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Engineering mammalian cytochrome P450 2B1 by directed evolution for enhanced catalytic tolerance to temperature and dimethyl sulfoxide

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The previously laboratory-evolved cytochrome P450 2B1 quadruple mutant V183L/F202L/L209A/S334P (QM), which showed enhanced H_2O_2-mediated substrate oxidation, has now been shown to exhibit a >3.0-fold decrease in $K_{m,HOOH}$ for 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) *O*-deethylation compared with the parental enzyme L209A. Subsequently, a streamlined random mutagenesis and a high-throughput screening method were developed using QM to screen and select mutants with enhanced tolerance of catalytic activity to temperature and dimethyl sulfoxide (DMSO). Upon screening >3000 colonies, we identified QM/L295H and QM/K236I/D257N with enhanced catalytic tolerance to temperature and DMSO. QM/L295H exhibited higher activity than QM at a broad range of temperatures (35–55°C) and maintained ~1.4-fold higher activity than QM at 45°C for 6 h. In addition, QM/L295H showed a significant increase in $T_{m,app}$ compared with L209A. QM/L295H and QM/K236I/D257N exhibited higher activity than QM at a broad range of DMSO concentrations (2.5–15%). Furthermore, QM/K236I/D257N/L295H was constructed by combining QM/K236I/D257N with L295H using site-directed mutagenesis and exhibited a >2-fold higher activity than QM at nearly the entire range of DMSO concentrations. In conclusion, in addition to engineering mammalian cytochromes P450 for enhanced activity, directed evolution can also be used to optimize catalytic tolerance to temperature and organic solvent.

Keywords: Cytochrome P450/directed evolution/heme accessibility/random mutagenesis/thermostability

Directed evolution has been successfully used to create a variety of industrial biocatalysts with enhanced catalytic efficiency, novel activities and enhanced tolerance of catalytic activity to temperature and organic solvents (Cherry, 2003; Turner, 2003). Xenobiotic-metabolizing mammalian cytochromes P450, which have broad substrate specificity, offer the possibility of vast applications in industrial synthesis, medicine and bioremediation (Coon, 2005; Kumar and Halpert, 2005; Bernhardt, 2006). However, mammalian cytochromes P450 require an expensive cofactor, NADPH, NADPH cytochrome P450 reductase (CPR) and often cytochrome b_5 (b_5). Compared with bacterial P450s, mammalian P450s generally have lower turnover, expression in *Escherichia coli*, and stability upon long-term storage. Therefore, enhanced tolerance of mammalian P450s to temperature and organic solvents and increased stability, in addition to efficient utilization of an alternate oxidant such as H_2O_2, will greatly facilitate industrial applications. Recent studies with the bacterial P450 enzymes have illustrated the potential of directed evolution for conferring such properties (Glieder *et al*., 2002; Cirino and Arnold, 2003; Salazar *et al*., 2003; Wong *et al*., 2004; Bernhardt, 2006; Urlacher and Schmid, 2006).

Directed evolution of several mammalian cytochromes P450 for enhanced activity has been established recently and the potential applications reviewed (Kumar and Halpert, 2005). Using this approach P450 1A2 yielded mutants with 5- and 10-fold enhanced catalytic efficiency with 7-methoxyresorufin and 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline, respectively (Kim and Guengerich, 2004a,b), and P450 2A6 yielded mutants that oxidize both 4- and 5-benzyloxyindole to colored products (Kim and Guengerich, 2005). More recently, we have developed a directed evolution approach to screen/select P450 2B1 mutants for enhanced H_2O_2-supported 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) *O*-deethylation in the background of an N-terminal truncated and C-terminal His-tagged construct and L209A mutation termed 2B1dH L209A (Scott *et al*., 2001, 2002; Kumar *et al*., 2005a). The evolved 2B1dH quadruple mutant V183L/F202L/L209A/S334P (QM) showed the highest activity for 7-EFC *O*-deethylation in an H_2O_2-supported reaction (Kumar *et al*., 2005a).

In this study, further characterization of QM revealed a >3.0-fold lower $K_{m,HOOH}$ for 7-EFC *O*-deethylation than the parental enzyme L209A. Upon random mutagenesis using a streamlined procedure involving error-prone PCR of the whole plasmid, we created QM/L295H and QM/K236I/D257N. QM/L295H exhibited an enhanced catalytic tolerance to temperature, whereas QM/L295H and QM/K236I/D257N exhibited an enhanced catalytic tolerance to dimethyl sulfoxide (DMSO). Furthermore, QM/K236I/D257N/L295H exhibited a >2-fold higher catalytic tolerance than QM at nearly the entire range of DMSO concentrations (5–30%).

Materials and methods

Materials

7-EFC was purchased from Molecular Probes, Inc. (Eugene, OR). NADPH, DMSO and polymyxin B sulfate were obtained from Sigma Chemical Co. (St Louis, MO). Recombinant CPR and b_5 from rat liver were prepared as described previously (Harlow *et al*., 1997). All other chemicals were of the highest grade available and were obtained from standard commercial sources. The creation/construction of P450 2B1 QM was described previously (Kumar *et al*., 2005a).

Random mutagenesis by error-prone PCR of the whole plasmid

Owing to a limited number of transformants and lengthy procedures, the standard error-prone PCR method used previously for P450 2B1 (Kumar *et al*., 2005a) was modified. Error-prone PCR of the cDNA was replaced by error-prone PCR of the whole plasmid in order to create several thousands of colonies without subcloning (Matsumura and Rowe, 2005). The whole plasmid error-prone PCR was carried out using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) under conditions that generate 1–2 mutations/kb as described previously (Kumar *et al*., 2005a). This is a very efficient way to create a large mutant library without optimizing primer length, selection of restriction sites, ligation conditions and transformation procedures. In brief, error-prone PCR of the whole plasmid was carried out by using 50 μg template, 25 mM $MgCl_2$, 10 mM dCTP, 10 mM dTTP, and 2 mM each of dATP and dGTP. The forward and reverse primers, which were targeted at the P450 2B1 cDNA sequences between 766 and 792, used were 5′-ATTGTGGAGAAGCACAGGGCCACCTTA-3′ and 5′-TAAGGTGGCCCTGTGCTTCTCCACAAT-3′, respectively.

Site-directed mutagenesis

QM/K236I/D257N/L295H was created using QM/K236I/D257N as a template, and 5′-TCCCTGCTCTCT**CAC**TTCTTTGCTGGC-3′ and 5′-GCCAGCAAAGAA**GTG**AGAGAGCAGGGA-3′ as forward and reverse primers, respectively, using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Nucleotides in bold indicate the site of mutation. To confirm the desired mutation and verify the absence of unintended mutations QM/K236I/D257N/L295H was sequenced at the University of Texas Medical Branch Protein Chemistry Laboratory (Galveston, TX).

High-throughput screening of 2B1dH QM mutants for enhanced tolerance to temperature and DMSO

Growth and induction of P450 and preparation of whole cell suspensions were conducted as described recently (Kumar *et al*., 2005a). Optimization of high throughput screening methods using a 96-well microplate was carried out as described earlier for P450 BM3 (Cirino and Arnold, 2003; Salazar *et al*., 2003; Wong *et al*., 2004) with slight modification. In brief, 50 μl of the substrate mixture (300 μM 7-EFC containing 4% methanol and 5 U/well polymyxin B sulfate) was incubated with 40 μl of whole cell suspension for 5 min at room temperature at various temperatures and concentrations of DMSO for 10 min to determine the T_{50} and $DMSO_{50}$ of QM. The T_{50} and $DMSO_{50}$ are the temperature and DMSO concentration, respectively, at which P450 retains 50% activity. Upon optimization of the screening assay method for tolerance of the catalytic activity to temperature and DMSO, QM random mutants were initially screened, and colonies with ±40% of the average template activity were selected. The selected colonies (~5% of the total number) were further treated at T_{50} and $DMSO_{50}$ for 10 min prior to the activity measurement to screen/select the mutants with ⩾2-fold higher activity than the control (room temperature and no DMSO). The measurement of the enzyme activity was done by first recording the background intensity at λ_{ex} = 405 nm and λ_{em} = 510 nm using a fluorescence microplate reader (Ascent Fluoroscan, Ramsey, MN). Then, the reaction was initiated by the addition of 10 μl H_2O_2 (10 mM final), and the formation of product was recorded at 2.5 min. Mutants with ⩾2-fold higher activity than the average template activity at T_{50} and $DMSO_{50}$ were sequenced at the UTMB Protein Chemistry Laboratory and were further characterized as described below.

Expression and purification of wild type and engineered enzymes

P450 2B1dH and mutants were expressed as His-tagged proteins in *E.coli* TOPP3 and purified using a Ni-affinity column as described previously (Kumar *et al*., 2005a). QM/L295H showed P450 expression very similar to QM, whereas QM/K236I/D257N and QM/K236I/D257N/L295H showed 2- and 4-fold lower P450 expression than QM, respectively. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA). The specific contents were between 12 and 15 nmol of P450 per mg protein.

Enzyme assay

H_2O_2- and NADPH-dependent 7-EFC *O*-deethylation was assayed as described previously unless otherwise stated in the figure legends (Scott *et al*., 2002; Kumar *et al*., 2005a). The reconstitution of P450 2B1dH (0.25 μM) with CPR and b_5 in the NADPH system was carried out at molar ratios of 1:4:2. P450 used in all the experiments was 0.25 μM. Steady-state kinetic parameters were determined by regression analysis using Kaleida graph (Synergy Software, PA). The k_{cat} and K_m values were determined using the Michaelis–Menten equation. The enzyme activity at different temperatures and DMSO concentrations, and at different time intervals was determined as described in the figure legends.

P450 heme-destruction assay

Determination of the kinetics of P450 2B1dH heme depletion in the presence of 60 mM H_2O_2 was conducted under conditions similar to those previously described for P450 3A4 (Kumar *et al*., 2005b). The reaction was carried out at 25°C in 100 mM HEPES buffer, pH 7.4, in a 1 ml semi-micro spectrophotometric cell with constant stirring. The reaction mixture contained 1 μM protein and 60 mM H_2O_2. Bleaching of the hemoprotein was followed by measuring a series of absorbance spectra in the 340–700 nm range. Each series contained at least 10 spectra. The measurements were done using a Shimadzu-2600 spectrophotometer. Determination of the total concentration of the heme protein was done by linear least square approximation of the spectra using a linear combination of spectral standards of P450 2B4 low-spin, high-spin and P420-states (Davydov *et al*., 2003). Fluctuations of the base line due to turbidity changes were compensated by polynomial correction in combination with principal component analysis (PCA) as previously described (Davydov *et al*., 1995; Renaud *et al*., 1996; Davydov *et al*., 2003). All data treatment and fitting of the titration curves were performed with our SpectraLab software package (Davydov *et al*., 1995).

Secondary structural and thermal denaturation studies using circular dichroism (CD)

Far-UV CD spectra were recorded in the 200–260 nm range as an average of five repetitive scans for each sample using a CD spectrophotometer equipped with a single cell Peltier system

(Aviv, Model 215) at 25°C. Thermal unfolding and refolding experiments were carried out by monitoring elipticity at 222 nm as a function of temperature between 25 and 92°C with a 10°C/min scan rate and a 10 s equilibration at each degree Celsius rise in temperature. The unfolding profiles were fit to a two-state model to obtain the mid-point of the thermal transition temperature as described previously (Muralidhara and Wittung-Staffshede, 2004). Refolding experiments were not successful owing to protein aggregation at higher temperatures, and hence the unfolding transition temperatures were termed apparent ($T_{m,app}$).

Molecular modeling

A 2B1 homology model was constructed using the Insight II software package (Molecular Simulations, Inc., San Diego, CA) and the crystal structure of a ligand-bound P450 2B4 complex (PDB entry 1SUO) (Scott *et al*., 2004). The sequence of 2B1 was obtained from SwissProt (accession number P00176). The coordinates of the conserved residues were assigned based on the corresponding residues of the 2B4 complex. The heme group was copied from 2B4 into the 2B1 model. For the 2B1 mutants, the coordinates of the corresponding residues were changed in the 2B1 3D model, and the resulting 2B1 mutants were energy minimized.

The structure of 7-EFC was constructed using the Builder module. During the docking calculations, the system energy minimization and molecular dynamics simulations were carried out with the Discover_3 program, using the consistent valence force field with a non-bond cutoff of 10 Å, to a maximum gradient of 5 kcal mol^{-1} $Å^{-1}$ as described previously (Kumar *et al*., 2003). 7-EFC was automatically docked into the 3D models of 2B1dH L209A and QM in a reactive binding orientation with the Docking module of Insight III, leading to *O*-deethylation. During the subsequent energy minimization process, the substrate molecule, along with the side chains of protein residues within 10 Å of the substrate, was allowed to move. The non-bond interaction energies were evaluated with the Docking module of Insight III, and the lowest energy orientation was obtained after molecular mechanics minimization of L209A and QM.

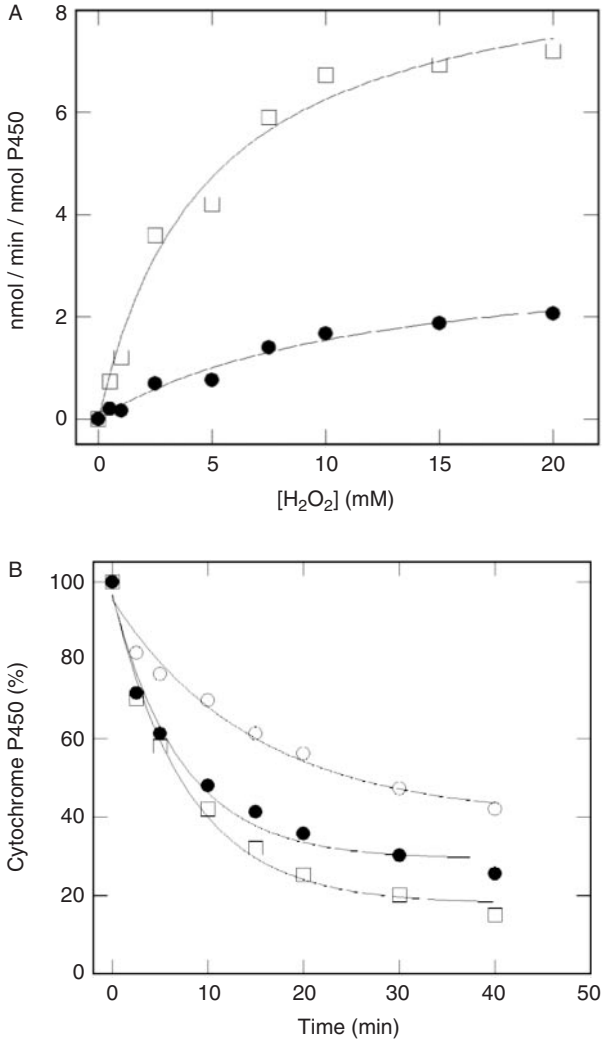

Fig. 1. (**A**) Steady-state kinetic analysis of H_2O_2-supported 7-EFC *O*-deethylation by P450 2B1dH mutants at 150 μM 7-EFC. The plots of the fit to the Michaelis–Menten equation of L209A (closed circles) and V183L/F202L/L209A/S334P (open squares) are presented. 2B1dH showed very low activity (~ 0.2 min^{-1} at 150 μM 7-EFC and 10 mM H_2O_2). (**B**) P450 2B1dH-heme depletion assays at 60 mM H_2O_2 and 2 μM P450. The plots of the fit to biphasic kinetics of P450 2B1dH (open circles), L209A (closed circles), and V183L/F202L/L209A/S334P (open squares) are shown.

Results

2B1dH QM shows decreased $K_{m,HOOH}$

In a previous study the engineered 2B1dH QM showed enhanced H_2O_2-supported oxidation of 7-EFC at 10 mM H_2O_2 (Kumar *et al*., 2005a). To assess the K_m for H_2O_2, QM was assayed at different concentrations up to 20 mM, above which H_2O_2 accelerates heme depletion (oxidative destruction of the heme). At 20 mM H_2O_2, the rate of 7-EFC *O*-deethylation is linear for up to 3 min (data not shown). At a saturating [7-EFC] and increasing [H_2O_2] QM showed a ⩾2.5-fold enhancement of k_{cat} compared with L209A (8.2 ± 0.6 min^{-1} versus 2.9 ± 0.3 min^{-1}) (Figure 1A). Interestingly, QM also showed >3-fold lower $K_{m,HOOH}$ than L209A (4.4 ± 0.2 mM versus 14 ± 3 mM). 2B1dH showed very low activity (~0.2 min^{-1} at 150 μM 7-EFC and 10 mM H_2O_2), and kinetic parameters could not be determined.

The increased catalytic activity of L209A compared with 2B1dH, and increased $k_{cat}/K_{m,HOOH}$ for QM compared with L209A, may suggest enhanced accessibility of H_2O_2 to the heme pocket. To test this hypothesis, we studied the H_2O_2-dependent destruction of the P450 heme at 60 mM H_2O_2 (Figure 1B). The kinetics fit the equation of a simple pseudo-first order reaction. L209A showed a 2-fold increase in the rate constant compared with 2B1dH (0.14 ± 0.02 min^{-1} versus 0.07 ± 0.01 min^{-1}), whereas QM did not show a significant further increase in the rate constant (0.16 ± 0.02 min^{-1} versus 0.14 ± 0.02), suggesting that H_2O_2 is more accessible to the heme in QM and L209A than 2B1dH. In addition, the rate constant of heme depletion at 60 mM H_2O_2 in the presence of testosterone, which is metabolized very slowly in the peroxide-dependent reaction, decreased significantly (0.016 ± 0.003 min^{-1}), further suggesting that the activity measurement with 7-EFC performed at 20 mM H_2O_2 is reliable.

Screening and selection of 2B1dH QM mutants for enhanced tolerance to temperature and DMSO

QM was used as the starting template for further directed evolution to screen and select mutants with enhanced tolerance

of the catalytic activity to temperature and DMSO. Compared with the average QM activity, QM displayed ~75% colony-to-colony variation in the activity under standard conditions (Figure 2A; closed circles) as well as at T_{50} (Figure 2A; open circles) and $DMSO_{50}$ (data not shown). These data suggest that random clones having ⩾2-fold higher activity than QM at T_{50} or $DMSO_{50}$ would correspond to mutants with enhanced tolerance of the catalytic activity to temperature or DMSO.

The modified error-prone PCR using the whole plasmid yielded several thousand colonies, of which 3000 colonies were screened first. Approximately 30–40% of the clones had ⩽20% of the average QM activity, and ~5% of the clones had ±40% of the QM activity (Figure 2B), suggesting a mutation rate of 1–2 bp/kb of the P450 coding sequence, similar to rates found earlier using error-prone PCR of the cDNA (Cirino and Arnold, 2003; Salazar *et al*., 2003; Kumar *et al*., 2005a). A second screen involving >150 colonies was further carried out individually at T_{50} and $DMSO_{50}$, and yielded a total of eight clones with ⩾2-fold higher activity than QM. Five of these had single or double mutations, while three were false positives (no mutation). Two potential candidates (QM/L295H and QM/K236I/D257N), which displayed enhanced activity at T_{50} and $DMSO_{50,}$ were subsequently expressed, purified and characterized as described below. Although, QM/L295H showed negligible change in P450 expression and P420 content, QM/K236I/D257N had decreased expression by ~2-fold compared with QM.

Steady-state kinetic analysis of the evolved P450 2B1 enzymes

The evolved P450 2B1 enzymes were assayed for H_2O_2-supported 7-EFC *O*-deethylation at 150 μM 7-EFC and at varying H_2O_2 concentrations (0–20 mM) (Table I). QM/L295H and QM/K236I/D257N showed a small change in k_{cat} (7.0 ± 0.2 and 7.0 ± 0.4 min^{-1} respectively, versus 8.9 ± 0.9 min^{-1} for QM). However, QM/K236I/D257N/L295H showed a >2-fold decrease in k_{cat} and a >2-fold increase in $K_{m,HOOH}$ leading to a >4-fold decrease in $k_{cat}/K_{m,HOOH}$ compared with QM (Table I). These results along with a 2- and 4-fold decreased P450 expression in QM/K236I/D257N/L295H compared with QM/K236I/D257N and QM/L295D, respectively, suggest that addition of L295H to QM/K236I/D257N is deleterious for P450 2B1 stability and activity.

2B1dH QM/L295H shows enhanced catalytic tolerance to temperature

The evolved P450 2B1 enzymes were assayed for 7-EFC *O*-deethylation after incubation at a wide range of temperatures (20–70°C) for 10 min (Figure 3A). P450 2B1dH wild-type showed very similar characteristics to L209A with respect to catalytic tolerance to temperature (data not shown). QM showed a modest enhancement in catalytic tolerance to temperature compared with L209A, whereas QM/L295H displayed a significant increase in tolerance of the catalytic activity to temperature at 40–50°C compared with QM. In addition, QM/L295H maintained ⩾75% residual activity at temperatures >5°C higher than the maximum temperature at which QM maintains ⩾75% residual activity (Figure 3A). However, QM/K236I/D257N showed only a modest enhancement in tolerance of the catalytic activity to temperature (Figure 3A). Time courses (0–360 min) at 45°C revealed that QM/L295H consistently exhibited ~1.4-fold higher activity than QM (Figure 3B),

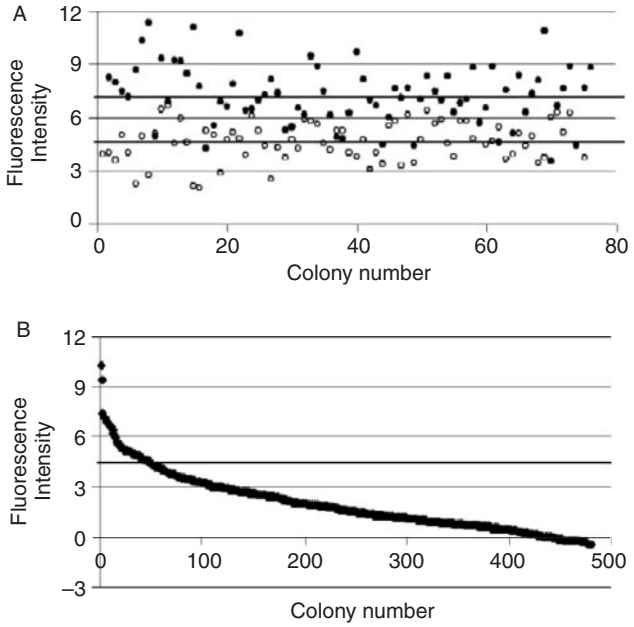

Fig. 2. Screening of P450 2B1 V183L/F202L/L209A/S334P (QM) random clones for the oxidation of 7-EFC. (**A**) Representative figure of activities determined for individual clones of QM under standard conditions (closed circles) and at 45°C (T_{50}) (open circles) are plotted in random distribution of the activity to demonstrate the range across the 96-well microplate. The average QM fluorescence intensity from the mean under standard conditions is 7.25 and at 45°C is 4.5 as shown by solid horizontal lines. (**B**) Representative figure of activities of individual clones of randomly mutagenized QM using error-prone PCR of the whole plasmid as plotted in decreasing order of activity. The average QM fluorescence intensity from the mean is 4.5 as shown by a solid horizontal line. The *x*-axis represents individual colony number, and the *y*-axis represents relative fluorescence intensity for the product, 7-HFC, at λ_{ex} = 405 nm and λ_{em} = 510 nm. The detailed methodology is described under Materials and methods.

Table I. Steady-state kinetics: H_2O_2-supported oxidation of 7-EFC by P450 2B1dH QM and mutants

P450 2B1dH	k_{cat} (min^{-1})	$K_{m,\ HOOH}$ (mM)	k_{cat}/K_m
QM	8.9 ± 0.9	6.1 ± 0.8	1.4
QM/L295H	7.0 ± 0.2	6.4 ± 0.4	1.1
QM/K236I/D257N	7.0 ± 0.7	6.4 ± 1.2	1.1
QM/K236I/D257N/L295H	4.0 ± 0.7	14 ± 4.0	0.29

Results are the mean ± standard deviation of at least three independent experiments.

which further suggests that QM/L295H possess enhanced catalytic tolerance to temperature compared with QM. QM/K236I/D257N/L295H showed the lowest catalytic tolerance to temperature, indicating that the addition of L295H to QM/K236I/D257N is deleterious for P450 thermostability, which is also consistent with the reduced P450 expression.

An effect of mutations on protein secondary structure and mid-point of apparent thermal transition ($T_{m,app}$) was monitored by CD. The results showed an increase in the secondary structure in QM/L295H and QM/K236I/D257N compared with L209A and QM (CD spectrum not shown). All four protein species clearly demonstrated a two-state transition (Figure 4A) with the $T_{m,app}$ of 58.6 ± 0.5°C, 61.6 ± 0.5°C, 63.4 ± 0.5°C and 63.0 ± 0.5°C for L209A, QM, QM/L295H and QM/K236I/D257N, respectively. Thus, the $T_{m,app}$ for QM/L295H and QM/K236I/D257N was ~2°C higher than

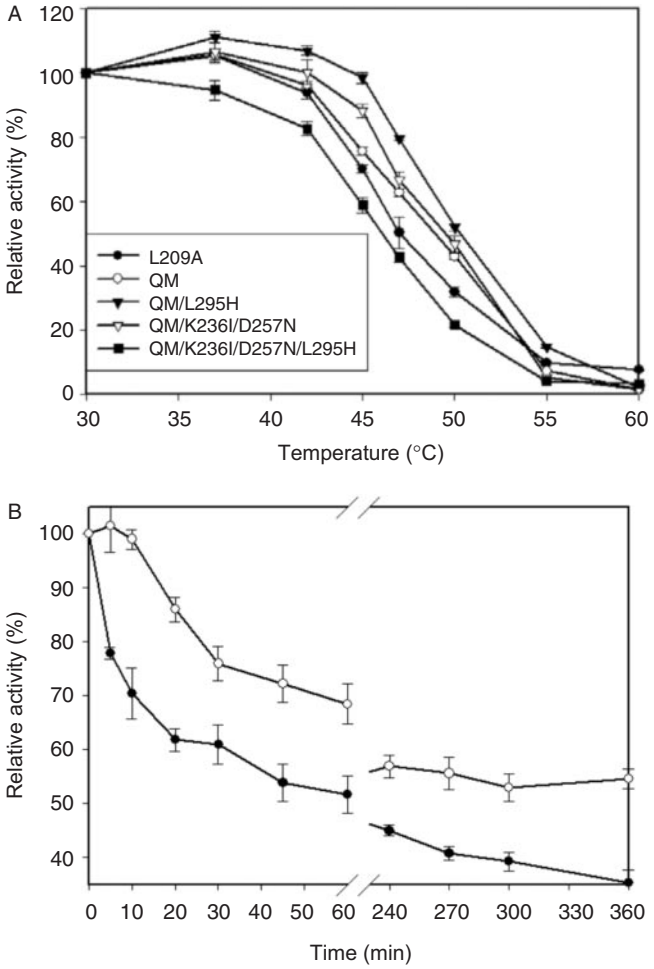

Fig. 3. (**A**) Temperature sensitivity profiles of the 2B1dH mutants: Relative activity is the ratio of the turnover (nmol/min/nmol P450) at various temperatures to that at 30°C. The samples were incubated at the temperature range between 30°C and 70°C for 10 min, chilled on ice for 15 min and finally incubated at room temperature for 25 min before carrying out the activity determination, as described in Materials and methods. (**B**) Time course profiles of QM and QM/L295H at 45°C. Relative activity is the ratio of the turnover (nmol/min/nmol P450) at various time intervals to that at 0 min. The time course experiment was carried out by incubating the enzyme samples at 45°C for 0–360 min prior to measuring the activity. QM (filled circles) and QM/L295H (open circles) are represented. Error bars represent the mean ± SD (n = 4).

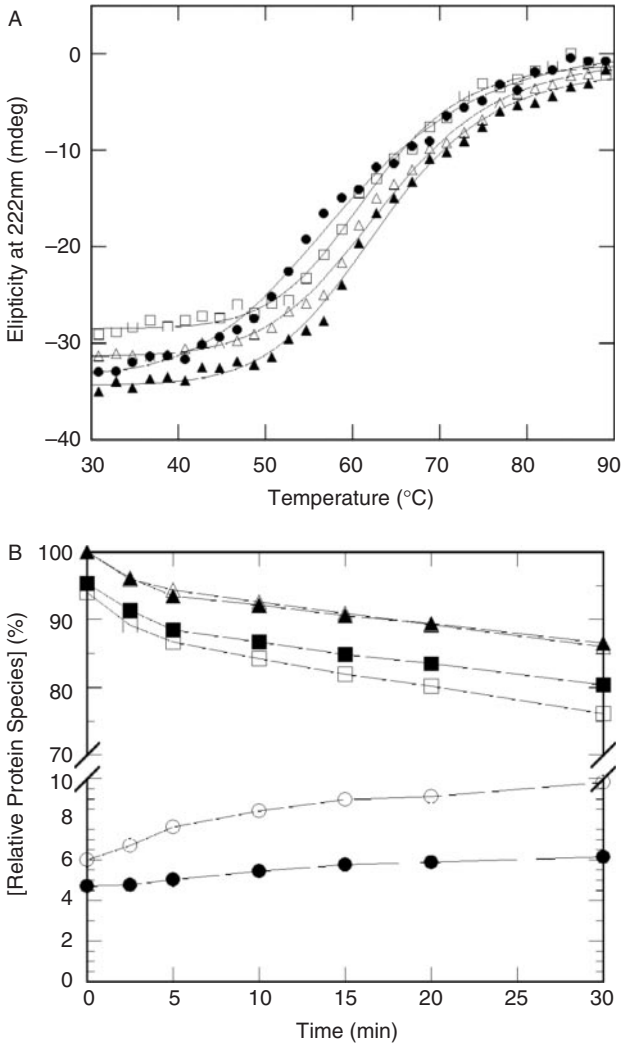

Fig. 4. (**A**) Thermal unfolding of 2B1dH mutants (5 μM) monitored by the decrease in elipticity at 222 nm as a function of temperature. The thermal transitions were fit (solid lines) to two-state transitions to obtain apparent T_m ($T_{m,app}$). Thermal unfolding for all the four proteins was irreversible, and hence the thermal transition temperature was termed apparent. L209A (filled circles), QM (open squares), QM/L295H (open triangles) and QM/K236I/D257N (filled triangles) are shown. (**B**) Time courses of inactivation of 2B1dH mutants (1 μM) at 45°C monitored by measuring a series of absorbance spectra in the 300–700 nm. Determination of the total concentration of the heme protein was done by linear least square approximation of the spectra by a linear combination of spectral standards of 2B4 low-spin, high-spin and P420-states. QM P420 (open circles), QM/L295H P420 (filled circles), QM P450 (open squares), QM/L295H P450 (filled squares), QM total protein (open triangles), and QM/L295H total protein (filled triangles) are shown.

the $T_{m,app}$ of QM, which in turn was ~3°C higher than L209A. There was minimal protein denaturation at 45°C, at which QM/L295H and QM/K236I/D257N showed highest tolerance of the catalytic activity (Figure 3 versus Figure 4A).

This subtle difference in protein stability suggests that the enhanced catalytic tolerance at 45°C for QM/L295H is mainly due to their enhanced resistance against P450 active site disruption via heme loss and/or conversion into inactive P420. To test this hypothesis, we monitored P450 and P420 contents in QM and QM/L295H at 45°C in the absence and in the presence of testosterone for 30 min (Figure 4B, data not shown). Expectedly, the substrate stabilized P450 in QM and QM/L295H initially by reducing the formation of P420 followed by the reduction of P450-inactivation. Although, in the presence of substrate, QM and QM/L295H both lost 14% of the total protein (P450 + P420), the loss of P450 in QM/L295H was relatively lower than QM (15% versus 18%; Figure 4B). Similarly, the formation of P420 was lower in QM/L295H than QM (1% versus 4%), which may reflect a marginal enhancement in the catalytic tolerance of QM/L295H at 45°C.

2B1dH QM/L295H, QM/K236I/D257N and QM/K236I/D257N/L295H show enhanced catalytic tolerance to DMSO

The residual activities of purified 2B1dH L209A, QM, QM/L295H and QM/K236I/D257N at various DMSO concentrations are shown in Figure 5. P450 2B1dH wild-type showed very similar characteristics to L209A with respect to catalytic tolerance to DMSO (data not shown). QM showed a modest enhancement in catalytic tolerance to DMSO compared with L209A, whereas QM/L295H and QM/K236I/D257N

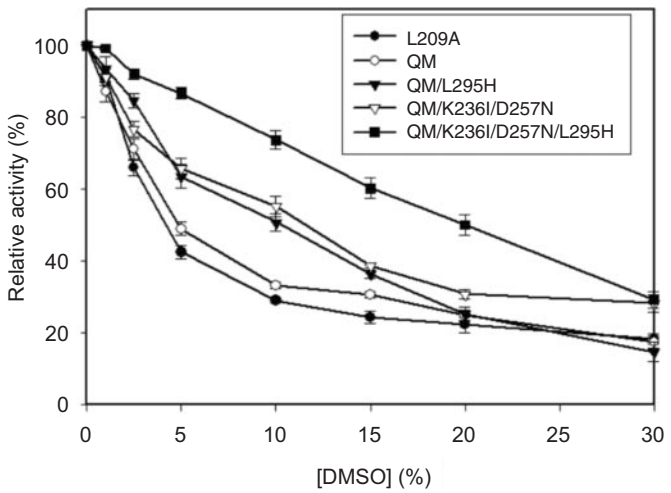

Fig. 5. DMSO sensitivity profiles of the P450 2B1dH mutants. Relative activity is the ratio of the turnover (nmol/min/nmol P450) at the various DMSO concentrations to that at 0% DMSO. The substrate mixture was made in organic solvents (DMSO, 0–40%). Enzyme samples were incubated with the substrate mixture for 10 min before carrying out the activity determination, as described in the Materials and methods. Error bars represent the mean ± SD (n = 4).

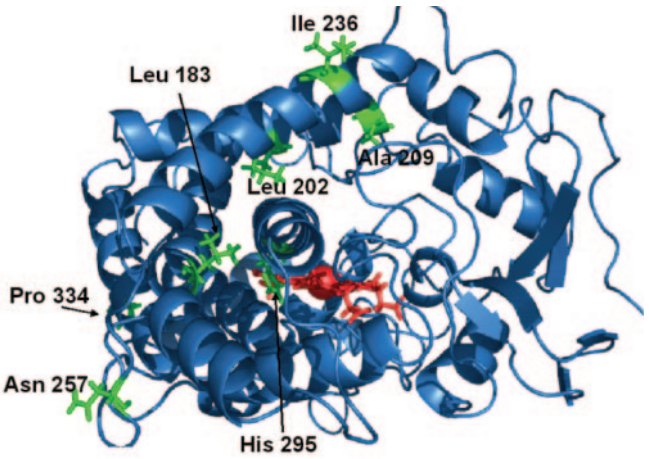

Fig. 6. Homology model of P450 2B1dH QM/K236I/D257N/L295H. The heme (red sticks) and mutant residues (green sticks) are shown.

displayed a significant increase in tolerance of the catalytic activity at a broad range of DMSO concentrations (2.5–15%) compared with QM. The catalytic tolerance of QM/L295H and QM/K236I/D257N at 10% DMSO was >1.6-fold higher than QM. Interestingly, QM/K236I/D 257N/L295H showed ~2-fold higher catalytic tolerance at nearly the entire range of DMSO concentrations, indicating that the effects of the amino acid substitutions is additive with DMSO unlike temperature. Furthermore, we also tested the catalytic tolerance of the evolved mutants to THF (data not shown). Although, QM showed enhanced tolerance of the catalytic activity to THF compared with L209A, the evolved mutants did not show further improvement in tolerance to THF.

Discussion

Improving utilization of H_2O_2, tolerance of catalytic activity to temperature and organic solvents, and structural stability have been a major focus of engineering bacterial cytochromes P450 in order to use these enzymes as industrial biocatalysts (Tee *et al*., 2005; Bernhardt, 2006; Urlacher and Schmid, 2006). Recent developments in directed evolution of xenobiotic-metabolizing mammalian cytochromes P450 enhance the possibility of designing them as industrial biocatalysts (Kumar and Halpert, 2005; Bernhardt, 2006). Recently, we have engineered mammalian cytochrome P450 2B1, termed QM, which displays enhanced k_{cat} in H_2O_2- and NADPH-supported reactions (Kumar *et al*., 2005a). Here we report the first study on engineering mammalian cytochrome P450 2B1 by directed evolution for enhanced tolerance of the catalytic activity to temperature and DMSO. The engineered P450 2B1 enzyme, QM/K236I/D257N/L295H had five mutations outside the active site as shown in Figure 6. This study can be considered as proof-of-principle and an important step forward for directed evolution to engineer these relatively unstable and dynamic proteins for such properties.

Given the limited understanding of the structural basis of peroxide-supported catalytic activity, directed evolution is currently the only possible way to engineer P450s for enhanced utilization of H_2O_2. Recently, bacterial enzyme P450 BM3 was designed by directed evolution to utilize H_2O_2 to catalyze hydroxylation of fatty acids (Glieder *et al*., 2002). 2B1dH QM, which was originally engineered for enhanced k_{cat} of H_2O_2-supported 7-EFC *O*-deethylation, also showed a similar enhancement in k_{cat} in the standard NADPH reconstituted system (Kumar *et al*., 2005a). Although, the mechanism by which QM acquires enhanced activity in both the reactions is difficult to predict, we suggest that the Phe^{202}→Leu substitution in the presence of V183L/L209A/S334P is a key determinant in enhancing the activity, because F202L alone showed lower activity than 2B1dH (Kumar and Halpert, 2005a). Docking of 7-EFC into a 2B1 model based on the X-ray crystal structure of 4-(4-chlorophenyl)imidazole-bound P450 2B4 showed that Leu-202 is in the active site of QM (4.5 Å from 7-EFC), while Phe-202 is outside the active site in L209A (7.2 Å from 7-EFC) (Figure 7). In addition, QM showed a $K_{m,HOOH}$ lower than L209A and similar to that obtained with P450 BM3 (Cirino and Arnold, 2003). The $K_{m,CuOOH}$ for P450 is usually associated with the accessibility of CuOOH to the active site heme pocket (Zhang and Pernecky, 1999). However, our data from the heme-depletion assay do not suggest an enhanced accessibility of H_2O_2 in QM compared with L209A, even though the accessibility of H_2O_2 in QM and L209A is increased compared with 2B1dH.

Cytochromes P450, especially mammalian P450s, are not easy to engineer for thermostability because of the fragile nature of the active site and heme cofactor (Giver *et al*., 1998; Scott *et al*., 2003; Scott *et al*., 2004; Muralidhara *et al*., 2006; Zhao *et al*., 2006). In one example, directed evolution of P450 BM3 enhanced the T_{50} from 43°C to 61°C (Cirino and Arnold, 2003). Although QM/L295H showed a small increase in T_{50} compared with QM, it displayed a significant tolerance in activity at the temperature range from 40°C to 50°C. In addition, a significantly higher activity of QM/L295H compared with QM at 45°C over the period of 6 h is important for enhanced shelf-life and usage in substrate metabolism at elevated temperature. 2B1dH enzymes demonstrated a higher $T_{m,app}$ (~60°C) than T_{50} (48°C) suggesting that P450s are first inactivated via P450 active sites and/or formation of inactive P420. Our studies of P450 inactivation at 45°C, in which P450 is mainly inactivated by heme destruction and conversion into

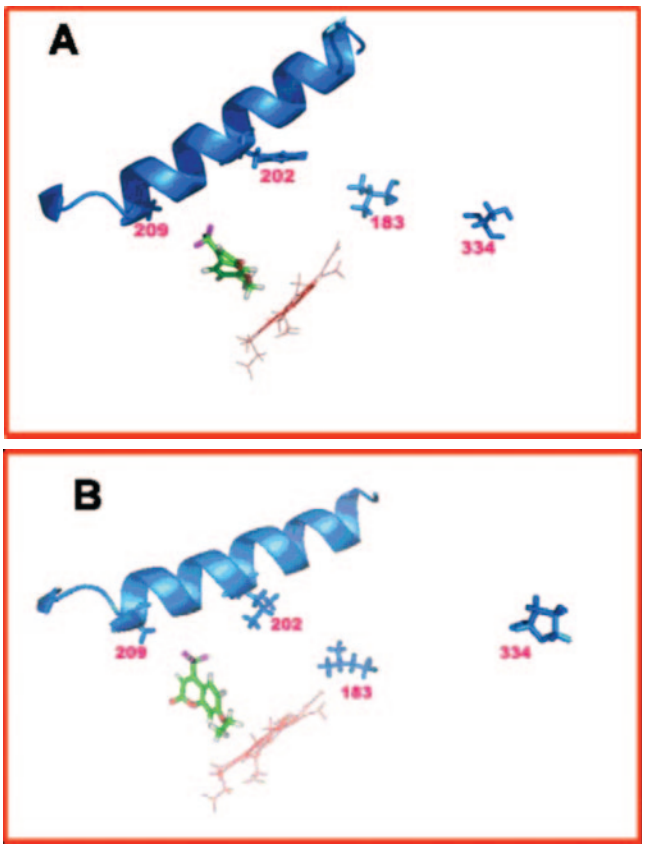

Fig. 7. Docking of 7-EFC into the active site of P450 2B1dH models of L209A (**A**) and 2B1dH V183L/F202L/L209A/S334P (**B**). The heme (red sticks), 7-EFC (green sticks), and the mutant residues 183, 202, 209, and 334 (blue sticks) are shown.

P420, is consistent with the hypothesis. Expression of P450s is usually associated with variable amounts of P420, and site-directed mutations and long-term storage further enhance the P420 content (Kumar *et al*., 2003). Therefore, stabilization of P450 against its degradation into P420 is critical. In addition, an increase in $T_{m,app}$ and secondary structure content of QM, QM/L295H and QM/K236I/D257N compared with L209A suggests that the engineered enzymes are slightly more robust in nature.

Since these enzymes act on hydrophobic substrates, tolerance to organic solvents is an important characteristic for potential use in industry. QM showed a negligible enhancement in the catalytic tolerance to DMSO compared with L209A, whereas QM/L295H and QM/K236I/D257N, which were isolated based on screening with DMSO, showed a further enhancement in the catalytic tolerance. The results suggest a reliable screening and selection system. Furthermore, an additive effect on catalytic tolerance to DMSO was observed when QM/K236I/D257N and L295H were combined, although QM/K236I/D257N/L295H showed lower expression, activity and catalytic tolerance to temperature than QM. To test whether the decrease in the catalytic tolerance to DMSO is associated with inactivation of the P450 heme and/or conversion into P420, we measured P450 and P420 species at 10% DMSO (data not shown). The results showed that the rate of inactivation of P450 was very low (<2% in 10 min) in QM without significant formation of P420, suggesting that DMSO does not inactivate the P450 heme and/or cause conversion into inactive P420.

The underlying mechanism of DMSO action on proteins, especially cytochrome P450, is not clear. An X-ray crystal structure of hen egg-white lysozyme in complex with DMSO showed substantial local conformational change and induced an Na^+ binding site in the protein without affecting the overall conformations of the complex (Mande and Sobhia, 2005). Therefore, we speculate that DMSO can access the active site and induces conformational transitions, and/or competes with water and/or substrates leading to decreased activity, which is consistent with molecular dynamic simulations of the heme domain of P450 BM3 in 14% DMSO. The study showed relatively more accessible DMSO in the active site via helices E, F and E–F loop (implicated in controlling the access channel) (Roccatano *et al*., 2005; Roccatano and Wong, 2006). The simulation study is consistent with the observation that P450 BM3 F87A exhibits decreased catalytic tolerance to DMSO, presumably because the Phe^{87}→Ala substitution (part of active site channel) may provide DMSO easy access to the active site (Salazar *et al*., 2003). Therefore, we suggest that Lys^{236}→Ile, Asp^{257}→Asn and Leu^{295}→His substitutions mask the access of DMSO to the heme pocket.

In conclusion, the evolved P450 2B1 enzyme for enhanced activity also shows retained or enhanced P450 expression, tolerance of the catalytic activity to temperature and organic solvents, and protein stability. In contrast, 2B1dH site-directed active site mutants from a previous study (Kumar *et al*., 2003) showed decreased expression and decreased catalytic tolerance to temperature and DMSO (Kumar *et al*., 2003; data not shown). Furthermore, this is the first report on designing mammalian P450s for enhanced catalytic tolerance to temperature and DMSO by directed evolution. As a future prospect, L295H and K236I/D257N can be created in other P450 2B enzymes such as rabbit P450 2B4, human P450 2B6 and dog P450 2B11 by rational mutagenesis to test whether these mutants possess similar characteristics in the related enzymes and to enhance their catalytic tolerance to temperature and DMSO. Re-engineering of P450s between subfamilies and among the same subfamily for catalytic activity, substrate specificity, and regio- and stereoselectivity has been well established (Domasnki and Halpert, 2001; Kumar *et al*., 2003). In addition, the proof-of-principle presented here can be utilized to engineer more important drug-metabolizing mammalian P450s such as P450 3A4 and P450 2C enzymes for enhanced catalytic efficiency and enhanced catalytic tolerance to temperature and organic solvent.

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