DOI: 10.1002/cbic.201402501

Directed Evolution of a Formate Dehydrogenase for Increased Tolerance to Ionic Liquids Reveals a New Site for Increasing the Stability

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The formate dehydrogenase (FDH) from *Candida boidinii* is a well-known enzyme in biocatalysis for NADH regeneration. Nevertheless, it has low activity in a water-miscible ionic liquid (1,3-dimethylimidazolium dimethyl phosphate, [MMIm][Me_2PO_4]). In this work, this enzyme was subjected to directed evolution by using error-prone PCR, and a mutant (N187S/T321S) displaying higher activity was obtained following selection based on the formazan-based colorimetric assay. The mutation N187S is responsible for improved activity both in aqueous solution and in [MMIm][Me_2PO_4], through an enhancement of the k_{cat} value by a factor of 5.8. Fluorescence experiments performed in the presence of a quenching agent revealed that the mutant does not unfold in the presence of 50% (*v*/*v*) [MMIm][Me_2PO_4] whereas the wild-type enzyme does. Molecular modelling revealed that the mutation is located at the monomer–monomer interface and causes an increase in the pK_a of residue E163 from 4.8 to 5.5. Calculation of the pK_a of this residue in other microbial FDHs showed that thermostable FDHs have a highly basic glutamate at this position (pK_a up to 6.2). We have identified a new site for improving FDH thermostability and tolerance to ionic liquids, and it is linked to the local charge of the enzymes in this class.

Introduction

Biocatalysis in neat or aqueous mixtures of ionic liquids (ILs) has been a growing research field since the 2000s.[1–4] ILs generally have low vapour pressures and expected low toxicities and they stabilise protein structures. Of course, these are general observations that can be influenced by the nature of the ions composing the IL itself. Numerous examples of enzyme-catalysed reactions are now being described, mostly with hydrolytic enzymes (i.e., lipases and esterases) and with ILs that are not miscible with water. In these cases, lipases and proteases were found to catalyse the same reactions with enhanced stability and activity relative to those that found in organic solvents. These reactions are often conducted in water-immiscible ILs—hence with low water content (up to 5%)—in order to prevent enzyme unfolding, but also to allow sufficient protein flexibility to achieve catalysis.

More recently, other classes of enzymes such as oxidases and dehydrogenases have been assayed in ILs.[5–8] The substrates (e.g., glucose, oxygen…) and co-substrates [e.g., NAD(P)$(H)^+$] of these enzymes need to form hydrogen bonds with the solvent in order to be efficiently solubilised.[9,10] Therefore, aqueous solutions of water-miscible ILs, or neat ILs able to form hydrogen bonds with the enzyme and the substrate, are often preferred. With a few exceptions, all the oxidoreductase-catalysed reactions in ILs are conducted in low-to-medium concentrations of ILs in aqueous solutions. In aqueous IL systems, it is now generally accepted that small chaotropic cations such as *N*,*N*-dimethylimidazolium ([MMIm]) or *N*-ethyl-*N*-methylimidazolium ([EMIm]) and large kosmotropic anions such as alkyl sulfates ([RO-SO_4]) or dialkyl phosphates ([RO,R'O-PO_2]) stabilise protein structures by following the Hofmeister series and the related B-coefficient of viscosity.[11–13] NAD(P)H-dependent enzymes in particular are frequently used in biocatalysis for the asymmetric reduction or reductive amination of ketones to alcohols or amines, respectively.[14,15] To be efficient, a dehydrogenase should be present to reduce the NADH in turn to NAD^+. This so-called recycling system is usually achieved with a formate dehydrogenase (FDH) or glucose dehydrogenase (GDH), to catalyse the oxidation of the inexpensive formate or glucose, respectively.

Another area of interest concerning ionic liquids is in increasing the solubilities of hydrophobic substrates with the aim of achieving high space-time yields (STYs). Therefore, the development of enzymes or methods to improve enzymatic

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201402501.

activities in high concentrations of ILs appears to be crucial. In a previous approach, we grafted hydroxylated cations (cholinium and (hydroxyalkyl)methylimidazolium) activated by 1,1′-carbonyldiimidazole onto the lysine residues of the FDH from *Candida boidinii* (*Cb*FDH) through carbamate linkages.[5,16] By this approach we were able to demonstrate that the stability of the FDH in [MMIm][Me_2PO_4] increases as the size of the grafted cation decreases. Inspired by this work, Camplo and co-workers modified lysozyme by using a phosphonium-based IL containing a succinimidyl activated ester.[17] Lipases from *Candida antartica* and from pork pancreas (PLL) were also modified with imidazolium-functionalised (carboxylated) ILs.[18,19] The resulting biocatalysts were found to show higher activities than the unmodified one. The chemical modification of subtilisin Carlsberg by the comb-shaped PEG (PM13), exhibiting anhydride moieties, allows for recovery of the transesterification activity of this enzyme in [EMIm][Tf_2N].[20] Additionally, the covalent grafting of horseradish peroxidase (HRP) in *N*,*N*′-methylenebis(acrylamide)/*N*-ethyl-*N*-vinylimidazolium copolymer ([VEIm]) leads to polymerised IL microparticles (pIL-MPs) that display improved activity due to the presence of the stabilising EMIm cation during polymerisation.[21] Recently, Nordwald and Karr have also proposed that enzymes could be stabilised in the presence of ILs when modifying the enzyme surface charge by chemical grafting of acetyl, succinyl, amine or ethyl moieties.[22,23]

Nevertheless, all of these methods require biocatalyst preparation before use, often through anhydrous activation procedures, and their feasibility on preparative or industrial scales will probably be limited. As an alternative, improvement of the biocatalyst might be achievable through mutagenesis of the protein sequence. The panel of available techniques to obtain new enzymes is now more extensive, ranging from site-directed mutagenesis (SDM), allowing the exchange of one amino acid for another, to more recent methods of directed protein evolution such as error-prone PCR (epPCR), iterative saturation mutagenesis (ISM) or DNA shuffling.[24] The choice of method should be related to the desired property. SDM and ISM, for example, are more appropriate when enzymes with enhanced activity or enantioselectivity are screened because mutations are mostly concentrated on or near to the enzyme active site. With regard to other properties, such as thermal stability or solvent tolerance, the locations of beneficial mutations are often far from the active site. The enzyme region where the mutation should be introduced is then difficult to predict. In such a case, epPCR, error-prone rolling cycle amplification (epRCA) and DNA shuffling are more appropriate.

Surprisingly, despite the number of published papers dealing with ILs or with directed evolution, and in view of the importance of formate dehydrogenase as a biocatalyst for cofactor recycling, we did not find any attempt to direct evolution of *Cb*FDH for improved properties in ILs. In fact, only a very few works deal with improved enzymatic activities in ILs. The first describes the inhibition of the monooxygenase P450 BM-3 by an IL cation (*N*-methyl-*N*-octylimidazolium [OMIm]).[25] The F87A variant obtained by a directed evolution strategy is less tolerant towards the IL, probably because OMIm binds more easily to the active site. Two cellulases—the thermophilic Cel^5A from *Thermotoga maritima* and Cel^2A, a cellulase isolated from the metagenomic library and related to *Clostridium cellulovorans* glycosyl hydrolase (family 9)—that are promising biocatalysts for the depolymerisation of cellulose were also evolved by use of carboxymethylcellulose or 4-methylumbelliferone-β-D-cellobioside, respectively, as reporter substrates.[26,27] ILs assayed were 1-ethyl-3-methylimidazolium acetate or a deep eutectic solvent (choline chloride/glycerol). In both cases, beneficial mutations were located far from the substrate binding and were related to an improved tolerance of higher ionic strength by the enzyme. Interestingly, the same mutation (H→R) is observed in both papers. Finally, improvement of the activity of a laccase from *Trametes versicolor* in the presence of 15% [EMIM][$EtSO_4$] has been described.[28]

With regard to *Cb*FDH, variants were produced essentially by site-directed mutagenesis in order to improve its resistance to shear stress or to use formate oxidation with $NADP^+$ instead of the naturally occurring substrate NAD^+. The in vitro evolution of *Cb*FDH has only been described once, with the aim of increasing the enzyme's resistance to acrylamide during polymerisation in an immobilisation procedure.[29] A variant displaying three mutations (E53V, K56R, C23S) showed a 4.4-fold increase in its activity once entrapped in 8% (*w*/*v*) polyacrylamide hydrogel. In this variant, the C23S mutation appears to confer the greatest improvement in acrylamide resistance, probably because the C23 residue no longer reacts to form the β-propionamide thioether derivate.

In continuation of our previous studies directed towards improved activity of dehydrogenases in [MMIm][Me_2PO_4], here we describe the directed evolution of *Cb*FDH. Moreover, relevant variants with improved activity in the presence of [MMIm][Me_2PO_4] are kinetically and structurally characterised.

Results and Discussion

Choice of evolution method

The *Cb*FDH gene was obtained from a commercial source and included codons optimised for expression in *Escherichia coli* cells (See Figure S1 in the Supporting Information). The choice of directed evolution strategy was aided by the mutagenesis assistant program (MAP, http://map.jacobs-university.de/MAP.html), with use of degenerate primer-based sequencing for the *Cb*FDH.[30] From our previous work dealing with the grafting of cations on the *Cb*FDH surface, we decided to favour a method that preferentially introduces charged amino acids onto the *Cb*FDH sequence, hypothesising that the enzyme–IL interactions could be optimised.[5,16] Five methods of random mutagenesis to introduce favourably charged amino acids into the FDH sequence were identified (Table 1): error-prone PCR methods in the presence of manganese (three methods differing by dNTP balance), PCR with nucleotide analogues (dPTP and 8-oxodGTP) and chemical mutagenesis (ethyl methanesulfonate). The chemical method was initially ruled out because it induces only two transversions (G→C and C→G). Similar considerations were taken into account for PCR with

Table 1. Chemical diversity indicator of *Cb*FDH obtained by five random mutagenesis methods.[a]

	Stop [%]	Charged [%][b]	Neutral [%][b]	Aromatic [%][b]	Aliphatic [%][b]
chemical distribution	0	29.40	21.43	7.97	41.21
Taq (+, G=A=C=T)[c]	+2.7	+1.5	−9.3	−10.8	+15.8
Taq (+, G≫A=C=T)[c]	+2.0	+1.6	−8.6	−11.4	+16.3
Taq (+, G=T, A=C)[c]	+2.4	+1.6	−8.6	−10. 8	+15.4
Taq (dPTP/8-oxodGTP)[c]	+0.8	+3.2	−8.3	−12.5	+16.8
EMS[c]	+2.3	+1.6	−6.7	−10.7	+13.6

[a] Data obtained with the MAP program. [b] Charged amino acids are D, E, H, K and R, neutral amino acids are C, M, P, S, T, N and Q, aromatic amino acids are F, Y and W, and aliphatic amino acids are G, A, V, L and I. [c] Random mutagenesis methods are summarised in Wong et al.[30]

nucleotide analogues: most of the transversions have low frequencies. Therefore, ep-PCR in the presence of manganese appears to be the most promising evolution method. Moreover, the introduction of stop codons is relatively low (≈2–3%) in comparison with the other methods. It should nevertheless be noted that *Cb*FDH contains a large number of charged amino acids (29.40%). As a result, the introduction of additional charged residues could be rather limited.

The directed evolution of *Cb*FDH was then conducted without a biased concentration of dNTP but in the presence of 250 μM Mn^{2+}. The sequencing of three variants revealed seven mutations corresponding to a mutation frequency of 0.25%: six of them are transitions (A→G×3, T→C×2, C→T×1), and the last is a transversion (C→G×1). These proportions are in accordance with the observed frequency for this mutagenesis method (A→G 32.7%, T→C 31.5%, C→T 5.2%, C→G 0.71%). With regard to the corresponding amino acids, three are aliphatic (T→A, S→G, A→V), two are charged (W→R and T→R), and one is neutral (I→T). The last mutation is silent and does not change the nature of the amino acid in the FDH sequence. These preliminary results show that the method correctly introduced charged amino acids into the *Cb*FDH sequence as proposed by the initial MAP analysis. Additionally no stop codons were observed; this suggests that the *Cb*FDH variants will be expressed as full-length proteins with ≈2.5 mutations per protein.

Screening of *Cb*FDH activity in the presence of [MMIm][Me_2PO_4]

It is commonly acknowledged that the success of a directed evolution study strongly depends on the screening assay. We decided to perform the screening as a two-step process. Firstly, cells expressing active FDH were selected on the agar plate after the colonies had been fixed between two agar layers. FDH activity was observed as large dark colonies, owing to the formazan precipitation in the presence of NAD^+ and formate. Among the 987 isolated clones transformed by the pET-28b-*Cb*FDH plasmid, only 94 (9.5%) showed activity. This low number of active clones is probably due to the way in which the *Cb*FDH binds the formate substrate: the formate is literally clamped between the side chains of N119 and R259 by only two hydrogen bonds, allowing direct electron transfer to NAD^+. Slight structural variations that modify these interactions will certainly lead to inactive variants. Positive clones were assayed for their activity in aqueous media in the 96-well format (Figure 1 A). Three colonies that express the wild-type *Cb*FDH (wells a–c) show specific activities of 35–56 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$. Negative controls (wells d–h) consisting of the empty vector (well e), extraction medium (well d) or inactive colonies in the solid-state screening do not show any significant activity (wells f–h). Nevertheless, some false negatives still occur: a clone in well h showed significant activity (29 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$) despite being considered inactive in the agar-plate assay. This was probably due to substrate diffusion limitations in the agar itself. Unfortunately, we were not able to obtain better screening performances by bypassing the fixation of the cells by the agar layer. Up to now, this is the limiting factor for this screen, which causes about one third of the active colonies to be considered inactive. Nearly all of the positive clones assayed exhibit significant activity (> 2.5 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$), and only 5.4% (five clones) are considered false positives (activity < 2.5 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$); this means that the agar plate assay is efficient for identifying positive clones.

These clones were assayed in the presence of 60% (*v*/*v*) commercial [MMIm][Me_2PO_4] (Figure 1 B). Lower concentrations of ionic liquids were not assayed because the wild-type FDH is active in up to 30% of this ionic liquid and its activity is even slightly enhanced. Moreover, we believed that using a screening assay of high stringency would exclude variants with only moderate improvements that might then be discarded. Under these experimental conditions, wild-type *Cb*FDH demonstrates only residual activity (0.6–5 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$), and negative controls do not present significant activity. Among 88 FDH variants active in aqueous solution, a single one shows significantly improved activity in this IL. Whereas *Cb*FDH has a specific activity of 2.3 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$ (≈5% of residual activity), this variant has a specific activity that decreases from 58 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$ in aqueous solution to 31 $Abs_{560\,nm}\,min^{-1}\,mg^{-1}$ in the presence of 60% [MMIm][Me_2PO_4] (53% of residual activity). This variant is therefore more tolerant and ≈70 times more active than the *Cb*FDH in 60% (*v*/*v*) [MMIm][Me_2PO_4]. Nevertheless, this assay remains a colorimetric titration of the diformazan precipitate and is not representative of the true enzymatic activity (i.e., the reduction of NAD^+).

In order to delve deeper into the enzyme mechanism, both *Cb*FDH and the variant with the gene were sequenced, and the mutation was identified before being over-expressed and purified to homogeneity (see Experimental Section).

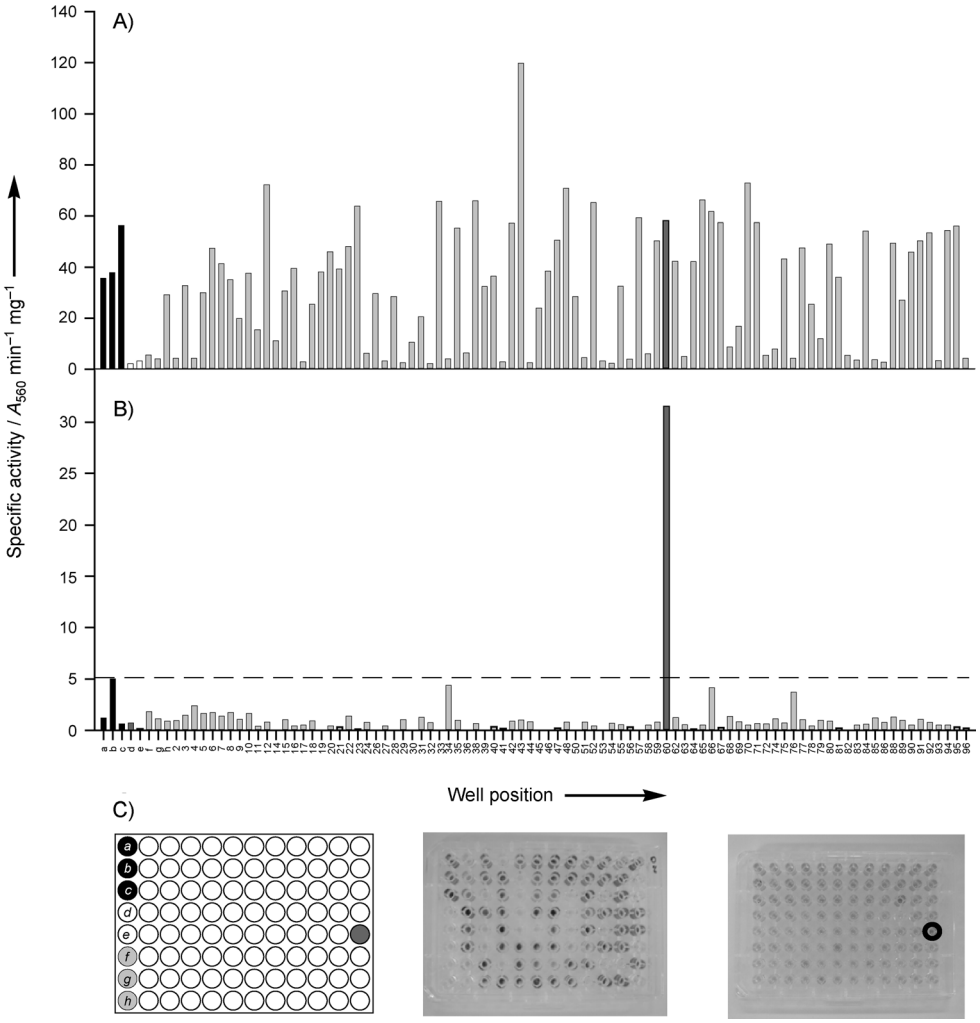

Figure 1. Screening of 88 FDH variants for improved FDH activity in [MMIm][Me_2PO_4], as determined by the formation of reduced formazan (see the Experimental Section). The specific activities of FDH variants were determined A) in PBS, or B) in the presence of 60% [MMIm][Me_2PO_4]. C) Layout of the microtitre plate is also depicted (left): photographs of the plate after 5 min reaction time in PBS (middle) and in 60% [MMIm][Me_2PO_4] (right). The dotted line in (B) represents minimum FDH activity in [MMIm][Me_2PO_4]. The FDH variant M60 is circled in (C, right). Wells a, b and c correspond to *Cb*FDH (positive control of FDH activity in PBS), well d contains only extraction buffer, well e contains cell extract from *E. coli* cells transformed with pET-28b (empty vector), and wells f–h are cell extracts of clones that do not present activity in the agar plate assay.

ose column and chromatography on a hydroxyapatite column.[29] SDS-PAGE analysis showed a homogenous protein band with a molecular mass of approximately 41 kDa for the *Cb*FDH and mutants; this is consistent with previous studies of monomeric FDHs.[29,31,32]

In the absence of the ionic liquid, the N187S/T321S and the N187S variants each show a threefold increase in specific activity relative to the *Cb*FDH and the T321S variant. The N187S is thus responsible for the improved recovered enzymatic activity. The kinetic constant analysis shows that, in aqueous solution, both enzymes are able to bind NAD^+ with similar $K_M^{NAD^+}$ values (75 ± 22 μM vs. 37 ± 20 μM), but that the k_{cat} of the variant N187S/T321S is three times higher than the value for *Cb*FDH (42.3 vs. 12.8 min^{-1}; Table 2). The variants T321S and N187S behave like the wild-type *Cb*FDH and N187S/T321S, respectively. The T321S mutation is more difficult to understand because it seems to contribute to the improvement of the k_{cat} value but also seems to restrain the improvement of k_{cat} due to the N187S mutation. Because of the only slight variation of the kinetics parameters due to the T321S mutation, this was not further investigated.

Characterisation in aqueous solution

Sequencing of the gene in the variant showed two base transitions, A→G and T→C, in positions 560 and 930, respectively; they correspond to the highest frequencies observed with this evolution method (Table 1). These mutations correspond to the N187S and T321S substitutions. Firstly, it is worth noting that none of the mutations led to an increase in the charge of the enzyme as we had previously expected. Secondly, both mutations led to serine residues.

In order to examine the reason for the increased activity shown by the double mutant (N187S/T321S) in the presence of [MMIm][Me_2PO_4], the single-site mutants N187S and T321S were isolated by site-directed mutagenesis. The proteins were purified in three steps: by using an aqueous two-phase system (ATPS), affinity chromatography with a Cibracon blue-Sepharose column and chromatography on a hydroxyapatite column.[29] SDS-PAGE analysis showed a homogenous protein band with a molecular mass of approximately 41 kDa for the *Cb*FDH and mutants; this is consistent with previous studies of monomeric FDHs.[29,31,32]

Table 2. Kinetic parameters of *Cb*FDH and variants.[a]

	*Cb*FDH	N187S/T321S	T321S	N187S
$K_M^{NAD^+}$ [μM]	75 ± 22	37 ± 20	84 ± 20	80 ± 21
k_{cat} [min^{-1}]	12.8 ± 0.2	42.3 ± 0.2	26.6 ± 0.2	75 ± 0.5
$k_{cat}/K_M^{NAD^+}$ [min^{-1} μM^{-1}]	0.17	1.14	0.31	0.93

[a] Determined from the Hanes–Woolf plot ($R^2 > 0.99$). Reaction conditions: phosphate buffer 20 mM, pH 7.2, [formate] 300 mM, [NAD^+] 0–5 mM, 30 °C.

Characterisation in [MMIm][Me_2PO_4]

Because the improved properties of mutants are related to an improved k_{cat} value and because K_m is only slightly affected, the purified *Cb*FDH and the variants were assayed in the pres-

ence of 0–70% (*v*/*v*) of [MMIm][Me_2PO_4] by directly measuring the formation of NADH at saturating substrate concentration (Figure 2).

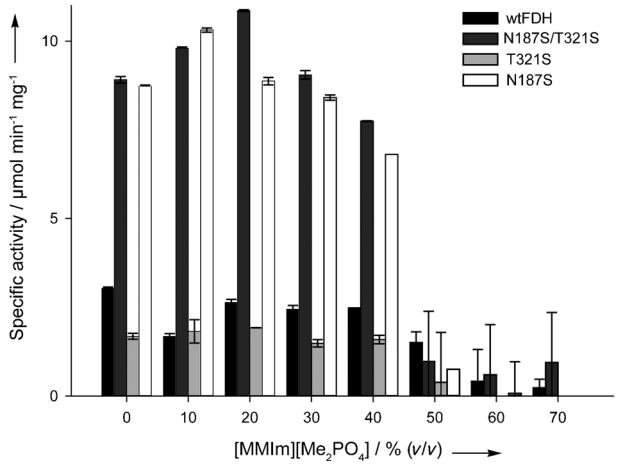

Figure 2. Specific activities of purified *Cb*FDH and N187S/T321S in the presence of 0–70% (*v*/*v*) [MMIm][Me_2PO_4]. [NAD^+] 5 mM, [formate] 300 mM, phosphate buffer pH 7.6, 20 mM.

The determination of enzymatic activity was conducted with [MMIm][Me_2PO_4] synthesised in our laboratory. Thanks to the low temperature used during the synthesis process (80 °C), and also to its protection from light, the resulting product is an achromatic liquid, unlike the yellow commercially available varieties (see the Supporting Information). In fact, commercially available ionic liquids are not sufficiently pure for these purposes and some contain contaminants that can impede FDH activity.[33,34] Our synthesised ionic liquid, however, was shown to have a purity of 97%. Furthermore, absorption spectroscopy experiments verified that the synthesised ionic liquid absorbs less than the commercially available versions and is therefore more optically pure. It is important also to mention that the presence of water in the medium would reduce the concentration of the ionic liquid. Absorption spectroscopy and ^{1}H NMR were used to determine the amount of water present by observing its absorbance at 975 nm and 983 nm. The results suggest that [MMIm][Me_2PO_4] is not significantly hydrated (see the Supporting Information).

At 30% (*v*/*v*) [MMIm][Me_2PO_4], the double mutant still possessed 100% of its activity, as shown by its specific activity of 9.04 $\mu mol\,min^{-1}\,\mu g^{-1}$ (Figure 2). Likewise, the single mutant N187S retained 96% of its activity. At this same concentration, the wild-type *Cb*FDH had lost 20% of its activity, whereas the single mutant T321S showed a decrease in activity of 12%. Moreover, in the presence of low concentrations of [MMIm][Me_2PO_4] (10–40%, *v*/*v*), both the wild-type *Cb*FDH and its mutants were active but the double mutant and the single mutant N187S were nearly four times more active, thus suggesting that the single mutation (N187S) is responsible for the tolerance of this medium by the double mutant. At high concentrations of [MMIm][Me_2PO_4] (50–70%, *v*/*v*), however, we could state that there is a slight improvement in the double mutant's activity in relation to the native *Cb*FDH and the single mutants, but it is likely that these results are not significant. Therefore, the selection of the mutant in the plate assay could be due to a high initial velocity (and consequently to higher product formation) rather than to a true tolerance of the IL.

Thermostabilities of FDH variants

Certain authors believe that a mutation that increases the tolerance of an enzyme in an ionic liquid could also favourably strengthen the enzyme's thermal stability.[26,35] We wanted to investigate further whether the mutations present in the double mutant, which had increased its activity in the presence of [MMIm][Me_2PO_4], might also have a positive effect on its thermal stability. We decided to test the thermal stabilities of the wild-type *Cb*FDH and of the double mutant at points above and below this range (see the Supporting Information). At 40 °C, the wild-type FDH showed a higher rate of reaction than the double mutant; however, the activities of both enzymes remained relatively stable for the first 90 min with no significant differences observed. After 2 h, the wild-type *Cb*FDH had retained 44% of its initial activity whereas the double mutant retained 62% of its initial activity. At 60 °C, both enzymes retained approximately 10% of their activity after 15 min. These results suggest that although the double mutant is more tolerant of the ionic liquid, its thermal stability in aqueous solution is comparable to that of the wild-type *Cb*FDH and shows no significant improvements, within experimental error.

[MMIm][Me_2PO_4] tolerance examined by steady-state fluorescence and molecular modelling

The double mutant and the mutant N187S proved to be more active than the wild-type FDH in [MMIm][Me_2PO_4] (10–40%, *v*/*v*), but their stabilities in high concentrations of the IL (50–70%, *v*/*v*) needed to be examined further. We thus decided to explore stability in terms of structural characteristics for the wild-type FDH and its mutants with the aid of fluorescence spectroscopy and structure modelling.

Possible structural modifications resulting from mutations were evaluated by fluorescence spectroscopy in the absence or in the presence of 50% (*v*/*v*) [MMIm][Me_2PO_4]. Beyond this concentration, the ionic liquid's absorption is too significant, and the fluorescence spectra for the enzyme are not exploitable.[35] Fluorescence quenching was assessed with acrylamide, and K_{sv} values were determined from the Stern–Volmer equation. In aqueous solution, the K_{sv} values were 7.3 M^{-1} for the wild-type FDH, 5.8 M^{-1} for the double mutant, 5.0 M^{-1} for the single mutant N187S and 5.1 M^{-1} for the single mutant T321S (Figure 3). These values are consistent with data previously obtained with commercial *Cb*FDH, which confirms that the enzymes are correctly overexpressed.[36] Fluorescence spectra show that in the absence of [MMIm][Me_2PO_4], the fluorophores for the wild-type FDH and its mutants are similarly exposed to the solvent, therefore their structures in aqueous solution are similar. However, in the presence of 50% (*v*/*v*) of ionic liquid,

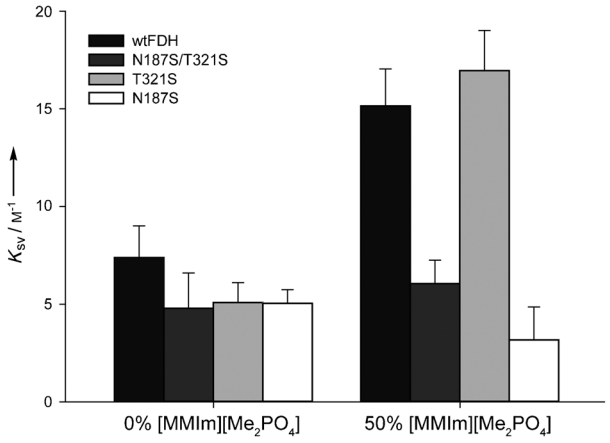

Figure 3. Intrinsic fluorescence attenuation of FDH variants in the presence of acrylamide in 0 and 50% [MMIm][Me_2PO_4] (*v*/*v*) in 20 mM phosphate buffer pH 7.6.

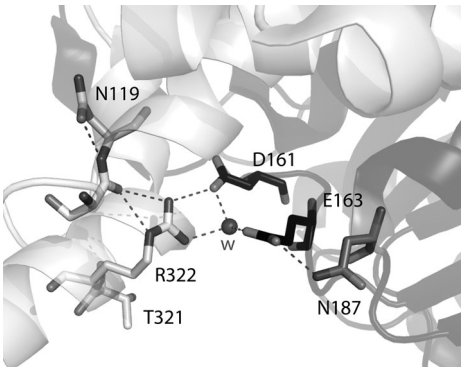

Figure 4. Structure of the interface between monomers in wild-type *Cb*FDH, showing the hydrogen bond network between N187 (dark grey), E163 and D161 (black) residues of one monomer and the catalytic N119 (light grey), R322 and S118 (white) residues of the other monomer. T321 is also depicted as white stick; "w" is a water molecule.

the K_{sv} values were 15.1 M^{-1} for the wild-type *Cb*FDH, 17.0 M^{-1} for the single mutant T321S, 6.1 M^{-1} for the double mutant and 3.2 M^{-1} for the single mutant N187S (Figure 3). The K_{sv} values for the wild-type FDH and the single mutant T321S are two to three times higher than those in aqueous solution, whereas those for the double mutant and the single mutant N187S are stable. These results imply that the wild-type FDH and the single mutant T321S have undergone important structural changes in the presence of [MMIm][Me_2PO_4], thus permitting the acrylamide to access buried tryptophan and tyrosine residues. In contrast, the K_{sv} values for the double mutant and the single mutant N187S suggest that the structural modifications of these enzymes are similar to those seen in aqueous solution. As a result, we are now able to conclude that both the double mutant and the single mutant N187S are more tolerant to the ionic liquid. This also reconfirms that the single mutant N187S is responsible for the double mutant's tolerance of [MMIm][Me_2PO_4].

Although we were unable to confirm a correlation between an increase in ionic liquid tolerance and thermal stability for the double mutant, we were able to use structural modelling to study a previously uncharacterised zone of amino acids surrounding the single mutation in N187S, which we believe is responsible for the double mutant's increased activity in [MMIm][Me_2PO_4]. We discovered that this zone is highly conserved among formate dehydrogenases and is made up (Figure 4) of amino acids forming Chain A [N119 (involved in formate fixation), S118 and R322] and Chain B (D161, E163 and N187). By using different models obtained with Swiss-model along with the PROPKA web interface, we were able to determine small but significant variances in the pK_a values of these amino acids. In particular, E163 in the wild-type *Cb*FDH (PDB 2FSS used as model) has a calculated pK_a of 4.88 (Figure 5). When positioned next to the single mutation T321S, its pK_a of 4.87 showed no discernible difference from that of the wild-type FDH. However, we observed an important increase in its pK_a after insertion of the single mutation N187S, thus rendering E163 less acidic. The pK_a values increased to 5.48 for the double mutant and 5.49 for the single mutant N187S. The hydrogen bond to E163 is clearly influenced by N187. Increased basicity of E163 means that the pK_a values become closer to those observed in already described thermally stable organisms such as *Pseudomonas* sp101 and *Thiobacillus* (pK_a value of 6.2 in each case).[32] This suggests that the thermostability increases with the disappearance of the local charge and therefore increases the hydrophobic interactions at the interface. The tolerance of the [MMIm][Me_2PO_4] could then be explained in terms of fewer ionic interactions between the IL ions and FDH residues located at the interface.

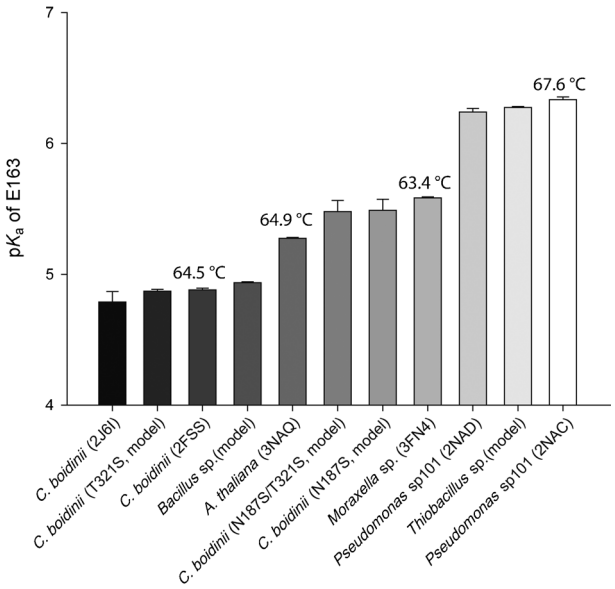

Figure 5. Estimation of E163 pK_a values (or of equivalent residue) of formate dehydrogenases from *Candida boidinii*, *Bacillus* sp., *Arabidopsis thaliana*, *Moraxella* sp., *Pseudmonas* sp101 and *Thiobacillus* sp. by PROPKA. Structures were obtained from the PDB when a PDB ID is reported between brackets or generated with SWISS-MODEL (labelled as model). Melting temperatures (T_m) determined by Sadykhov are reported at the tops of the bars (if available).[37] Error bars were calculated by use of the two pK_a values of each residue of the dimer.

From these results, it is possible that the single mutant N187S and the double mutant are more thermally stable than the wild-type *Cb*FDH. The T_m value of N187S would be expected to be around 1.5 °C higher than that of the wild-type *Cb*FDH, but such a small increase could not be observed with our experimental setup (Figure 5 and the Supporting Information). More sensitive methods should be used in the future to allow full understanding of the thermal stabilities of these enzymes. These results tend to confirm the viewpoint that mutations that improve tolerance of ionic liquids also improve thermostability, and vice versa. Literature describing improved thermostability in formate dehydrogenases is nevertheless rare and, to the best of our knowledge, thermostability is not rationally explained for FDHs. In this study, the E163 residue, together with the dimer interface, have been identified for the first time as relevant actors in the tolerance of FDHs to ionic liquids and thermostability. A more comprehensive model is currently under investigation.

Conclusions

In conclusion, this study has characterised a formate dehydrogenase obtained from *Candida boidinii* by directed evolution and displaying enhanced activity in a water-miscible ionic liquid. The double mutant N187S/T321S and the single mutant N187S were four times more active in aqueous solution and in [MMIm][Me_2PO_4], and catalyse the reaction more efficiently and more rapidly than the wild-type FDH. This explains why this mutant was selected from the selection assay. Fluorescence spectroscopic analysis confirms that the double mutant and the single mutant N187S showed improved tolerance of [MMIm][Me_2PO_4] in comparison with the wild-type FDH. The single mutant N187S seems to increase tolerance of [MMIm]-[Me_2PO_4] by influencing ionic bond formation and reinforcing the dimer stability. These results clearly demonstrate that the activity and stability of a biocatalyst in an ionic liquid can be greatly improved by using our methods. This strategy can be applied to enhance other enzymes with poor performance in water-soluble ionic liquids.

Experimental Section

Materials: Methylimidazole (99%) and trimethyl phosphate (99%) were from Acros. [MMIm][Me_2PO_4] (98%) was from Iolitec GmbH (Denzlingen, Germany). Sodium azide was from Prolabo (Paris, France). Bradford reagent was from Bio-Rad. Phenazine ethosulfate (PES), Nitroblue tetrazolium chloride (NBT) and all other chemicals were from Sigma–Aldrich.

Synthesis of *N*,*N*-dimethylimidazolium dimethyl phosphate: Typically, methylimidazole (1.47 g, 0.018 mmol) was added dropwise under argon to ice-cold trimethyl phosphate (2.52 g, 0.018 mmol). The reaction mixture was allowed to warm to room temperature before being heated at 80 °C for 36 h under argon and protected from light.[33] After cooling to room temperature, the remaining substrate was removed under vacuum over 24 h at room temperature with stirring. The optical quality of the resulting product, [MMIm][Me_2PO_4], was analysed by fluorescence and absorption spectroscopy (see the Supporting Information). The formed product (yield of the isolated product = 97%) was a transparent colourless viscous liquid and was characterised by mass spectrometry (ESI+) with a THERMO LCQ Advantage instrument and by NMR with a Bruker DRX 300. The ^1H, ^{13}C and ^{31}P NMR spectra were recorded in CD_3CN. ^1H NMR (300 MHz, CD_3CN, 25 °C): $\delta = 3.34$ (d, $J_{H,P} = 10.37$ Hz, 6H; CH_3N), 3.85 (s, 6H; CH_3O), 7.72 (d, $J_{H,H} = 1.45$ Hz, 2H; CH=CH), 9.21 ppm (s, 1H; CH); ^{13}C NMR (400 MHz, CD_3CN, 25 °C): $\delta = 36.3$, 52.26 (d, $J_{C,P} = 6.11$ Hz), 124.49, 139.74 ppm; ^{31}P NMR (300 MHz, CD_3CN, 25 °C): $\delta = 1.82$ ppm; MS (EI+): *m/z* 97.1 (100) [$MMIm^+$]; MS (EI$^-$): *m/z* 125.1 (100) [$Me_2PO_4^-$].

Cloning: The *Cb*FDH gene (AJ245935, 1095 base pairs) was commercially synthesised (GenScript, Piscataway, NJ, USA) with the additional sequences 5′-ggatccataaaaggagatatacc-3′ and 5′-ctcgagtctaga-3′ at the 5′- and 3′-extremities of the gene sequence, respectively (see the Supporting Information). The sequence introduced at the 5′-extremity contains a restriction site for BamHI (G/GATC), a stop codon, the Shine–Dalgarno sequence and seven extra nucleotides before the open reading frame. The sequence introduced at the 3′-extremity includes restriction sites for XhoI (C/TCGAC) and XbaI (TCTAGA). The gene was provided as the pUC57-FDH plasmid by the supplier. After amplification and isolation of the pUC57-FDH plasmid by standard procedures, the FDH gene was cloned into the pET-28b expression vector. In brief, pUC57-FDH (2.5 μg) or pET-28b (1 μg) were digested with BamHI (20 Units, 10 U μL^{-1}, Euromedex, Souffelweyersheim, France) and XhoI (20 Units, 20 U μL^{-1}, Ozyme, Montigny-le-Bretonneux, France) in Tris·HCl buffer (pH 7.9, 10 mM), NaCl (100 mM), $MgCl_2$ (5 mM), DTT (1 mM) and BSA (100 μg mL^{-1}) in a final volume of 300 μL for 1 hour at 37 °C. The insert coding for the FDH was isolated by agarose gel electrophoresis and use of the QIAquick gel extraction kit (Qiagen). The insert and digested pET-28b were desalted (QIAquick kit, Qiagen) and incubated together in ratios of 1:1 (85 ng/18 ng) or 1:3 (85 ng/54 ng; vector/insert) in the presence of one unit of T4 DNA ligase (Invitrogen) in Tris·HCl buffer (50 mM, pH 7.6), $MgCl_2$ (10 mM), ATP (1 mM), DTT (1 mM) and PEG-8000 (5%, *w/v*). Ligation medium (2 μL) was used to transform competent *E. coli* BL21 cells by thermal shock (45 s, 42 °C). Cells transformed by the pET-28b-FDH were selected on lysogeny broth (LB)/agar/kanamycin (100 μg mL^{-1}). The presence of the insert was verified by double digestion (using BamHI/XhoI) of the plasmid DNA of six clones (Nucleospin kit, Macherey–Nagel, Hoerdt, France), and the nucleotide sequence was determined by Eurofins MWG Operon (Courtaboeuf, France).

Directed evolution by Ep-PCR in the presence of manganese: Error-prone PCR was performed on pET-28b-FDH vector with the 5′-ttaatacgactcactatagg-3′ and 5′-ccaaggggttatgctag-3′ primers (5 μL, 1 pmol μL^{-1}, Eurofins MWG Operon). The PCR reaction medium consisted of Taq polymerase (2.5 μL, 1 U μL^{-1}, Roche), dNTP mix (5 μL, 2.5 mM each, Invitrogen), pET-28b-FDH (3 μL, 0.267 ng μL^{-1}) and $MnCl_2$ (250 μM final) in Tris·HCl buffer (10 mM, pH 8.3), $MgCl_2$ (1.5 mM) and KCl (50 mM). The PCR product was desalted (QIAquick kit), digested (BamHI/XhoI) and inserted into pET-28b (same protocol as above). Chemically competent *E. coli* BL-21 (New England Biolabs) cells were transformed by thermal shock, cultured in LB liquid medium (960 μL) and selected on LB/agar/kanamycin (100 μg mL^{-1}). The mutation frequency was determined by sequencing three arbitrary chosen clones (not tested for FDH activity).

Selection of active clones: The screening of active clones was adapted from the method described by Ansorge-Schumacher et al.[29] Transformed *E. coli* cells were selected on LB/agar/kanamycin (100 μg mL^{-1}) and replicated on LB/agar/kanamycin (100 μg mL^{-1}) supplemented with IPTG (1 mM) before incubation

overnight at 37 °C. Induced cells were covered with an agar solution in potassium phosphate buffer (pH 7.4, 0.1 M), Triton X-100 (0.2%) and EDTA (10 mM) at a maximum temperature of 70 °C. Once the agar had cooled down to room temperature, cells were permeabilised with potassium phosphate buffer (pH 7.4, 0.1 M, 5 mL), Triton X-100 (0.1%) and EDTA (200 mM). This step was done three times with gentle stirring for about 15 min. The plates were washed three times with potassium phosphate buffer (pH 7.4, 0.1 M) before addition of the substrate solution [3.5 mL, formate (1.25 M), NAD^+ (5 mM) in potassium phosphate buffer (pH 7.4, 0.1 M)]. The reaction was allowed to proceed for 5 min with stirring. NADH formation was revealed by the addition of PES (60 mM, 120 μL) and NBT (6 mM, 200 μL). The reaction was allowed to take place in the dark for 20–30 min. Cells were washed with water, and positive clones appeared as dark spots.

Construction of single mutants: The single mutant N187S was constructed by digesting the pET-28b expression vectors (2 μg) containing the wild-type FDH gene or the double mutant M60 gene with with Pac I and Xho I [2 units each, 20 U μL^{-1}, New England Biolabs, in Bis·Tris·propane·HCl buffer (1 mM, pH 7), $MgCl_2$ (1 mM) and DTT (0.1 mM)] in a final volume of 100 μL, with agitation for 2 h at 37 °C. The insert coding for the FDHwt and the vector coding for the double mutant M60 containing only the N187S mutation were isolated by agarose gel electrophoresis and use of the QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). The insert and the vector were desalted (QIAquick kit, Qiagen) and incubated together in ratios of 1:1 (85 ng/18 ng) or 1:3 (85 ng/54 ng; vector/insert) in the presence of T4 DNA ligase (1 Unit, Invitrogen, Cergy-Pontoise, France) in Tris·HCl buffer (40 mM, pH 7.6), $MgCl_2$ (10 mM), ATP (0.5 mM) and DTT (10 mM) for a final volume of 20 μL for 1 hour at 37 °C. Ligation medium (1 μL) was used to transform competent *E. coli* DH10B cells by electric shock (2.5 kV during 5.7 ms). Cells transformed by the pET-28b-N187S were selected on LB/agar/kanamycin (100 μg mL^{-1}). The correct orientation of the insert was verified by PCR screening of the plasmid DNA of 12 clones (Nucleospin kit, Macherey–Nagel), and the nucleotide sequence was determined by Eurofins MWG Operon (Courtaboeuf, France). The single mutant T321S was constructed in a similar manner by digesting the plasmid DNAs coding for the FDHwt and the double mutant M60 with BamHI (2 Units, 10 U μL^{-1}, New England Biolabs) and Pac I (2 Units, 10 U μL^{-1}, New England Biolabs) and subsequently using the same protocol as above to isolate the single mutation.

Colorimetric screening assay: Cells presenting FDH activity on the agar plate were cultured overnight in LB/kanamycin (1 mL, 100 μg mL^{-1}) from the mother plate in a 96-well culture plate. Protein expression was induced with IPTG (1 mM) for 5 h. Cells were harvested by centrifugation at 2000 *g* for 15 min at 4 °C and resuspended in PBS buffer (500 μL, 0.1 M, pH 7.4, potassium salt), Triton X-100 (0.1%) and EDTA (200 mM, sodium salt) and centrifuged again at 2000 *g* (15 min, 4 °C). The supernatant was used for the screening assay as follows: the reaction medium was composed of supernatant (10 μL), substrate solution [10 μL, NAD^+ (25 mM), formate (1.5 M), PES and NBT solution (10 μL, 140 μM and 1 mM, respectively)] and either 0 or 60% (*v*/*v*) of $[MMIm][Me_2PO_4]$. The final volume was adjusted to 100 μL with PBS buffer (pH 7.2). The formation of NBT diformazan blue precipitate was detected after 5 min by measuring the absorbance at 560 nm. The activity was normalised by determining the protein content in each supernatant by a Bradford assay by the standard procedure. The colorimetric assay was preferred in the screening due to the high absorbance of the IL in the UV region.

FDH purification: Wild-type and mutant FDHs were purified by modifications of protocols by Ansorge-Schumacher et al.,[29] who incorporated a PEG-based ATPS and by Schütte et al.,[31] who used hydroxyapatite gel chromatography and Cibracon blue Sepharose affinity chromatography. Bacterial pellets were resuspended in potassium phosphate buffer (1 mL, 0.1 M, pH 7.6). PEG 400 (30%, *v*/*v*) was added to the suspension, which was homogenised. The mixture was heated for 2 h at 37 °C and then allowed to cool to room temperature (5–10 min). Dipotassium phosphate salt (K_2HPO_4, 5%, *w*/*w*) and water (21%, *w*/*w*) were added. After K_2HPO_4 dissolution, PEG 1550 (7% *w*/*w*) and sodium chloride (6%, *w*/*w*) were added. The solution was mixed until homogenised, and separation into two phases was achieved after 2 h. The upper phase was recovered and mixed with PEG 6000 (20%, *w*/*w*) and water (10%, *w*/*w*). The enzyme precipitated from this solution after 2 to 3 h at room temperature and was recovered by centrifugation at 10000 *g* for 10 min before dissolution in potassium phosphate buffer (60 mM, pH 7.6). This solution was concentrated three times in potassium phosphate buffer (60 mM, pH 7.6) with centrifugal filters (Ultracel YM-30, Millipore, Molsheim, France) before purification by Cibracon blue Sepharose affinity chromatography. After washing with potassium phosphate buffer (60 mM, pH 7.6), the enzyme was eluted by addition of NADH (1 mM) and concentrated three times in potassium phosphate buffer (5 mM, pH 7.6) by the same protocol as before. The enzyme was then purified by hydroxyapatite chromatography. After washing with potassium phosphate buffer (5 mM, pH 7.4), the enzyme was eluted by use of a step gradient in potassium phosphate buffer (20–100 mM, pH 7.6). The FDH was recovered in potassium phosphate (60 mM). Once the protocol was optimised for wild-type FDH, the enzymes were purified five to 24 times with overall yields of 20–70%, depending of the mutation.

Enzyme activity assays: Enzyme activity was measured from the NADH absorbance at 340 nm ($\varepsilon_M^{340\,nm} = 6.22 \cdot 10^3\ M^{-1}\,cm^{-1}$) with a Tecan Infinite M200 (Salzburg, Austria) microtitre plate reader at 30 °C. A single well contained enzyme solution (10 μL, 0.4 U mL^{-1}), NAD^+ solution (10 μL, 50 mM), sodium formate (10 μL, 3 M) and $[MMIm][Me_2PO_4]$ (0–70%, *v*/*v*) in potassium phosphate buffer (20 mM, pH 7.6). Optical pathways were determined by measuring the absorbance of water at 975 nm in each individual well ($\varepsilon_M^{975\,nm} = 3.05\ 10^{-3}\ M^{-1}\,cm^{-1}$). Activities are average values of at least three measurements and are expressed in μmol min^{-1} mg^{-1}. The kinetic parameters (K_m, k_{cat} and k_{cat}/K_m) of the different enzymes were determined with a final concentration of formate at 300 mM and final concentrations of NAD^+ ranging from 25 μM to 5 mM. The parameters were extracted from the Hanes–Woolf plot.

Effect of temperature and of $[MMIm][Me_2PO_4]$ on the stabilities of wild-type FDH and the double mutant: Thermal stabilities were determined by incubating the enzymes (0.04 mg mL^{-1}) at 40 and 60 °C in sodium phosphate buffer (20 mM, pH 7.6). The concentrations of NAD^+ and sodium formate were 5 mM and 300 mM, respectively. Aliquots (160 μL) in 1.5 mL Eppendorf tubes were placed in a water bath (40 or 60 °C). Every 15 min, a sample (90 μL) was removed, and its residual activity was assayed spectrophotometrically as described above. Enzyme stability in the presence of $[MMIm][Me_2PO_4]$ was assayed with a 96-well plate. The enzyme was incubated (0.04 mg mL^{-1}) in sodium phosphate buffer (pH 7.6, 20 mM) containing $[MMIm][Me_2PO_4]$ (62.5%, *v*/*v*). Every 12 h, the residual enzyme activity was determined spectrophotometrically. The concentrations of NAD^+ and sodium formate were 5 and 300 mM, respectively.

Fluorescence: Protein unfolding was evaluated by quenching of the protein fluorescence with acrylamide as previously reported.[36]

Wild-type and mutant FDHs were diluted in potassium phosphate buffer (20 mM, pH 7.6) until a final concentration of 0.32 U mL^{-1} was reached (2.61 μM), for use in all experiments. Enzyme samples were excited at 280 nm (bandwidth = 5 nm), and emission spectra were recorded between 310 and 600 nm (bandwidth = 20 nm). Quenching experiments were performed with various concentrations of acrylamide (0–300 mM), as well as in 0 or 50% [MMIm]-[Me_2PO_4] (*v*/*v*). In all experiments, the fluorescence of the medium devoid of enzyme was subtracted from the enzyme spectra in order to allow for the influence of the solvent on the enzyme fluorescence spectrum. The maximum fluorescence intensities are average values of at least two experiments. The fluorescence intensity at 330 nm was used for K_{sv} determination.

Structure modelling of the wild-type FDH and mutants: Swissmodel (http://swissmodel.expasy.org/) was used to predict structures by identifying FDH structural homologues.[38] Homology modelling was performed with use of the crystal structure of the formate dehydrogenase from *Candida boidinii* (PDB ID: 2fss) as a template for *Cb*FDH mutants. Uniprot accession numbers used are D6CJK0 and Q76EBZ for the generation of structures of FDH from *Bacillus* sp. and *Thiobacillus* sp., respectively. The server PROPKA 3.1 (http://propka.ki.ku.dk/) was used to evaluate pK_a values of individual residues.

Acknowledgements

The French Ministry of Education, Research and Technology is gratefully acknowledged for fellowships (M.B. and J.L.L.C.). Mathieu Deygas is kindly acknowledged for practical help. This work was financially supported by the Réseau de Recherche 2 of the CNRS and by the GDR Synthèse et Procédés durables pour une Chimie éco-compatible.

Keywords: biocatalysis · directed evolution · ionic liquids · pK_a · protein stability

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Received: September 3, 2014
Published online on ■■ ■■, 0000

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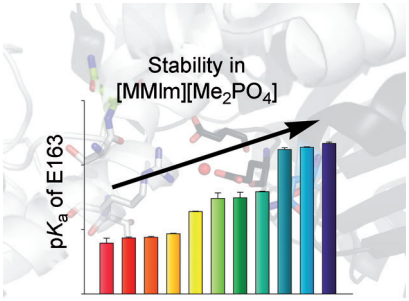
Directed Evolution of a Formate Dehydrogenase for Increased Tolerance to Ionic Liquids Reveals a New Site for Increasing the Stability



A(n ionic) liquid diet: A mutation that improves the stability of formate dehydrogenases in ionic liquids and causes a 5.8-fold enhancement of the k_{cat} value was identified. The site is located at the monomer–monomer interface and influences the local pK_a values, including an increase in the pK_a of residue E163 from 4.8 to 5.5.