzymatic properties of JGW, such as the preference for short-chain secondary alcohols and the inability to use methanol or alcohols with negatively charged groups, further confirm that JGW has preserved the SDR's active site and reaction mechanism. The fact that JGW cannot effectively use $NADP^+$ also indicates that the change of Lys41Glu did not alter coenzyme usage. However this change, combined with others, such as Arg103Ala, likely contributes to the different affinities of ADH and JGWs to NAD^+.

Comparative studies of structure and enzyme kinetics of JGW and ADH show that adaptive amino acids replacements outside the active site contribute to changes in catalytic functions (Fig. 1). With the possible exception of Arg103Ala which may contact the coenzyme directly, amino acid replacements in JGW affect functions by virtue of second and third shell interactions with residues that directly contact the substrates. These replacements may cause subtle conformational changes, for example by altering hydrogen-bond networks, modifying protein electrostatics, and/or influencing conformational flexibility (Golding and Dean 1998). Indeed, changes outside active sites are commonly recovered in laboratory-directed evolution experiments (Hsu et al. 2005; Oue et al. 1999; Rothman et al. 2004). Our analysis of JWG shows that adaptive changes outside the active site can similarly affect enzyme functions. While consistent with JGW as an adaptively evolving enzyme, whether the changes in substrate specificity are directly subject to selection, or the consequence of adaptation in other functional properties, has yet to be determined.

Unlike the parental ADH, which is expressed in the fat body and digestive tract, JGW is expressed exclusively in the testis of *D. teissieri* and more broadly in *D. yakuba*. This suggests that JGW may have different species-specific functions (Wang et al. 2000). Sequence analysis also shows a clear indication of adaptive evolution following the birth of *jgw*. The evolutionary patterns of *jgw* gene are: (1) a fast rate of amino acid replacement from *jgwI* to *jgwII* and (2) asymmetrical distributions of substitutions in the two lineages of *D. yakuba* and *D. teissieri* after speciation. As shown by a χ^2 test, a significant excess of amino acid substitutions were seen in the lineage to *D. yakuba* JGW, suggesting that the *jgw* genes in the two species have pursued different adaptive routes (Table 3). While both JGWs share the same overall catalytic mechanism, the

Table 3 Accelerated evolution of *jgw* in *D. yakuba*

	D. teissieri	*D. yakuba*
Amino acid replacements	9	28
Synonymous changes	10	11

The χ^2 test indicates that *jgw* evolved with significant difference rates ($P = 0.003$) between two species. Those changes N-terminal encoding region plus ADH derived part of *jgw*

different responses to the detergents and the difference in pH tolerance indicate a further functional evolution after speciation. Nevertheless, reconstructing the entire evolutionary history of JGW requires further experiments, such as reconstructing the ancestral JGWs and comparing their biochemical functions with parental ADH and current JGWs. Similar study has been done in studies of the evolutionary history of isopropylmalate dehydrogenase (IMDH) (Lunzer et al. 2005) and adaptation of bacterial β-lactamase to the novel antibiotic cefotaxime (Weinreich et al. 2006).

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