Thermostable variants constructed via the structure-guided consensus method also show increased stability in salts solutions and homogeneous aqueous-organic media

E. Vazquez-Figueroa1, V. Yeh2, J.M. Broering1,3, J.F. Chaparro-Riggers1,4 and A.S. Bommarius1,2,5

1School of Chemical and Biomolecular Engineering and, 2School of Chemistry and Biochemistry, Parker H. Petit Institute for Bioengineering and Bioscience, 315 Fert Drive, Atlanta, GA 30332-0363, 3Department of Bioengineering, Stanford University, 318 Campus Drive, Clark Center, W300, Stanford, CA 94305-5440 and 4Now at Rinat Laboratories, Pfizer, Inc., 230 East Grand Avenue, South San Francisco, CA 94080, USA

Introduction

Enzymes are extraordinary catalysts that often provide unsurpassed fidelity and selectivity under ambient and near ambient conditions of pH, temperature and solvent composition. As target compounds in pharma become more complex and processes more environmentally demanding, biocatalysts are increasingly favored over non-biological catalysts (Panke et al., 2004; Constable et al., 2007; De Wildeman et al., 2007; Moore et al., 2007; Pollard and Woodley, 2007; Ran et al., 2008). However, despite their indisputable favorable qualities, insufficient protein stability in reaction media has hampered biocatalyst implementation in organic synthesis. Recognizing this shortcoming, a number of approaches have been taken to develop highly stable proteins for biocatalysis applications, either by isolating and expressing novel proteins with exceptional stability (Haki and Rakshit, 2003) or engineering the protein environment through the addition of co-solutes (Davis-Searles et al., 2001; Broering and Bommarius, 2005; Polizzi et al., 2007), chemical modifications (Polizzi et al., 2007) and immobilization of the protein in solid or gel-like matrices (Kandimalla et al., 2006; Kim et al., 2006; Polizzi et al., 2007). Alternatively, protein engineering methods have been used to modify the protein scaffold to improve hydrophobic interactions (Kumar and Nussinov, 2001), electrostatic-surface interactions (Wunderlich et al., 2005; Strickler et al., 2006), internal packing (Filikov et al., 2002, Korkegian et al., 2005) and increase rigidity (Matthews et al., 1987; Mansfeld et al., 1997; Gaseidnes et al., 2003) to withstand denaturing conditions. Although comprehensive, generalizable guidelines for engineering stable proteins have not been achieved, these innovative approaches have produced stable proteins with significant success (Lehmann and Wyss, 2001; Eijsink et al., 2004, 2005) while also making significant contributions to our understanding of protein stability.

The stability of proteins in organic solvents is one particular problem that continuously receives attention due to increasing interest for industrial applications, in particular fine chemicals and pharmaceuticals (Dordick, 1989; Gómez-Puyou et al., 1992; Ran et al., 2008; Carrea and Riva, 2008; Serdakowski and Dordick, 2008). Increased biocatalyst tolerance to organic solvents can allow for an increased concentration of substrates and products to be employed. This in turn allows a wider range of operating conditions, such as reactant concentrations, to be utilized to optimize reactions by shifting reaction equilibrium, reduce/eliminate hydrolysis or polymerization side reactions, and avoid microbial contamination (Dordick, 1989). This results in increased productivity and thus more economically attractive processes. Though much work has gone into understanding and predicting organic solvent effects on enzymatic activity based on water activity (Valiyet et al., 1992, 1994), log P (Laane et al., 1986; Laane, 1987) and solvent polarity (Gupta et al., 1997; Kim et al., 2000), most medium optimization of biocatalysts is still performed on a case-by-case basis. Khmelnitsky et al. (Khmelnitsky et al., 1991, 1994) have studied enzyme behavior in the combined presence of...
salts in organic solvents and progress has been summarized elsewhere (Serdakowski and Dordick, 2008). In this work, salt and organic solvent effects on enzyme behavior will be studied independently.

Most interestingly, it has been noted that an improvement in protein stability to higher temperatures can result in a simultaneous increase in stability in the presence of organic solvents (and other denaturants) (Owusu and Cowan, 1989; Cowan, 1997). Cowan et al. (Owusu and Cowan, 1989; Cowan, 1997) showed a positive correlation between thermal stability and tolerance to organic solvents in naturally occurring homologs and Hao et al. demonstrated how a thermostable fructose bisphosphate aldolase found via directed evolution also featured increased stability in organic solvents (Hao and Berry, 2004).

In our previous work, we improved the thermostability of glucose 1-dehydrogenase (GDH) from Bacillus subtilis (B. subtilis) strain 168 via a structure-guided consensus concept (Vazquez-Figueroa et al., 2007). This approach makes use of the consensus sequence (Steipe et al., 1994) in addition to a protein structure (or homology model) to sieve through and pick potential amino acid mutations. The ease of use and predictive power of consensus-based sequences consistently allow protein engineers to identify more stable protein variants with >30% success rate (Lehmann et al., 2002; Polizzi et al., 2006; Vazquez-Figueroa et al., 2007)—significantly outperforming other approaches such as directed molecular evolution.

GDH catalyzes the oxidation of glucose to gluconolactone (which spontaneously hydrolyses to gluconic acid) with the concomitant reduction of NAD(P)⁺ to NAD(P)H (Fig. 1). GDH is an enzyme of great interest since it utilizes an abundant and inexpensive substrate, it accepts both NAD⁺ and NADP⁺, it has very high activity (>100 U/mg) and it is not thermodynamically limited due to the spontaneous hydrolysis of the gluconolactone. Currently, this enzyme is widely employed, with further great potential in the regeneration of nicotinamide cofactors for keto-reductions (De Wildeman et al., 2007; Moore et al., 2007), Baeyer–Villiger oxidations (Mihovilovic, 2006), alkane/alkene hydroxylation (Wong et al., 2004), carbon–carbon double bond reductions (Chaparro-Riggers et al., 2007; De Wildeman et al., 2007; Hall et al., 2007; Pollard and Woodley, 2007) and keto acid reductions via amino acid dehydrogenases (Bommarius et al., 1998; De Wildeman et al., 2007). In this work, we characterized four variants of GDH from B. subtilis with different kinetic and thermodynamic stabilities, in different stabilizing and destabilizing salts and a variety of organic cosolvents. We intend to demonstrate the potential of the structure-guided consensus method toward developing enzymes tolerant to denaturing salts and organic cosolvents.

**Experimental section**

**Materials**

*Bacillus subtilis* strain 168 was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH, USA). Primers (oligonucleotides) for amplification and mutagenesis were obtained from MWG-Biotech (Huntsville, AL, USA). pET28a was purchased from Novagen (Madison, WI, USA). *Escherichia coli* BL21(DE3) were obtained from Invitrogen (Carlsbad, CA, USA). Ni-nitrilotriacetic acid resin for 6xHis-tag purification was purchased from Qiagen (Valencia, CA, USA). NaI, NaNO₃, NaHCO₂, NaF, Na₂SO₄, NaCH₃COO, reduced β-nicotinamide adenine dinucleotide (NAD⁺), isopropyl β-D-1-thiogalactopyranoside (IPTG), NaH₂PO₄·H₂O and imidazole were purchased from Sigma (St Louis, MO, USA). NaBr, NaCl and D-glucose were purchased from EMD Biosciences (San Diego, CA, USA). Dialysis was carried out using a 11.5 mm diameter, 3500 MWCO Spectrum®, Spectra/Por® molecular porous membrane tubing (San Diego, CA, USA).

**Cloning, PCR, site-directed mutagenesis via overlap extension and purification**

His-tag GDH variants were cloned, mutated and purified as described elsewhere (Vazquez-Figueroa et al., 2007). Purified variants were then dialyzed in 50 mM sodium phosphate pH 8.0 at 4°C to remove imidazole and sodium chloride. The concentration of the purified His-tag GDH variants was measured via Bradford assay using commassie protein reagent from Pierce (Rockford, IL, USA).

**Enzyme activity assay**

Enzymes were assayed using a Beckmann-Coulter DU®800 spectrophotometer (Fullerton, CA, USA). Enzyme was added to 1 ml of assay solution containing 100 mM D-glucose, 1 mM NAD⁺ in 50 mM sodium phosphate pH 8.0. Enzyme activity was determined by monitoring NADH formation at 340 nm. *kd,obs* was calculated from first-order deactivation kinetics.

**Hofmeister series, kinetic stability**

Concentrated, pure His-tag GDH was diluted to 50 μg/ml with the different salts of interest (i.e. NaI, NaNO₃, NaBr, NaCl, NaHCO₂, NaF, Na₂SO₄ and NaCH₃COO₂H) at pH 8.0 in 50 mM sodium phosphate and 0.95 and 0.99 water activity. Enzyme–salt mixtures were deactivated by incubating at 65°C and periodically assaying residual activity.

**NaCl, GDH temperature dependence**

For varying NaCl concentrations, a stock of 4 M NaCl with the different salts of interest (i.e. NaI, NaNO₃, NaBr, NaCl, NaHCO₂, NaF, Na₂SO₄ and NaCH₃COO₂H) at pH 8.0 in 50 mM sodium phosphate and 0.95 and 0.99 water activity. Enzyme–salt mixtures were deactivated by incubating at 65°C and periodically assaying residual activity.

**Circular dichroism (CD)**

Circular dichroism (CD) ellipticity was measured with a Jasco J-810 (Easton, MD) with a Peltier-controlled multi-cell holder using a quartz cuvette with a 1 mm path length. Samples were heated at approximately 3°C/min and change in CD ellipticity at 222 nm was monitored. These data were then used to calculate an apparent Tₘ.

![Fig. 1. Cofactor reduction by glucose 1-dehydrogenase.](image-url)
Kinetic stability at different temperatures

Pure, dialyzed GDHs at 50 µg/ml were incubated at the different temperatures for 1 h. After incubation, the residual activity was measured as described earlier.

Half-life in organic solvent For half-life experiments, samples were prepared in 20% v/v organic solvent and incubated at 25°C. Residual activity was measured—without organic solvent—until approximately 10% enzymatic activity remained. Half-life was determined by first-order deactivation kinetics.

Kinetic stability in organic solvent C°

A combination of: (i) concentrated NAD\(^+\) and D-glucose in pH 8.0 50 mM sodium phosphate, (ii) pH 8.0 50 mM sodium phosphate, (iii) organic solvent and (iv) pure, dialyzed enzyme were mixed to obtained different aqueous-organic cosolvent compositions. Mixture was assayed immediately upon enzyme addition.

Kinetic stability in organic solvent C°

Different proportions of 5 mM sodium phosphate (to minimize buffer precipitation) and organic solvent by %v/v were made and pre-cooled to 4°C. Pure enzyme was then added to result in 50 µg/ml and the desired %v/v of organic solvent. Samples were incubated for 1 h at 4°C after which the residual activity was measured.

Results and discussion

Four GDH variants (Vazquez-Figueroa et al., 2007) were selected to study salt and organic solvent effects: triple (P45A, F155Y and V227A), single (E170K), double (E170K, Q252L) and septet (P45A, N46E, F155Y, E170K, V227A, W230F and Q252L) variants, termed GDH1, GDH2, GDH3 and GDH4, respectively. These variants were selected since they all had enhanced stability when compared to the wild-type, with half-lives of ~0.05, ~0.02 and ~5000 min at 65°C (300 mM NaCl, 250 mM imidazole, 50 mM sodium phosphate pH 8.0) for GDH1, GDH2 and GDH3/GDH4 (GDH3 and GDH4 have similar kinetic stability under these conditions). The wild-type was not selected due to its low stability and thus difficulty to study at high temperatures, in the presence of destabilizing salts, or in organic cosolvents. These variants will allow us to test a broad range of kinetic stability and to study their behavior in the presence of salts and organic cosolvents. All GDHs studied contain an N-terminal His-tag to facilitate purification.

Salts effects on glucose dehydrogenase

Previous work demonstrated that enzyme deactivation in chaotropic solutions correlated exponentially with anion B-viscosity coefficients, a measure of ion hydration (Broering and Bommarius, 2005). Moreover, an asymptotic stability is observed in the kosmotropic regime (Broering and Bommarius, 2005), which suggests an optimal stability obtainable in salt solutions. For characterization in salt solutions, we decided to test GDH in sodium salts along the Hofmeister series: I\(^-\) (−0.068 M\(^-1\)), NO\(_3\)\(^-\) (−0.046 M\(^-1\)), Br\(^-\) (−0.032 M\(^-1\)), Cl\(^-\) (−0.007 M\(^-1\)), HCO\(_3\)\(^-\) (0.052 M\(^-1\)), F\(^-\) (0.1 M\(^-1\)), SO\(_4\)\(^2-\) (0.208 M\(^-1\)) and CH\(_3\)CO\(_2\)\(^-\) (0.25 M\(^-1\)).

B-viscosity coefficient values are shown in parenthesis for each anion—chaotropes have B<0 and kosmotropes B>0. Residual activities were measured at 65°C and the observable deactivation rate constant (k\(_{d,obs}\)) calculated based on first-order deactivation kinetics. The logarithm of the deactivation constant, log (k\(_{d,obs}\)), was plotted against the B-viscosity coefficients (Fig. 2). GDH1 was too unstable at 65°C to be appropriately characterized.

It is evident from the data that the GDH variants do not behave entirely as observed previously (Broering and Bommarius, 2005), with the characteristic negative linear correlation in the chaotropic regime with B-viscosity coefficient and constant log (k\(_{d,obs}\)) in the kosmotropic regime. Though both GDH3 and GDH4 appear to exhibit a lower bound for the value of log (k\(_{d,obs}\)), consistent with prior observations (Broering and Bommarius, 2005), only the strongest chaotrope tested (NaI) significantly decreased protein stability [i.e. increased log (k\(_{d,obs}\))]. In addition,
GDH3 and GDH4 show a slight decrease in stability, up to a 2-fold increase in $k_{d,\text{obs}}$ (or a 10% difference in log ($k_{d,\text{obs}}$)) with increasing B-viscosity coefficient in the kosmotropic regime. The more thermostable variants (GDH3 and GDH4) demonstrate high stability regardless of salt-type at both water activities $a_w$ of 0.99 and 0.95. Only NaI solutions demonstrate significant destabilizing effects at 0.95 water activity, again consistent with previous observations of a decrease in kinetic stability with increasing chaotropic salt concentration (Broering and Bommarius, 2005). The kinetic stability of GDH2 depended strongly on salt type. The $k_{d,\text{obs}}$ value changed up to 511-fold between salts ($\Gamma$ versus $\text{Cl}^-$ at 0.95 water activity). The same behavior—though much smaller in magnitude—is observed for GDH3 and GDH4 at $a_w = 0.95$. From these data, we can rank the tolerance of GDHs to deactivation by $\Gamma$ as GDH4 > GDH3 > GDH2.

All GDH variants had increased stability at lower water activity ($a_w$) — increased salt concentrations—in all salts except in iodide. In addition, this stability dependence on salt concentration is clearly observed for GDH2 and less pronounced for GDH3 and even less for GDH4. All in all, it is evident that GDH variants with increased kinetic stability (GDH3 and GDH4) demonstrate less susceptibility to variations in stability based on salt type and salt concentration.

**Stability of glucose dehydrogenase as a function of salt concentration**

The thermodynamic and kinetic stability of GDH1–GDH4 were tested in solutions with NaCl concentrations ranging from 50 mM to 3 M. To test apparent thermodynamic stability, apparent melting temperatures ($T_m$) were recorded at 222 nm via CD. All variants are stabilized with increasing NaCl concentration (Supplementary data are available at PEDS online, Figure S1). Variants with apparent thermodynamic stability rank in a similar order as previously observed in the case of kinetic stability: GDH4 > GDH3 > GDH2 > GDH1.

If the influence of changing salt concentration on proteins fit a Debye–Hückel model, either the log $K$ or the $T_m$ values would yield a straight line when plotted against the square root of the ionic strength: $\log K = A \cdot e^{-\rho \cdot \text{protein} \cdot z_{\text{salt}}} (1)^{1/2}$ (Hine, 1962). $T_m$ values were normalized with respect to the most labile variant, GDH1 (P45A, F155Y and W230F): $T_{m,o} \approx 46.7^\circ C$ (319.9 K) without salt. The ratios $T_m/T_{m,o}$ were then plotted against the square root of the ionic strength of NaCl (Fig. 3A). Excellent linear behavior was observed for the three most stable variants (GDH2–GDH4) with $R^2 > 0.99$. The intercepts rank in the order of thermal stability (from 1.00 for GDH1 to 1.11 for GDH4), while the slopes of the three most stable variants are the same within measurement error. Therefore, thermal stabilization of GDH does not occur via changes of the interaction of the protein surface with the surrounding medium but rather by enhancing cohesive forces in the interior of GDH.

The test of kinetic stability as a function of salt concentration reveals that GDH2 is strongly stabilized by salt concentrations up to 1 M (Fig. 3B). While this trend has been observed earlier on wild-type GDH from *B. megaterium* (Wong et al., 1985), a close relative of GDH from *B. subtilis* utilized here, the exact functional relationship is different. The strong salt dependency of activity of GDH2 (E170K) matches findings by Baik et al. (Baik et al., 2003, 2005). The kinetic stability of GDH3 and GDH4 in the presence of NaCl was virtually identical (Fig. 3B) (Vazquez-Figueroa et al., 2007), whereas the apparent thermodynamic stability (as expressed by apparent $T_m$) of GDH4 was considerably higher than that for GDH3 (Fig. 3A). As has been observed for deactivation of GDH upon changes in pH values, kinetic stability is determined by dissociation of the obligate tetramer into subunits—upon changes in pH values, GDH dissociates into dimers (Baik et al., 2005). In contrast, melting points as a basis for apparent thermodynamic stability register unfolding events that apparently are more difficult to accomplish for GDH4 than for GDH3.

**Organic solvent effects on glucose dehydrogenase**

The stability of the GDH variants was studied in water-miscible organic solvent mixtures. The use of such solvents allows a wide range of aqueous-organic compositions to be considered and avoids deactivation effects due to interfacial denaturation at the aqueous-organic interphase (Owusu and Cowan, 1989; Ghatorae et al., 1994). Other groups have performed extensive studies of the effect of both miscible and immiscible organic solvents on enzymes (Martinek et al., 1977, 1980; Klibanov et al., 1978; Semenov and Martinek, 1980; Semenov et al., 1980). Of interest was the ability to rank miscible solvents based on denaturation capacity (DC)
Furthermore, the approach described by Khmelnitsky et al. (Khmelnitsky et al., 1991) and the observed characteristic sigmoid deactivation behavior (Mozhaev et al., 1989; Khmelnitsky et al., 1991). This sigmoidal drop-off in activity has also been observed in residual activity experiments to estimate $T_\text{50}^0$ values—where values represent the temperature at which 50% activity (subscript) remains after 60 min incubation (superscript)—commonly used for quick screening of enzymes (Nagao et al., 1989; Baik et al., 2003; Reetz et al., 2006). To start, $T_\text{50}^0$ values for GDH1–GDH4 were determined (Fig. 4). GDH demonstrated sigmoidal drop-off behavior and in addition, the $T_\text{50}^0$s increased with increasing apparent $T_\text{m}^0$ (Supplementary data are available at PEDS online, Figure S2). Then a range of six organic solvents were selected along the range of DCs (Khmelnitsky et al., 1991): ethylene glycol, 1,2-propanediol, ethanol, acetonitrile, acetone and 1,4-dioxane—arranged in increasing order of DC (Table I). Reactions containing increasing concentration of organic cosolvent in pH 8.0 sodium phosphate buffer at 25°C were performed. For all solvents except acetonitrile, the initial rate of GDH decreased with increasing organic solvent concentration in a linear matter with consistent $R^2$ values $>0.90$ (Supplementary data are available at PEDS online, Figure S3), unlike the deactivation behavior reported by Khmelnitsky et al. (Khmelnitsky et al., 1991). For the case of acetonitrile, a minimum in rate was observed around 40% volume (%v/v) for two GDH variants. Using the linear fits, $C^0_{505}$—the concentration of organic solvent by %v/v where 50% in initial rate is retained—were determined for all solvents except acetonitrile (Supplementary data are available at PEDS online, Table S1). Although it is evident that the most labile variant (GDH1) has significantly lower stability (as determined by $C^0_{505}$) in ethanol, acetone, and 1,4-dioxane than the others, no particular trend is observed between variant thermostability, organic solvents and $C^0_{505}$. Furthermore, the approach described by Khmelnitsky et al. (Khmelnitsky et al., 1991) is unsuitable for GDH due to the low solubility of glucose, cofactor and buffer at high organic cosolvent concentrations.

Half-lives were obtained to determine kinetic stability in the presence of organic cosolvents. The difficulty encountered was finding appropriate conditions of temperature and organic solvent that would allow appropriate characterization of all the variants—the half-lives of the select variants differ by five orders of magnitude under previously studied conditions (300 mM NaCl, 250 mM imidazole, 50 mM sodium phosphate pH 8.0) (Vazquez-Figueroa et al., 2007). GDH variant half-lives determined at 25°C in 20%v/v organic solvent are shown in Table I. Under these conditions, no deactivation of the three more stable GDH variants was observed in ethylene glycol and 1,2-propanediol. In the case of 1,4-dioxane and acetonitrile, the most labile variants denatured too quickly to measure half-lives effectively. However, for ethanol, acetonitrile, acetone and 1,4-dioxane, the more thermostable variants show increased stability (as demonstrated by half-lives) at 20%v/v organic solvent.

Next, study deactivation by the organic solvent without interference from thermal effects, protein deactivation was characterized at 4°C. This temperature was selected to prevent the sample from freezing (some freezing was observed upon sample perturbation at $>95\%$ v/v for 1,4-dioxane which has a freezing point of 12°C (Smallwood, 1996)), and at this temperature no significant loss of activity was observed for all variants after incubating for 1 h (Fig. 4). GDH was added to increase organic solvent concentrations, incubated for 1 h at 4°C and the residual activity measured in aqueous buffer (pH 8.0, 50 mM sodium phosphate)—analogous to residual activity on the temperature scale, $T_\text{50}^0$. The residual activity was then normalized with respect to the activity measured after 1 h without organic solvent (Fig. 5). It is evident that a minimum in activity is observed around 50%v/v for all variants in all solvents studied and all GDH variants show significant activity in $>80\%$v/v of organic solvent. The inverted bell-shape has also been observed by Griebenow and Klibanov (Griebenow and Klibanov, 1996) for subtilisin and lysozyme. The authors studied $\alpha$-helix content via FTIR in THF, 1-propanol and acetonitrile. They concluded that in aqueous-organic solvent mixtures two effects simultaneously act on protein stability: (i) the propensity of a protein to denature increases with the increase in organic solvent content and (ii) as water content decreases, protein conformational mobility decreases and thus its ability to acquire the thermodynamically dictated conformation. We surmise that, analogous to lysozyme and subtilisin, GDH has a higher propensity to denature at high organic solvents but it is kinetically entrapped in a folded and thus active state. This is further corroboration of Griebenow and

**Table I.** Half-lives (min) for GDHs at 25°C in 20%v organic solvent

<table>
<thead>
<tr>
<th>Solvent (DC)</th>
<th>GDH1 (P45A V155F E170K V227A W230F Q252L)</th>
<th>GDH2 (E170K)</th>
<th>GDH3 (E170K Q252L)</th>
<th>GDH4 (P45A N46E F155Y E170K V227A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene glycol (18.7)</td>
<td>291</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1,2-propanediol (38.8)</td>
<td>462</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ethanol (54.4)</td>
<td>28.5</td>
<td>37.5</td>
<td>430</td>
<td>27397</td>
</tr>
<tr>
<td>Acetonitrile (64.3)</td>
<td>$&lt;1^c$</td>
<td>$&lt;1$</td>
<td>1.69</td>
<td>19.0</td>
</tr>
<tr>
<td>Acetone (78.2)</td>
<td>3.9</td>
<td>10.0</td>
<td>311</td>
<td>11533</td>
</tr>
<tr>
<td>1,4-dioxane (92.1)</td>
<td>$&lt;1$</td>
<td>15.2</td>
<td>101</td>
<td>182</td>
</tr>
</tbody>
</table>

$^a$Denaturing Capacity as calculated by Khmelnitsky et al. (Khmelnitsky et al., 1991).

$^b$No deactivation was observed after 20 days.

$^c$Deactivation occurred immediately upon addition of enzyme to aqueous-organic medium.
Klibanov’s (Griebenow and Klibanov, 1996) findings, with a larger, more complex, homotetrameric enzyme. In the regime of low organic solvent content (<50%v/v), all GDH variants seem to decrease in residual activity with increasing organic solvent content. In this regime, $C_{50}^{60}$ data at 4°C (analogous to $T_{50}^{60}$)—the concentration in which 50% activity remained (subscript) after 60 min incubation (superscript)—were obtained (Supplementary data are available at PEDS online, Table S2). These $C_{50}^{60}$ values were then plotted against the apparent $T_m$ and $T_{50}^{60}$ values (Fig. 6). A strong correlation between thermal stability and organic solvent stability exists, as was noted by Cowan et al. (Owusu and Cowan, 1989; Cowan, 1997) for homologs found in nature, with correlation coefficients $R^2 > 0.80$ and $>0.95$ between $C_{50}^{60}$ values and $T_m$ and $T_{50}^{60}$, respectively. To our knowledge, this is the first time a linear correlation ($R^2$ values $>0.95$) between $T_{50}^{60}$ and $C_{50}^{60}$ has been reported and that an enzyme variant constructed via a consensus-based approach resulted in improved organic solvent and chaotropic-salt stability.

**Conclusions**

In summary, we have demonstrated that previously generated thermostable GDH variants constructed via the structure-guided consensus method (Vazquez-Figueroa et al., 2007) show increased stability in the presence of increasingly high levels of salt and organic cosolvents. The most temperature-stable variants, GDH3 and GDH4, even showed increased stability in the presence of $I^-$, $NO_3^-$ and $Br^-$, three chaotropic anions.

As previously observed, increased NaCl concentrations clearly stabilized GDH (Wong et al., 1985; Nagao et al., 1989; Baik et al., 2003; Baik et al., 2005), as demonstrated by increased $T_m$ and lower $k_{d,obs}$ values. However, in the presence of NaCl, the higher apparent $T_m$ observed for GDH4 versus GDH3 did not translate to lower $k_{d,obs}$ values—a minimum in kinetic stability was obtained. In the absence of NaCl, GDH4 is more stable kinetically ($T_{50}^{60}$) and thermodynamically ($T_m$) than GDH3. Under previous experimental conditions (300 mM NaCl, 250 mM imidazole, pH 8.0 50 mM sodium phosphate), this was not observed due to the presence of NaCl (Vazquez-Figueroa et al., 2007). Consequently, the incorporation of five additional mutations (N45A, N46E, F155Y, V227A and W230F) did in fact result
in overall increased stability, further proof of the predictive power of consensus-based methods.

The most stable variant, GDH4 (P45A, N45E, F155Y, E170K, V227A, W230F and Q252L) showed a >2500-fold increase in tolerance—as determined by half-lives—to 20%v/v acetone, acetonitrile and 1,4-dioxane at 25°C when compared to GDH1 (P45A, F155Y and V227A). In addition, a 1.7-, 3.5- and 5.7-fold improvement in $\epsilon_{50}$ values at 4°C were observed for ethanol, acetone and 1,4-dioxane for GDH4 relative to the GDH1.

While our results do not elucidate the factors governing GDH activity at high temperature, in salt solutions, or organic solvents, we think that the concept of corresponding states is applicable to GDH (Jaenicke and Zavodszky, 1990; Zavodszky et al., 1998; Griebenow et al., 2001). It has been shown that enzyme conformational mobility is a key parameter allowing catalysis under given, distinct conditions. Zavodszky et al. (Zavodszky et al., 1998) and Griebenow et al. (Griebenow et al., 2001) have demonstrated how enzyme function is maintained—at high temperatures and in the presence of organic solvents, respectively—as long as native-like conformational mobility and stability is preserved. Consequently, thermostable GDH4 is able to preserve its mobility and stability under denaturing conditions due to increased structural stability incurred previously via mutagenesis (Vazquez-Figueroa et al., 2007). Importantly, kinetically very stable GDH variants, here GDH4, show the least dependence on variation of salt type and concentration or the level of water-miscible organic solvent.

The existence of a correlation between temperature and organic solvent has been shown in naturally occurring homologs of enzymes (Owusu and Cowan, 1989; Cowan, 1997) and for a thermostable enzyme variant obtained via directed evolution (Hao and Berry, 2004). However, this is the first time a consensus-based method has been demonstrated successfully to improve enzyme stability in the presence of organic solvents and chaotropic salts. In contrast to traditional directed evolution methods where a large library has to be generated, tested for diversity, and screened for improved stability, consensus-based methods are of interest due to their predictive power, limited number of variants generated, high success rate and ease of use. The aforementioned results demonstrate how consensus-based methods show promise in the design of organic solvent and chaotropic salt-tolerant enzymes. This is of great value to the protein engineer since enzyme deactivation in chaotropic salts and organic solvents is prevalent and deactivation pathways are not yet well understood.

Acknowledgements

The authors would like to thank the Bacillus Genetic Stock Center (Daniel R. Zeigler, Columbus, OH, USA) for providing B. subtilis strain 168. The authors would also like to acknowledge Aaron Englehart and Professor Nicholas V. Hud for training and advice on CD. V.Y. gratefully acknowledges assistantships from the Presidential Undergraduate Research Award (Georgia Institute of Technology) and the Undergraduate Research Scholar Program (Parker H. Petit Institute for Bioengineering and Bioscience). E.V.-F. gratefully acknowledges assistantships from the Goizueta Fellowship and the NSF Graduate Research Fellowship.

References


