Journal of Molecular Catalysis B: Enzymatic 65 (2010) 73–78

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Chemical modifications by ionic liquid-inspired cations improve the activity and the stability of formate dehydrogenase in [MMIm][Me_2PO_4]

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ARTICLE INFO

Article history:
Available online 2 February 2010

Keywords:
Formate dehydrogenase (FDH)
Ionic liquid
Chemical modification
Stability

ABSTRACT

The formate dehydrogenase (FDH, EC: 1.2. 1.2) from *Candida boidinii* was found to be inactivated and unstable in the presence of high concentration (>50%) of the water soluble dimethylimidazolium dimethyl phosphate ([MMIm][Me_2PO_4]) ionic liquid. In order to circumvent this problem, the enzyme was chemically modified by cations usually present in ionic liquids: cholinium (**1**), hydroxyethyl-methylimidazolium (**2**) and hydroxypropyl-methylimidazolium (**3**) cations were activated with carbonyldiimidazole before being reacted with the FDH leading to a heterogeneous population of 6–7 biocatalysts. FDH modified by (**1**) or (**3**) led to 3–9 modifications while FDH modified by (**2**) led to 6 proteins presenting 7–12 grafted cations. Specific activity of the modified enzymes was decreased by a 2.5–3-fold factor (0.10–0.15 μmol min^{-1} mg^{-1}) compared to the non-modified FDH (0.33 μmol min^{-1} mg^{-1}) when assayed in carbonate buffer (pH 9.7, 25 mM). After modification, the FDH still present 0.06 μmol min^{-1} mg^{-1} in 70% [MMIm][Me_2PO_4] (v:v) (30–45% of their activity in aqueous buffer) while the native enzyme is inactive at this ionic liquid concentration, proving the efficiency of this strategy. The half-life of the modified enzyme is also increased by a 5-fold factor after modification by (**1**) ($t_{1/2}$ of 9 days) and by a 3-fold factor after modification by (**2**) or (**3**) ($t_{1/2}$ of 6 and 5 days respectively) in aqueous solution. When stored in 37.5% [MMIm][Me_2PO_4] (v:v), both modified and unmodified FDH have an increased half-life ($t_{1/2}$ of 6–9 days). This grafting strategy is found to be good methods to mimic and study the stabilizing effect of ionic liquids on enzymes.

1. Introduction

Ionic liquids are proposed as solvent replacement for biocatalytic application since the beginning of the 2000's, thanks to their low vapour pressure, expected low toxicity [1] and stabilizing effect on protein structures [2,3]. Numerous examples of enzyme-catalysed reactions performed in ionic liquids are now described, mostly involving hydrolytic enzymes (e.g. lipases and esterases) and mainly with water-unsoluble ionic liquids [4]. Enzyme-catalysed oxidoreduction reactions involving oxidases and dehydrogenases are also frequently used in biocatalysis but are still little developed in ionic liquids [5]. In theses processes, a cofactor regeneration system (e.g. the oxidation of NAD(P)H to $NAD(P)^+$ by glucose dehydrogenase or formate dehydrogenase) is often required in order to limit the costs and to draw equilibrium toward complete conversion of the substrate [6,7]. Usually, the low solubility of NAD(H) in organic solvent is also observed with ionic liquids, restricting dehydrogenase-catalysed reaction to water miscible ionic liquids. However, these ionic liquids are also the most deleterious for the enzymatic activity.

To prevent the unfolding of enzymes in ionic liquids, different approaches are used including rational design of the ionic liquid [8,9], chemical modification of enzyme by polyethylene glycol (PEG) [10] or inclusion in water-in-ionic liquid microemulsions [11,12]. However these two last approaches are only performed with water insoluble ionic liquids.

A few dehydrogenases are nevertheless found to be active in ionic liquids: the formate dehydrogenase of *Candida boidinii* presents up to 98% of its initial activity in 75% [MMIm][$MeSO_4$] [13] and morphine dehydrogenase is found to be active in the highly viscous [HO-PrMIm][Glycolate] [14]. In this last case, the high solubility of the cofactor and of the enzyme is achieved due to the expected hydrogen bounds between the ionic liquid ions and the enzyme or the cofactor. On the other hand, this highly viscous ionic liquid leads to low reaction rate.

In the present work, FDH from *C. boidinii* is chemically modified by 3-hydroxyethyl-trimethylammonium (cholinium, (**1**)), hydroxyethyl-methylimidazolium (**2**) or hydroxypropyl-methylimidazolium (**3**) cations (Scheme 1) in order to improve its

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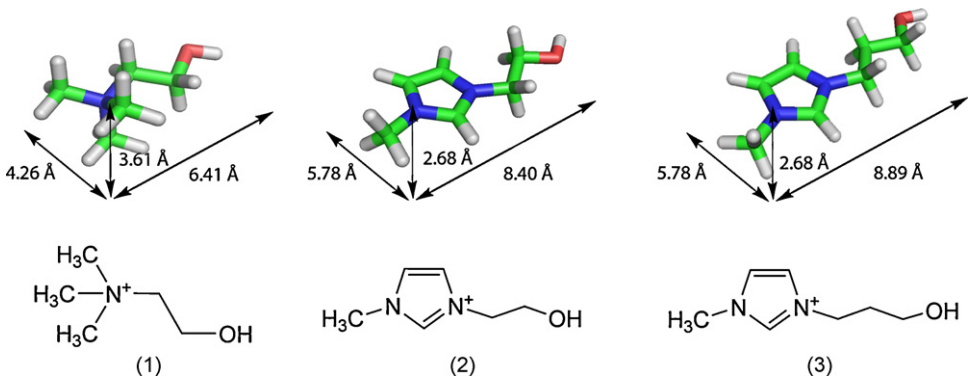
doi:10.1016/j.molcatb.2010.01.028



Scheme 1. Structures (down) and dimensions (up) of hydroxyalkyl cations (**1**–**3**) used to chemically modify the FDH. (**1**) Cholinium, (**2**) hydroxyethyl-methylimidazolium, (**3**) hydroxypropyl-methylimidazolium.

activity and stability in dimethylimidazolium dimethyl phosphate ([MMIm][Me_2PO_4]), a water miscible ionic liquid.

2. Experimental

2.1. Materials

Formate dehydrogenase from *Candida boidinii* (FDH; E.C. 1.2.1.2; 70 U ml^{-1}) was from Jülich Fine Chemicals (Jülich, Germany). 1,3-Dimethylimidazolium dimethyl phosphate ([MMIm][Me_2PO_4]) was from Iolitec (Denzlingen, Germany). (2-Hydroxyethyl)trimethylammonium dimethyl phosphate (**1**) and hydroxyethyl-methylimidazole chloride (**2**) were from Solvionic (Toulouse, France). All others chemicals were from Sigma–Aldrich (St-Quentin-en-Fallavier, France).

2.2. Synthesis of hydroxypropyl methyl imidazolium chloride (*3*)

Hydroxypropyl-methylimidazolium chloride (**3**) was prepared according to standard procedures. 1-Methylimidazole (31.3 mmole, 2.6 g, 1 equiv.) and 3-chloro-1-propanol (31.3 mmole, 2.98 g, 1 equiv.) were mixed in a round flask and warmed-up to 80 °C under reflux and argon atmosphere for 48 h. Reaction product was washed extensively with diethylether, in order to remove un-reacted compounds and dried over vacuum. The resulting solid salt (5.34 g, Yield 95%) was used without further purification. ^{1}H NMR (300 MHz, D_2O): δ = 2.33 (2H, m), 3.84 (2H, *t*, *J* = 6.0 Hz), 4.12 (3H, s), 4.52 (2H, *t*, *J* = 7.02 Hz), 4.98 (2H, s), 7.73 (1H, s), 7.73 (1H, s). ^{13}C NMR: 32.039, 36.134, 46.838, 58.295, 122.661, 123.977. MS (EI^+): m/z 141 (100%, $HOPrMIm^+$).

2.3. Chemical modification of the formate dehydrogenase

Cations (1–3) was dissolved in anhydrous DMSO for a final concentration of 1.36 M. Carbonyldiimidazole were dissolved in anhydrous DMSO for a final concentration of 1.36 M. The two solutions (250 μl of each for a final concentration of 680 mM) were mixed and let react for 4 h at room temperature in order to activate the hydroxyl functions of the cations. Activated cations (100 μl of the 680 mM solution) were added in excess to 900 μL of a FDH solution (1.5 μM) in carbonate buffer (pH 9.7, 25 mM) for a final concentration of 68 mM and incubated 24 h at room temperature. The modified FDH was concentrated over a 10 kDa membrane (Ultracell YM-10, Millipore, Molsheim, France) and unsalted with micro biospin 6 column (Bio-Rad, Marnes-la-Coquette, France). Enzymes were stored in solution at 4 °C or used directly after modification. Enzyme concentration was measured by the Bradford reagent and the number of modifications was determined by electrospray mass spectrometry (ESI^+, API 165 Applied Biosystems).

2.4. Enzyme assay

The activity of FDH was measured by following the NADH apparition at 340 nm ($\varepsilon_M^{340\ nm} = 6.22 \times 10^3\ M^{-1}\ cm$) with a Tecan Infinite M200 (Salzburg, Autria) microplate reader. The reaction media was composed of 10 μl FDH solution, 10 μl NAD^+ solution (50 mM) and 0–70% (v:v) of [MMIm][Me_2PO_4]. Reaction was initiated by injection of 10 μl of sodium formate (3 M). The final volume is 100 μl. All solutions are prepared in $NaHCO_3$ buffer (pH 9.7, 25 mM). Optical pathways were determined by measuring absorbance of water at 975 nm in each individual well ($\varepsilon_M^{975\ nm} = 3.35 \times 10^{-3}\ M^{-1}\ cm^{-1}$). Specific activities were an average value of at least 3 measurements and are expressed in μmol min^{-1} mg^{-1}.

2.5. Enzyme stability

Samples of the native and modified FDH (0.05 U ml^{-1}) were incubated in a microtiter plate at ambient temperature in carbonate buffer (pH 9.7, 25 mM) with or without [MMIm][Me_2PO_4] (37.5% or 50%, v:v). The activity of three samples was measured everyday as previously described. The final concentration of the ionic liquid, if any, was 37.5% or 50% (v:v). The half-life ($t_{1/2}$) was obtained from the exponential decay of the activity over the time considering the enzyme inactivation follows a first order kinetic.

2.6. Mass spectrometry analysis

The determination of the average number of modification was determined by mass spectrometry. Briefly, 1 ml of the FDH sample (0.4 U/ml corresponding approximately to 1.5 μM) is concentrated over a 30 kDa membrane (Ultracell YM-30, Millipore, Molsheim, France) and unsalted with micro biospin 6 column previously equilibrated with deionised water (Bio-Rad, Marnes-la-Coquette, France). The proteins are then diluted in water:methanol 50:50 (v:v), formic acid 0.1% (v:v) and injected at 5 μl/min in an API 165 Applied Biosystems electrospay (ESI) facility equipped with a simple quadripole.

3. Results and discussion

3.1. Chemical modifications

Our initial hypothesis was that the activity of an enzyme could be enhanced in ionic liquids if aggregation and unfolding could be prevented. The ionic liquid [MMIm][Me_2PO_4] was chosen in this study because (i) it is water miscible, (ii) its viscosity is much lower than the ionic liquids allowing NADH dissolution like [HOPrMIm][Glycolate] for example [8] and (iii) it is composed of a small chaotropic cation and a large (bulky) kosmotropic anion which are

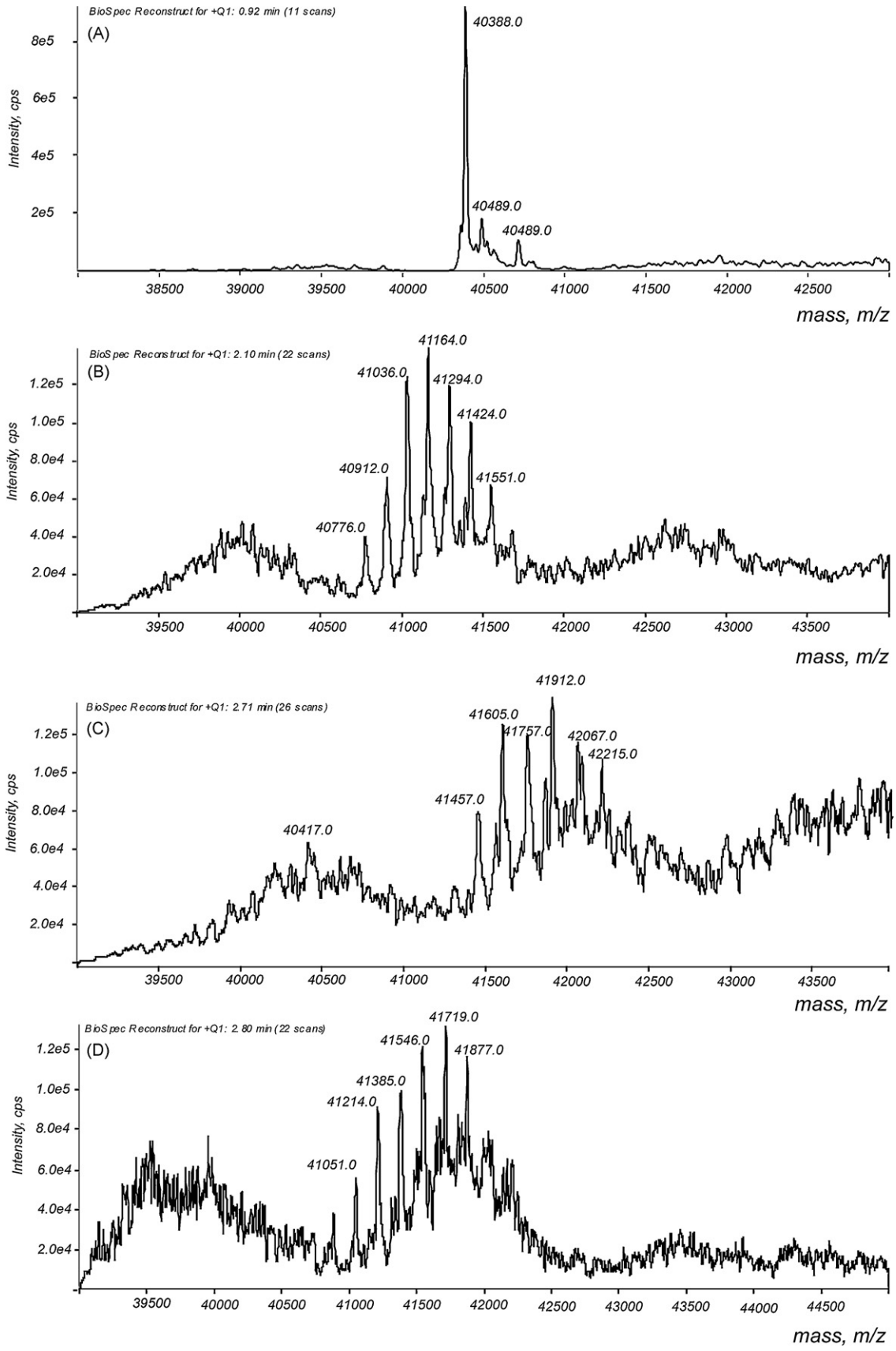


Fig. 1. ESI-MS spectra of wild-type FDH (A) and chemically modified FDH by cholium cation (B), by hydroxyethyl-methylimidazolium (C) and by hydroxypropyl-methylimidazolium (D) cations.

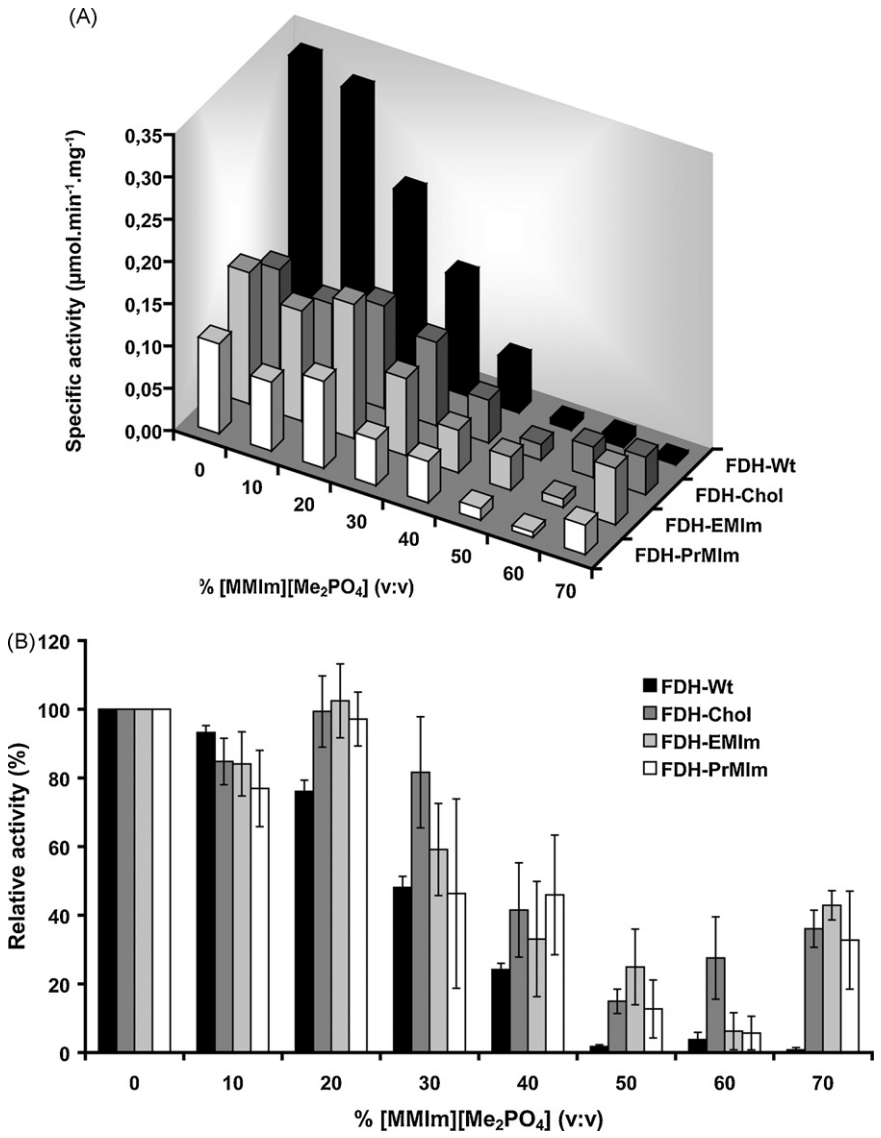


Fig. 2. Specific (A) and relative (B) activities of the wild-type and of the modified FDH in presence of 0–70% [MMIm][Me_2PO_4] (v:v). FDH-Wt: native FDH, FDH-Chol: FDH modified by cholinium cation (**1**), FDH-EMIm: FDH modified by hydroxyethyl-methylimidazolium cation (**2**), FDH-PrMIm: FDH modified by hydroxypropyl-methylimidazolium cation (**3**).

expected to stabilize the enzyme according to the theory developed by Zhao et al. [15,16].

Taking into account this hypothesis, a new approach was developed based on the grafting onto the enzyme surface of cations which usually composed ionic liquids. This was expected to help enzyme solubilization and therefore to prevent aggregation. Therefore, the cations (**1**–**3**) presented in Scheme 1 were activated with carbonyldiimidazole before being covalently linked onto the enzyme lysine residues via carbamate linkages according to published procedure [17]. These short-length cations were chosen to modify the lysine residues as they have structure similarity with the most commonly used ionic liquids (e.g. imidazolium rings and tetramethylammonium moieties). It should be notice that their grafting onto lysine residue will not change the overall charge of the protein.

The number of modified lysine residues has been determined by mass spectrometry (ESI^+) as shown in Fig. 1. The mass spectrum of the native FDH present a single (main) peak at m/z 40,388 (Fig. 1A) (ExPASy proteomics server mass: 40,370 Da; entry O13437). The grafting of cations (**1**–**3**) onto the FDH always led to a heterogeneous population of 6 to 7 proteins, differing by a mass corresponding to the grafted cations: each cholinium, ethyl-methylimidazolium and propyl-methylimidazolium led to an increase of the mass of 129, 153 and 168 respectively, including the carbamate linkage. FDH modified by (**1**) or (**3**) contains 3–9 modified lysine residues while 7 to 12 lysine residues are modified with cation (**2**). This higher number of grafted residues could partially be explained by lower steric hindrances induced by the size of the cation and the flexibility of the alkyl chain. The imidazolium-based cations (**2**) in particular have a smaller height than cation (**1**) and a shorter length than cation (**3**) as shown in Scheme 1. Therefore, during the time-course of the grafting, in the case of (**2**), the modification of a lysine residue located at the proximity of a previously modified lysine is allowed. Indeed, the surface of the enzyme (pdb entry 2fss) present a lysine-rich cluster (residues 2, 76, 81, 83, 84, 86, 108 and 109) at its surface in which ε-amine function of the lysine residues are at a distance of less than 20 Å one from the other, suggesting our hypothesis is plausible.

3.2. Activity in ionic liquid

The specific activities of the wild type FDH and of the FDH grafted by cations (**1**–**3**), as well as their residual activities, in the same conditions are showed in Fig. 2A and B respectively.

Activity of the wild type FDH was sharply reduced in the presence of increasing concentrations of [MMIm][Me_2PO_4] (Fig. 2A). From 0 to 40% [MMIm][Me_2PO_4], the activity dropped from 0.33 to 0.06 μmol min^{-1} mg^{-1}, which correspond to the disappearance of 76% of the initial activity (Fig. 2B). Above 40% (v:v) of the ionic liquid, FDH is almost inactive (about 2% of residual activity, Fig. 2B), probably due to the denaturing effect of ionic liquid.

The grafting of cations (**1**–**3**) onto the FDH led to a decrease of the specific activity from 0.33 to 0.10–0.15 μmol min^{-1} mg^{-1} (Fig. 2A), (30 to 45% of the initial activity, Fig. 2B), depending of the cation.

Interestingly, the cation (**2**) which is coupled with more lysine residues than the other cations led to the less deleterious effect.

When assayed in presence of increasing concentration of ionic liquid, the grafted enzymes showed in some extent a decrease of their specific activity (Fig. 2A). In the presence of 20% of [MMIm][Me_2PO_4], they remained unaffected while the native enzyme already lost 24% of its initial activity and in the presence of 40% of ionic liquid, they retained 33–46% (vs. 24% for the native enzyme, Fig. 2B). The main beneficial effect of theses modifications was observed above 40% of ionic liquid. While the native enzyme was almost inactive in 70% [MMIm][Me_2PO_4], the grafted enzyme retained 30% of its activity when modified by the cation (**3**) (corresponding to 0.034 μmol min^{-1} mg^{-1}) and up to 43% (corresponding to 0.06 μmol min^{-1} mg^{-1}) for the enzyme modified by the cation (**2**).

From these results it appeared that the modification by cation (**2**) led to the best improvement of the enzyme activity in this ionic liquid. Despite the modification of the FDH by the activated cation (**2**) led to a partial enzyme inactivation (Fig. 2A), this new biocatalyst is more resistant to the deleterious effect of this ionic liquid than the wild-type enzyme. This was probably related to the higher number of modified lysine residues compared to the modification by cation (**1**) or (**3**), which is suspected to prevent more efficiently the unfolding of the protein and to solubilize it more efficiently in this imidazolium-based ionic liquid. The specific impact of the modifications on the protein structure is currently under study.

3.3. Stability in ionic liquids

In order to evaluate the influence of the ionic liquid and of the modifications on the FDH stability, the half-lives of the biocatalysts were determined in aqueous solution and in presence of [MMIm][Me_2PO_4] (37.5 and 50%, corresponding to 2.2 M and 3 M).

The half-life of the wild-type FDH in aqueous solution, determined from its residual activity, was 1.5 ± 0.6 days (Fig. 3). The enzyme stored in 37.5% [MMIm][Me_2PO_4] (v:v) leads to an increase of its half-life by a nearly 5-fold factor and reached 7.6 ± 0.6 days but at a higher concentration (50%) the half-life drop drastically to 0.37 ± 0.04 days. The stabilizing effect of [MMIm][Me_2PO_4] has already been described for the alcohol dehydrogenase (ADH) from *Rhodococcus erythropolis* at low ionic liquid concentration. In the presence of only 10% of [MMIm][Me_2PO_4], the half-life of ADH is increased by a 2.3-fold factor, but this same ionic liquid was also showed to slightly decrease the half-life of glucose dehydrogenase (by a 1.2-fold factor) used as a cofactor recycling enzyme. In such a process, the FDH will appear more appropriate for cofactor recycling [18]. The stabilization of the enzyme structure in this water-miscible ionic liquid is still a matter of discussion. It is not clear how the ionic interactions between the salts and the protein molecules could explain the preservation of the enzyme activity

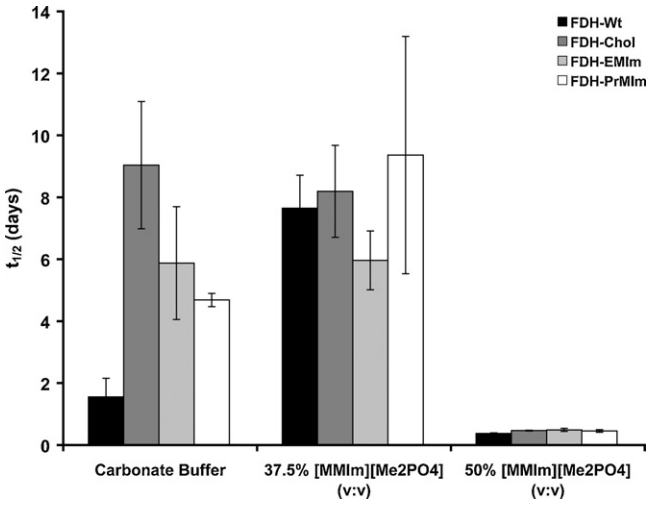


Fig. 3. Stability of the wild-type and of the modified FDH in carbonate buffer (25 mM, pH 9.7) in absence and in presence of 37.5% [MMIm][Me_2PO_4] (v:v). FDH-Wt: native FDH, FDH-Chol: FDH modified by cholinium cation (**1**), FDH-EMIm: FDH modified by hydroxyethyl-methylimidazolium cation (**2**), FDH-PrMIm: FDH modified by hydroxypropyl-methylimidazolium cation (**3**).

(e.g. the enzyme structure) [3,19,20]. The high salt concentration could promote the salting-in effect on the enzyme, favouring its solubilisation and preventing the formation of aggregates due to hydrophobic interactions between enzymes molecules [21]. Both ion-pairing and electrostatic repulsion have to be considered. The increase of the half-life of the native FDH in presence of 37.5% [MMIm][Me_2PO_4] is likely to be explained by the position of both the cation and the anion of the ionic liquid in the Hofmeister series, related to their chaotropic or kosmotropic efficiency [15,16]. Recently, the viscosity *B*-coefficient is proposed to determine the chaotropicity or the kosmotropicity of an ion in aqueous solution [22]. Small cations like [MMIm] tend to be considered as chaotropes (low *B*-coefficient) and to stabilize protein structures. Larges (bulky) anions, like [Me_2PO_4], which are considered to be have a high kosmotropicity (high *B*-coefficient), tends to stabilize both the water network and the protein structure [23]. Therefore [MMIm][Me_2PO_4] was chosen as a good candidate to increase the stability of the FDH, which is verified experimentally (Fig. 3). At higher ionic liquid concentration (50%), the enzyme is nevertheless found to be unstable. In theses conditions, other parameters like ionic strength or hydrophobic interactions acting directly on the enzyme three-dimensional structure could overrule the ionic liquid stabilizing effect. The grafted enzymes possess an increased half-life compared to unmodified FDH when stored in carbonate buffer. Their half-life reached 4.7–9 days, corresponding to a 3–6-fold improvement with the highest stability obtained with the cation (**1**).

As discussed before, small organic cations tend to be chaotropic, e.g. they disorganize the water network in the solution. The mechanism of the enzyme stabilization is not clearly defined but the water shell of the enzyme is likely to be also disorganized as the concentration of the cation concentration increase. In this study, the cations are grafted covalently onto the enzyme surface. Looking to our results, we suppose the water shell of the FDH is disorganized with a similar mechanism to the one of soluble cations with the exception of the cation is retained at the enzyme surface and does not diffuse in the bulk solution. Therefore the stabilizing effect of the ionic liquid due to the cation could be achieved if the ionic liquid (or one of its ions) remain confined at the enzyme surface,e.g. if small organic cations are grafted at the enzyme surface, as we did. As shown in Fig. 3, the higher stabilizing effect is obtained with cation (**1**),

while it decreases with cations (**2**) and (**3**). The *B*-coefficient of the cation (**1**) appears to be the smallest (based on structure similarity with Me_4N^+, $B = 0.380\,dm^3\,mol^{-1}$) and it increases for cation (**2**) (similarity with EMIm, $B = 0.491\,dm^3\,mol^{-1}$) and for cation (**3**) (*B* estimated to be $\sim 0.516\,dm^3\,mol^{-1}$) (*B*-coefficients are from Ref. [22]). Theses results suggest that more chaotropic the grafted cation is, higher is the stabilizing effect in aqueous environment, mimicking the effect of an ionic liquid in solution.

The grafted enzymes did not present any significant improvement on their stability on 37.5% [MMIm][Me_2PO_4] compared to the experiments in carbonate buffer except for the cation (**3**). The half-life of the unmodified or grafted enzyme reached 6–9 days vs. 1.5 days for unmodified enzyme in carbonate buffer. Therefore there is no apparent synergistic effect between the stabilizing effect of the grafted cations and of the ionic liquid under our experimental conditions. In such a case, the stabilizing effect of the grafted chaotropic cation on the enzyme surface should be sufficient to stabilize the enzyme structure and additional [MMIm] cations in the bulk solution do not improve the stability anymore.

This should be modulated when the protein is grafted by the cation (**3**). In this special case, a synergistic effect could be observed when the enzyme is store in 37.5% [MMIm][Me_2PO_4]. The half-life of the grafted enzyme was increased by a 2-fold factor (from 4.7 to 9.4 days). The grafted cation (**3**) has a high *B*-coefficient, therefore its influence on the disorganization of the water shell of the enzyme is less pronounced than for cations (**1**) and (**2**) and consequently its stabilizing effect is lower. In presence of the soluble ionic liquid, the [MMIm] and [Me_2PO_4] ions seems to have their normal stabilizing effect, complementing the action of the grafted cation (**3**).

Only a slight improvement of the activity is observed in presence of 50% of the ionic liquid (0.45 ± 0.08 to 0.5 ± 0.99 days) compared to the wild type enzyme and are mostly unstable, probably for the same reasons.

4. Conclusion

The modification of the formate dehydrogenase from *Candida boidinii* by cations inspired from ionic liquids structures led to biocatalysts retaining 33–43% of their initial activity in 70% [MMIm][Me_2PO_4] in contrary to the native enzyme which was almost inactive in these experimental conditions. The most interesting properties were obtained when the enzyme is modified by hydroxyethyl-methylimidazolium cation (**2**). This was explained by a higher number of modified lysine residues compared to the two other cations. The stability of modified FDH in aqueous solution was also sharply increased by a 3–6-fold factor. These results strongly suggest, based on the stabilizing effect of chaotropic cations, that immobilization of ionic liquid-inspired cation onto the protein surface could mimic the effect of the ionic liquid on the water shell of the enzyme. The increased stability in 37.5% [MMIm][Me_2PO_4] (v:v) of the wild type FDH was nevertheless not further enhanced by the modifications but a grafted enzyme with a moderate increase in stability could be further improved by the ionic liquid itself.

Acknowledgment

The financial support from the Cluster de recherche Chimie de la Région Rhône-Alpes is duly acknowledged.

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Loïc J. Blum, born in 1955, received the Doctorat de spécialité (1983) in Biochemistry and the Doctorat d'Etat ès Sciences (1991) from the université Lyon 1. He is presently Professor of Biochemistry and Biotechnology at the same university. His research work is connected to heterogeneous biocatalysis and with the development of Nanobiotechnologies-related topics. He is the head of both the "Laboratoire de Génie Enzymatique et Biomoléculaire (LGEB)" of the université Lyon 1 and the CNRS-université Lyon 1 "Institut de Chimie et Biochimie Molécuaires et Supramoléculaires" ICBMS). Since 1983 author or co-author of over 160 articles and book chapters.