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Contribution of Dynamic and Static Quenchers for the Study of Protein Conformation in Ionic Liquids by Steady-State Fluorescence Spectroscopy

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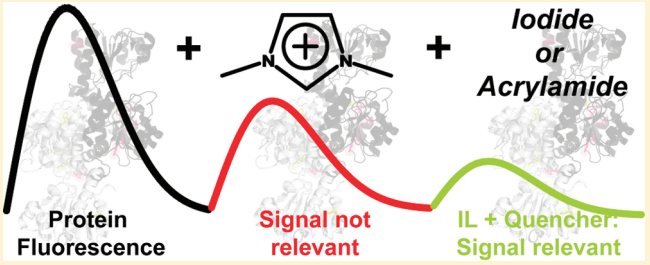
Supporting Information

ABSTRACT: The study of protein conformation in ionic liquids (ILs) is crucial to understand enzymatic activity. Steady-state fluorescence is a proven, rapid and easy method to evaluate the protein structure in aqueous solutions, but it is discussed when used in ILs. In this work, the structure of the formate dehydrogenase from *Candida boidinii* (FDH, EC: 1.2.1.2) in three imidazolium-based ILs (dimethylimidazolium dimethylphosphate [MMIm][Me_2PO_4], 1-butyl-3-methylimidazolium acetate [BMIm][CH_3COO], and dimethylimidazolium methylphosphonate [MMIm][$CH_3HPO_2(OCH_3)$]) is studied by fluorescence spectroscopy. The UV−vis spectroscopic analysis shows that the decrease of the FDH fluorescence is not only due to the high light absorption of these ILs. The Stern−Volmer analysis clearly shows that these ILs are quenchers of the indole fluorescence, while this quenching property is not found when imidazole is used. Fluorescence spectra of the FDH in the presence of the ILs show that a maximal ionic liquid concentration (MILc), which could be used for steady-state fluorescence study, should be defined. Therefore, FDH conformation could not be directly related to the decrease of its fluorescence in ILs. Nevertheless, the structure of the FDH could be evaluated with dynamic and static quenchers like iodide or acrylamide, used below the MILc, demonstrating the relevance of this parameter. The Stern−Volmer constants (K_{SV}^{Q}), calculated in the presence of the different ILs, demonstrate that these ILs are strong denaturing agents, each one acting with a different mechanism. This report provides a suitable and easy-to-apply method to study any enzyme structures in ILs by steady-state fluorescence.



INTRODUCTION

Ionic liquids (ILs) are salts that exhibit a melting point below 100 °C and in many cases, below room temperature. They are commonly composed of an organic cation such as alkyl-substituted imidazolium, ammonium, or pyrrolidinium and of a more compact anion such as halide, tetrafluoroborate, or fluoroalkyl-based anions. At the end of the last century, ILs have gained a lot of attention thanks to their low vapor pressure, expected low toxicity, and stabilizing effect on protein structures. These properties depend on the anion and on the cation of the IL. Therefore, a large number of applications involving ILs have been proposed in electrochemistry, organic synthesis, and material sciences, but also in biocatalysis.[1,2]

Numerous examples of enzyme-catalyzed reactions performed in ILs are now described,[2−4] but further applications of ILs as a solvent for enzyme-catalyzed reactions require more comprehensive studies. Lipases and proteases, the enzymes primarily employed in nonaqueous enzymology, were found to catalyze similar reactions in ILs and in organic solvents with enhanced stability and activity.[1−3,S−7] These reactions are often conducted in water-immiscible ILs with low water content (less than 5%) in order to prevent enzyme unfolding and to allow sufficient protein flexibility to achieve catalysis. The solvatation state depends on the water content and on the presence of aliphatic or polar nanodomains at the protein surface.[8]

More recently, other classes of enzymes have been studied in ILs such as oxidases and dehydrogenases.[9−13] Theses enzymes do not present apolar surfaces, and it is unlikely they are soluble in apolar ILs. Moreover, to be available for the reaction, the substrates (e.g., glucose, oxygen, etc.) and cosubstrates (e.g., NAD(P)$(H)^+$) of these enzymes should form H-bonds with the solvent. Aqueous solutions of water miscible ILs or neat ILs are able to form H-bonds with the enzyme and the substrates and are often preferred. With a few exceptions, all the oxidoreductase-catalyzed reactions in ILs were conducted in low-to-medium concentrated ILs in aqueous solution.[9,14,15]

It is agreed that the deleterious or the beneficial effect of ILs on the enzyme activity is related to the impact of these salts on the enzyme structure. The effect of ILs, dissolved in aqueous solution, on the enzymatic activity generally follows the Hofmeister

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series: kosmotropic anions (such as $[CF_3CO_2]$ and $[CF_3SO_3]$) and small chaotropic cations (such as 1-methyl- ([MMIm]) and 1-ethyl-3-methylimidazolium ([EMIm])) preserve the enzyme activity, while the chaotropic anions (such as $[BF_4]$ and $[PF_6]$) and large kosmotropic cations (such as 1-butyl- ([BMIm]) and 1-hexyl-3-methylimidazolium ([HMIm])) inactivate the biocatalyst.[16−19] In such a case, the viscosity B-coefficient, which roughly represents the hydration of the ions in a moderately concentrated solution, was found to be a suitable, but empirical, value to describe the kosmotropic/chaotropic behavior of an IL on a protein. In highly concentrated or pure IL, the B-coefficient is no longer useful because of the strong ion−ion coordination within the IL, and the kosmotropicity/chaotropicity-protein stability relationship is no longer applicable. At this stage of knowledge, no theory can totally predict the effect of a particular IL on a specific enzyme.

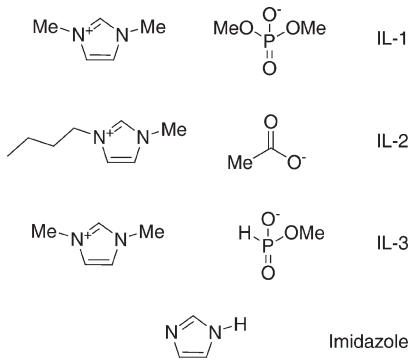
The enzyme conformation in ILs or in aqueous-IL solutions is now investigated by numerous spectroscopic methods, but probably due to experimental difficulties and ambiguous data interpretations, a simple and reliable methodology is still lacking to the scientific community.[20−23] Circular dichroism in the far-UV region, which requires high enzyme concentration to be satisfactory (>5 mg ml^{-1}),[24,25] is difficult to use due to the high UV-light absorption of imidazolium-based ILs and the presence of impurities (DNA, aggregates, particles, etc.).[26] It should be probably restricted to the study of proteins with ligands or cofactors (e.g., heme) at wavelengths where the ILs does not absorb. So considered, cytochrome *c* (cyt *c*) was studied by circular dichroism thanks to the Soret band of its heme (350−450 nm). In aqueous solutions of alkylammonium formate, cyt *c* conformation was found to remain stable up to 50−70% of these ILs (v/v).[27] In the neat $[EMIm][EtSO_4]$, the secondary structure of cyt *c* remains largely intact, while the tertiary structure is significantly altered upon solubilization.[28] Up to now, reliable far-UV CD spectra were only obtained with helical and trp-cage model peptides, which conserve their structure in butyl-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide ($[BMPy][NTf_2]$).[28] On the contrary, a β-hairpin model peptide is destabilized in the same IL.[29] Attempts have been made using Fourier transform infrared spectroscopy (FTIR) to study cyt *c* in nonimidazolium-based ILs[30,31] or protease P6 in imidazolium-based ILs.[18] FTIR was also used to evaluate the *Candida antartica* lipase B (CALB) structure in ether-functionalized ILs and in formate- and acetate-based ILs associated with [BMIm].[20] Nevertheless, this technique requires the removal of the solvent background and of the water vapor with a special care, often leading to misinterpreted results.[32] Results appear to be even more ambiguous when the second derivative of the FTIR spectrum is presented and when amide bands are attributed to secondary structures on the basis of the bands defined in water. Original spectra are too often omitted to judge of the quality of the measurement. Nevertheless, the amide II band was observed in $[EMIm][EtSO_4]$ with highly concentrated cyt *c* (18 mg ml^{-1}) but no fine analysis of the secondary structure could be achieved. The thermal stabilization of cyt *c* by choline dihydrogene phosphate ($[Chol][H_2PO_4]$) (80% v/v) was also observed by ATR-FTIR following the apparition of a band at 1626 cm^{-1} attributed to the formation of β-sheets assumed to be due to the enzyme aggregation.[30,31] Other studies show that water-miscible ILs containing chaotropic anions induce the unfolding of the enzyme, while the nonmiscible ILs have less effect on the secondary structure.[17,20,30] It can be noted that enzymes often used in organic media, such as CALB, are less sensitive to the deleterious effect of the ILs as proven by the recovered activity.[20,21]

The most accomplished studies of enzymes conformations in ILs deal with immiscible ILs and with methods that cannot be routinely used in most of the laboratories. They include small angle neutron scattering (SANS),[33,34] time-resolved fluorescence spectroscopy,[35] molecular dynamics simulation,[8,36] and solvent jump experiments.[37]

Steady-state fluorescence spectroscopy of proteins is a proven, rapid and easy method for evaluating structures and conformations, but it remains a controversial method in ILs. The inactivation of the cellulase from *Trichoderma reesei* in the water-miscible IL [BMIm][Cl] was suggested to be due to the unfolding of the enzyme on account of the drastic decrease of intrinsic tryptophan fluorescence at concentrations of IL higher than 20% (v/v).[38] Similar results were obtained with α-amylases in [BMIm][Cl] and [HMIm][Cl].[39] These experimental evidence were in accordance with SANS experiments, which show the unfolding of cyt *c*, of GFP (green fluorescent protein), and of HSA (human serum albumin) in [BMIm][Cl].[33,34] Intrinsic fluorescence studies have also been conducted in the presence of immiscible ILs in order to demonstrate the thermal stabilization of α-chymotrypsin by 85% (v/v) $[EMIm][NTf_2]$,[22] of CALB by 98% (v/v) [EMIm]-$[NTf_2]$ or butyltrimethylammonium ([BTMA]) $[NTf_2]$,[21] and of a single tryptophan molecule (monellin) by 98% (v/v) [BMPy]-$[Ntf_2]$.[40] On the contrary, fluorescence spectroscopy failed to evaluate the influence of $[MMIm][Me_2PO_4]$ on the structures of chymotrypsin, of trypsin, and of V8 protease due to the interferences of this IL at concentrations higher than 50% (v/v).[37] It was suggested that the IL molecules interact with the tryptophanyl residues of these enzymes causing the loss of the fluorescence (quenching).[20] It was also observed that 1-methylimidazole quenches the fluorescence of tryptophan molecules in solution that meant the loss of fluorescence in imidazolium based-ILs was not necessarily a signature of protein unfolding.[41]

The covalent attachment of the fluorescent probes acrylodan[42] or tetramethylrhodamine (TMR)[43] on the unique free cysteine of HSA or of cyt *c*, respectively, was also performed. The anisotropy and the polarity-dependent fluorescence of the HSA-acrylodan conjugate was investigated during thermal denaturation experiments.[42] The authors found that the conjugate reorganizes into a closer conformation in [BMIm]-based ILs as the temperature increases, while in aqueous solution, the conjugate unfolds. In another work, the quenching of the TMR fluorescence by the heme of cyt *c* is abolished when this protein is placed in the presence of ∼1.5 M of [BMIm]-based ILs.[43] This observation is assigned to the unfolding of cyt *c*. At higher IL concentrations, a refolding of the protein is even observed with some ILs. These methods are probably the most accomplished in the study of protein structure in ILs by steady-state fluorescence. Nevertheless, they could only be applied to proteins presenting a single site for labeling (e.g., a single cysteine located at the protein surface) or are limited to heme-containing proteins. For example, they probably cannot be applied to CALB, one of the most studied enzymes in ILs, which contains six cysteinyl residues involved in three disulfide bridges, no heme, and five tryptophanyl residues. Considering the high number of enzymes and the significant combination of anions and cations that could compose an IL, a general, efficient, and easy-to-apply methodology to evaluate the influence of an IL on a protein structure is necessary, especially concerning enzymes classically used in syntheses applications.

Scheme 1. ILs Used in This Study: [MMIm][Me_2PO_4] (IL-1), [BMIm][CH_3COO] (IL-2), [MMIm][$CH_3(O)PO_2H$] (IL-3), and Imidazole



This report aims to evaluate whether steady-state fluorescence can nevertheless be used to study any fluorophore-containing protein in water-miscible ILs. We emphasize that no chemical modifications of the enzyme or expensive equipment are required. After underlining the limitations of steady-state fluorescence in such studies, we propose to use an old but complementary approach to evaluate more accurately the enzyme conformations in ILs, e.g., with the help of additional quenching agents.

As a model, the conformational behavior of the formate dehydrogenase from *Candida boidinii* (FDH) in three different ILs is investigated. The FDH was chosen because dehydrogenases in ILs have become a focus of research due to their industrial use. More precisely, this FDH is involved in the cofactor regeneration during enzymatic reduction of ketones.[10,44] The FDH from *Candida boidinii* is a homodimer of 364 amino acid subunits containing 5 tryptophanyl, 16 tyrosinyl, and 8 phenylalaninyl residues (Supporting Information, SI 1). Therefore, it could be excited at 280 nm to evaluate the overall protein conformation (e.g., the signal is mostly due to tryptophan and tyrosine) but also at 295 nm to study mainly the tryptophan environment. The FDH is studied in three imidazolium-based water-miscible ILs: [MMIm][Me_2PO_4] (**IL-1**), [BMIm][CH_3COO] (**IL-2**), and [MMIm][CH_3OPO_2H] (**IL-3**) (Scheme 1), and structural aspects are obtained by quenching the FDH fluorescence by iodide or acrylamide. The fluorescence results are correlated with the enzyme activity in ILs.

EXPERIMENTAL SECTION

Material. Formate dehydrogenase from *Candida boidinii* (E.C. 1.2.1.2; 77 U ml^{-1}; batch-no, Z70911.01) is from Jülich Fine Chemicals (Jülich, Germany). Dimethylimidazolium dimethylphosphate ([MMIm][Me_2PO_4]) is from Iolitec GmbH (Denzlingen, Germany). 1-Butyl-3-methylimidazolium acetate ([BMIm][CH_3COO]) and dimethylimidazolium methylphosphonate ([MMIm][CH_3OPO_2H]) are from Solvionic (Toulouse, France). Bradford reagent is from Bio-Rad (Marnes-la-Coquette, France). All others chemicals are from Sigma-Aldrich (St-Quentin-Fallavier, France). Absorption and fluorescence experiments have been performed with a Tecan Infinite M200 (Salzburg, Austria) micro plate reader.

UV−Vis Absorption Experiments. UV−vis absorption spectra of the FDH, in buffer or in the presence of 0−70% (v/v) of IL, were measured from 230 to 600 nm. Phosphate buffered saline (PBS) buffer (pH 7.2; NaCl 8 g L^{-1}; KCl 0.2 g L^{-1}; Na_2HPO_4 1.44 g L^{-1}; KH_2PO_4 0.24 g L^{-1}) was used as buffer for the aqueous/IL binary mixtures and for diluting the FDH. The final concentration of FDH is fixed at 0.32 U mL^{-1} (2.61 μM). In every experiment, the contribution of the medium was subtracted.

Scheme 2. Reaction Catalyzed by the FDH[a]

$$HCOO^- + NAD^+ \xrightarrow{FDH} CO_2 + NADH\ (340\ nm)$$

$$NADH + PES_{ox.} \longrightarrow NAD^+ + PES_{red.}$$

$$PES_{red.} + NBT \longrightarrow PES_{ox.} + Formazan\ (560\ nm)$$

[a] The first reaction is the oxidation of the formate by the FDH. The two other reactions correspond to the chemical oxidation of NADH by phenazine ethosulfate (PES) oxidized in turn by nitrobluetetrazolium (NBT).

Enzyme Activity. The measurement of the enzyme activity by spectrophotometric assays in IL could also be subjected to interferences, especially when the formation of NADH is measured at 340 nm, a wavelength at which all the ILs of this study absorb. In such complex media, the oxidation of NADH by a chromogenic substrate like formazan (maximum absorption at 560 nm) is often preferred (Scheme 2). Fortunately, at 560 nm, these ILs do not present significant absorption properties ($\varepsilon_M^{560\ nm} < 0.05\ M^{-1}\ cm^{-1}$ for **IL-1** and **IL-2** and $\varepsilon_M^{560\ nm} = 1.3\ M^{-1}\ cm^{-1}$ for **IL-3**) meaning that the absorbance at 560 nm, due to the apparition of formazan, is representative of the enzymatic activity. Enzyme activity was measured by following the NADH apparition at 340 nm ($\varepsilon_M^{340\ nm} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$) or the formazan apparition at 560 nm ($\varepsilon_M^{560\ nm} = 14\ 103\ M^{-1}\ cm^{-1}$). A single well is composed of 10 μL of enzyme solution (0.4 U mL^{-1}, 5.38 μM), 10 μL of NAD^+ solution (50 mM), 10 μL of formate (3 M), and 0−70% (v/v) of ILs in PBS buffer (pH 7.2). A medium composed of 10 μL of enzyme solution (0.4 U mL^{-1}, 5.38 μM), 5 μL of NAD^+ solution (50 mM), 5 μL of sodium formate (3 M), 5 μL of nitroblue tetrazolium (NBT, 2 mM), 5 μL phenazine ethosulfate (PES, 0.24 mM), and 0−70% (v/v) of ILs in PBS buffer (pH 7.2) is used if the activities are measured at 560 nm. Optical pathways are determined by measuring the absorbance of water at 975 nm in the near IR region for each individual well ($\varepsilon_M^{975\ nm} = 3.05 \times 10^{-3}\ M^{-1}\ cm^{-1}$). Activities are an average value of at least 3 measurements and are expressed in μmol min^{-1} mg^{-1}.

Fluorescence. FDH is diluted in PBS buffer and the final concentration is fixed at 0.32 U mL^{-1} (2.61 μM) in all the experiments. Enzyme samples are excited at 280 or 295 nm (bandwidth = 5 nm), and emission was recorded between 310 and 500 nm or between 320 and 500 nm (bandwidth = 20 nm) in the presence of 0−70% (v/v) ILs or 0−359 mM imidazole in PBS, pH 7.2 (imidazolium/imidazole is 40/60 at pH 7.2). Quenching experiments by iodide or acrylamide are performed in the presence of 0−200 mM NaI or 0−350 mM acrylamide, in the presence or in the absence of 0−60% (v/v) of ILs or 0−5 M urea in PBS, pH 7.2. In all experiments, the fluorescence of the medium in the absence of the enzyme is subtracted from the enzyme spectra. The maximal intensities of fluorescence are an average value of at least three experiments. The quenching experiments of the tryptophan solution are performed at a final

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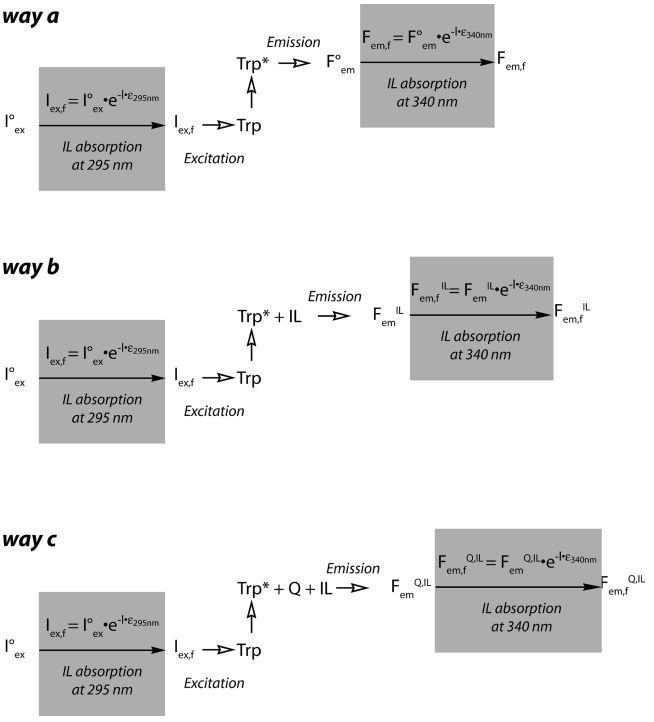
Scheme 3. Modification of the Tryptophan Fluorescence by IL Absorption (way a), IL Quenching (way b), and IL with an Additional Quencher (way c)



concentration of 50 μM in PBS, pH 7.2, and the intensities at 340 nm are used for the K_{SV} determination.

THEORY

Proteins usually reemit light between 300 and 350 nm after excitation at 280 nm (due to all the fluorophores of the protein) or at 295 nm (mostly due to tryptophanyl residues). The fluorescence intensity is the sum of the light emitted by each individual fluorescent residue of the protein. The (partial) unfolding of the protein increases the interactions of fluorescent residues with the solvent (usually water). Additionally, the indole ring of tryptophan could interact with the side chains of the other residues of the protein.[45,46] Both phenomena lead to the decrease of the fluorescence intensity and sometimes to a red shift of the emission peak, which is used as a signature for protein unfolding in aqueous solution.

The introduction of ionic liquids, e.g., of imidazolium cations in this study, usually lead to a decrease of the protein emission peak,which is attributed to protein unfolding by some authors and to interferences by others.[20,38,39] Scheme 3 describes the possible events that could lead to the decrease of the fluorescence of a single tryptophan molecule or of a tryptophanyl residue within a protein. Obviously, the same events occur for any other fluorophore (e.g., tyrosine or phenylalanine), considering appropriate wavelengths.

In the simplest case (Scheme 3, way a), the decrease of the fluorescence in the presence of an IL is due to the absorption of both the excitation and the emission lights (inner filter effect). In such a case, the attenuation of the light intensity can be described by the Lambert−Beer law considering the absorption of the excitation light (eq 1) and of the emitted fluorescence (eq 2) by the IL and considering a function of the fluorescence of the fluorophore itself (eq 3)[47]

$$I_{ex,f} = I^0_{ex}\, e^{-l\varepsilon_{ex}} \tag{1}$$

$$F^0_{em,f} = F^0_{em}\, e^{-l\varepsilon_{em}} \tag{2}$$

$$\frac{F_{em,f}}{I^0_{ex}} = f(\text{Fluo})\, e^{-C_{IL}l(\varepsilon_{ex}+\varepsilon_{em})} \tag{3}$$

where I^0_{ex} is the original nonfiltered light intensity, $I_{ex,f}$ the filtered light intensity, which participates in the excitation of the fluorophore, F^0_{em} the nonfiltered light intensity emitted by the fluorophore, $F^0_{em,f}$ the emission light intensity filtered by the media, ε the molar extinction coefficients of the IL at the excitation and emission wavelengths (M^{-1} cm^{-1}), C_{IL} the IL molar concentration (M), and f(Fluo) an intrinsic fluorescence constant of the fluorophore, which depends on its physicochemical environment (in solution or buried inside an enzyme core, for example). Therefore, in this case, a linear relationship between $\log(F_{em,f}/I_{ex}{}^0)$ and C_{IL} should be observed, and it becomes obvious that the decrease of the observed fluorescence is not due to protein unfolding but only to light absorption. These relationships should be first verified for any fluorophore in the presence of a light absorbing species. At constant IL concentration, the inner filter effect is constant enabling the study of the protein unfolding with steady-state fluorescence spectroscopy by another physical agent (temperature, by another chaotropic agent, etc.). If eq 3 is not verified (e.g., the inner filter effect is not the only reason of the fluorescence attenuation), the quenching of the fluorescence by solutes (ILs salts) should be first considered (Scheme 3, way b).

Quenching experiments can be interpreted differently depending on the exposition of a fluorophore in a protein. In the simplest model, the quencher affects the fluorescence of all the fluorophores of the protein, and therefore, all the fluorophores could be seen as a single population. In this case, the quenching can be studied by the Stern−Volmer relationship (eq 4)

$$\frac{F^0_{em,f}}{F_{em,f}} = 1 + K_{SV}[Q] \tag{4}$$

$F^0_{em,f}$ is the filtered emission fluorescence intensity in the absence of quencher (way a), $F_{em,f}$ the filtered emission fluorescence in the presence of quencher (way b), $K_{SV}{}^{Q}$ the Stern−Volmer constant (M^{-1}),[34] and [Q] the quencher concentration (M).

When a fraction of the fluorophore is buried into the protein core, the quencher affects only a fraction of the fluorophore, and the quenching experiments should be interpreted by the Lehrer equation (eq 5)[48] where α is the fraction of fluorophore affected by the quencher

$$\frac{F^0_{em,f}}{(F^0_{em,f} - F_{em,f})} = \frac{1}{\alpha} + \frac{1}{\alpha K_{SV}[Q]} \tag{5}$$

ILs could potentially act as collisional quenchers (Scheme 3, way b) reacting in a bimolecular reaction with excited fluorophores. It becomes important to define two Stern−Volmer constants: $K_{SV}{}^{Q}$, the Stern−Volmer constant describing the quenching of the fluorescence by a classical quencher (iodide or acrylamide, for example), and $K_{SV}{}^{IL}$, the Stern−Volmer constant describing the quenching by the IL. Therefore, the attenuation of the protein fluorescence due to the increase of the

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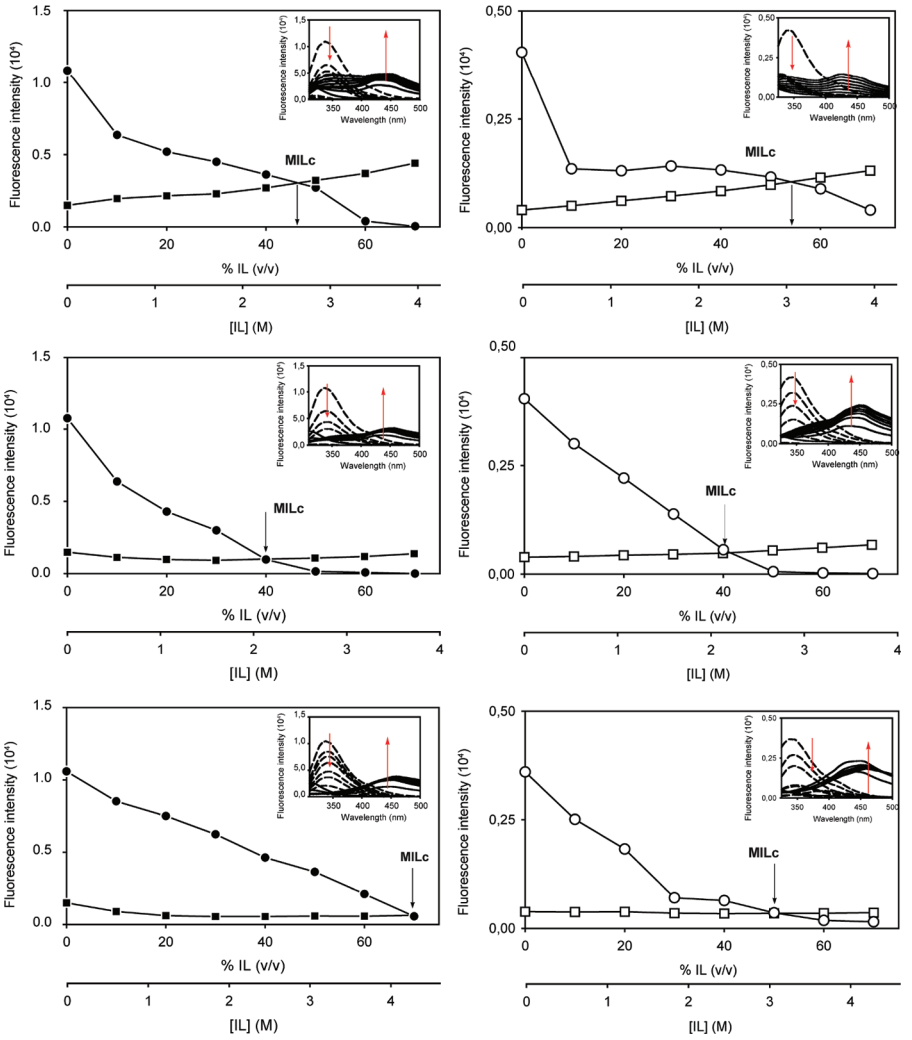

Figure 1. Fluorescence intensities of the FDH (●) and of ILs (■) at 335 nm after excitation at 280 nm (left). Fluorescence intensities of tryptophanyl residues (○) and of ILs (□) at 340 nm after excitation at 295 nm (right). (a, d) **IL-1**, (b, e) **IL-2** and (c, f) **IL-3**. Insets: Original fluorescence spectra corrected by the IL contribution (dashed lines) and of the ionic liquid itself (solid lines). The arrow shows the spectra at increasing IL concentration.

ionic liquid concentration cannot be strictly related to the protein unfolding but simply to a collisional quenching process if one of the relationships, eq 4 or 5, is experimentally verified considering the IL as the quenching species.

The introduction of a second quenching agent of the protein fluorescence (iodide or acrylamide) in the medium, at a fixed IL concentration, allows the determination of a K_{SV}^{Q} value representative of the fluorophore accessibility to the second quencher and therefore of the protein folding (Scheme 3, way c). The presence of two quenchers also supposes that iodide (or acrylamide) should not significantly quench the fluorescence of the IL or that the IL should not quench the fluorescence of iodide (or acrylamide), if any. Nevertheless, this last case does not occur as iodide (or acrylamide) does not reemit fluorescence (data not shown).

A K_{SV} value depends of the fluorophore lifetime (τ_0) and of the collision kinetic constant (k_q), which depends itself of the viscosity of the medium (η).[49] The individual fluorophore lifetime should be ideally determined in the absence of a quenching agent. ILs acting potentially as a quencher; their influence on the lifetime is implicit. As the viscosity of the medium increases with the IL concentration, the Ksv^{Q} value should decreases if the protein does not unfold, with respect to the light absorption of the media. On the contrary, an increase of the Ksv^{Q} value is necessarily related to a higher accessibility of fluorescent residues by the quencher, and consequently, this is associated with the protein unfolding.

RESULTS

According to the theoretical part described above, the investigation of the conformation of the FDH from *Candida boidinii* in three water-miscible ILs (Scheme 1) is performed in order to evaluate the possibility to use steady-state fluorescence spectroscopy as an unambiguous method.

We first determine the fraction of accessible fluorophores and of accessible tryptophanyl residues in the native FDH with the help of a dynamic quencher (e.g., iodide) and eqs 4 and 5. The Lehrer plot obtained after excitation at 280 nm allows the calculation of an accessible fraction of fluorophore of 0.99 (e.g., $\alpha \approx 1$) (Supporting Information, SI 2). The K_{SV}^{Iodide} values determined with the Lehrer plot are similar to those determined with the Stern−Volmer analysis (4.48 and 4.28 M^{-1}, respectively).

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Therefore, all the fluorescent residues of the native FDH could be considered as homogeneously accessible by the dynamic quencher, and none of them should be considered as deeply buried inside the protein core. Second, the tryptophan accessibility obtained after specific excitation at 295 nm leads to an accessible fraction of 0.99 ($\alpha \approx 1$), and the K_{SV}^{Iodide} values obtained with the Lehrer and with the Stern−Volmer plots are similar (3.67 and 3.49 M^{-1}). The tryptophanyl residues do not behave differently than the other fluorophores of the FDH. In this protein (Supporting Information, SI 1), none of these residues appear to be deeply buried inside the FDH core. Consequently, in this work, the quenching experiments are interpreted with the Stern−Volmer relationship (eq 4), as the simplest model, instead of the model described by Lehrer (eq 5).

The steady-state fluorescence spectra of the FDH (2.61 μM) in the presence of 0−70% (v/v) of **ILs**-**1**−**3**, after excitation at 280 nm and at 295 nm, are given in Figure 1 (insets). The fluorescence spectra of the ILs alone under the same conditions are provided in the same figure. After excitation at 280 nm, the intensity of the FDH peak located at 335 nm decreases as the IL concentration increases. Despite the fact that the ILs strongly absorb in the UV region (Figure 2), the fluorescence of the FDH is clearly visible up to 47% (v/v) of **IL**-**1**, 40% (v/v) of **IL**-**2**, and 70% (v/v) of **IL**-**3**. At higher IL concentrations, the FDH fluorescence is lower than the signals of the ILs alone, and no relevant FDH fluorescence spectra could be obtained. After excitation at 295 nm, a similar behavior is observed. The maximum fluorescence intensity decreases with the IL concentration and the tryptophanyl emission spectra could only be measured up to 54% (v/v) of **IL**-**1**, 40% (v/v) of **IL**-**2**, and 50% (v/v) of **IL**-**3**.

Consequently, we define a maximum IL concentration (MILc) for which a reliable protein (or tryptophanyl) fluorescence spectrum could be obtained. Obviously, this parameter depends on the nature of the ionic liquid, of the protein concentration, and on the presence of impurities. The ILs of this study are commercial ones, used without further purification. Therefore, as shown by others, they contain some impurities, due to the IL preparation and history (heating, starting material, etc.), which have a strong influence on their spectroscopic properties (UV, fluorescence, etc.).[26,50] The IL concentration range, which could be used for protein conformation studies with such IL, is consequently limited, and the MILc will be lower than expected. Further studies will require ILs of higher purities with low UV−vis absorbance and consequently higher MILc. If such an IL is not accessible, the determination of the MILc appears to be essential for relevant analysis. The MILc value could also be improved by using higher protein concentration. For example, if the FDH is used at a concentration of 13 μM and excited at 295 nm, the MILc is more than 70% (v/v) with **IL**-**3** but only ∼50% with **IL**-**2** (data not shown). Therefore, the MILc could not be simply bypassed by using high protein concentrations but should be determined at the beginning of a new study for each IL/enzyme couple. This also implies that, even for ILs displaying a high fluorescence, there should exist an IL concentration range in which the protein conformation could be evaluated by steady-state fluorescence. All further experiments will be conducted at IL concentrations below the MILc.

The strong decrease of the protein fluorescence below the MILc could be improperly attributed to the protein unfolding. Nevertheless, light absorption by the IL and/or quenching of the protein fluorescence could also explain this observation.

Light Absorption by the ILs. In order to determine if the decrease of the fluorescence is only due to the light absorption by the ILs (eq 3), the UV−visible absorption spectra of the FDH (2.61 μM) in PBS (pH 7.2) were measured in the presence of different concentrations of the different ILs (Figure 2). In the absence of IL, the FDH absorption peak is clearly visible at 280 nm. In the presence of ILs, the solutions are yellowish and translucent at medium-to-high IL concentration (high light absorption around 420 nm), but no precipitates or aggregates are observed. The ILs of this study strongly absorb UV-light between 230 and 400 nm, and the absorption peak of the FDH around 280 nm is clearly hidden by the IL signal even at 10% (v/v) of IL. The molar extinction coefficients of these ILs at the wavelength used for fluorescence and FDH activity studies (280, 295, 335, 340, and 560 nm) are provided in Table 1.

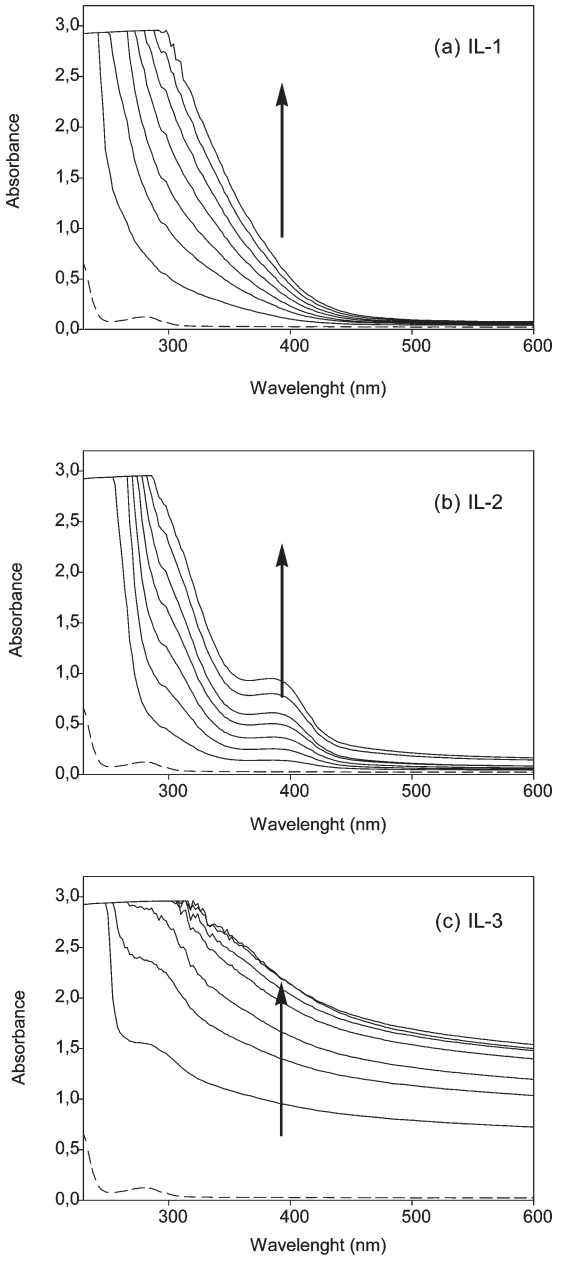

Figure 2. UV−vis absorption spectra of the FDH in PBS buffer pH 7.2 (dotted line) and in the presence of 10−70% (v/v) (steps of 10%) of (a) **IL**-**1**, (b) **IL**-**2**, or (c) **IL**-**3** (solid lines). The arrow shows the spectra at increasing IL concentration.

Table 1. Molar Extinction Coefficient of the ILs at 280, 295, 335, 340, and 560 nm

ILs	$\varepsilon^{280\ nm}$ (M^{-1} cm^{-1})	$\varepsilon^{295\ nm}$ (M^{-1} cm^{-1})	$\varepsilon^{335\ nm}$ (M^{-1} cm^{-1})	$\varepsilon^{340\ nm}$ (M^{-1} cm^{-1})	$\varepsilon^{560\ nm}$ (M^{-1} cm^{-1})
IL-1	2.03	1.56	0.92	0.81	0.02
IL-2	2.04	1.66	0.63	0.73	0.04
IL-3	2.95	2.92	2.2	1.87	1.27

Table 2. Stern−Volmer Constants of the Quenching of FDH and Tryptophan Fluorescence by ILs-1−3

IL	FDH K_{SV}^{IL} (M^{-1})	tryptophan K_{SV}^{IL} (M^{-1})
IL-1	0.88	30
IL-2	1.56	35
IL-3	0.55	41

Considering these values, the light intensity for the excitation of the FDH (at 280 or at 295 nm) will be lower than in an aqueous solution. Similarly, the light emitted by the FDH (at 335 nm or at 340 nm) will be absorbed by the IL leading to a reduced intensity of the fluorescence spectra (as described in Scheme 3, way a). This also means that a fluorescence spectrum obtained in an IL could only be strictly compared to another one if both are recorded at the same IL concentration and with the same IL batch because a reliable correction taking into account the IL concentration and purity seems difficult to apply.

The dependency of the emitted fluorescence of the tryptophanyl residues to the IL light absorption (Scheme 3, way a) is evaluated by eq 3 using an excitation wavelength of 295 nm and an emission of 340 nm (Supporting Information, SI 3). Considering the three ILs tested in this work, eq 3 cannot be verified with certainty as no linear behavior is observed ($R^2 < 0.90$). Moreover, if eq 3 was validated, the slopes calculated from Supporting Information, Figure SI 3, should be $l(\varepsilon^{295\ nm} + \varepsilon^{340\ nm})$ and should follow this order **IL-3** > **IL-1** ≈ **IL-2**. Obviously, this is not verified. The same analysis performed on the overall FDH fluorescence, using 280 nm as excitation wavelength and 335 nm as fluorescence emission wavelength, does not lead to a linear behavior ($R^2 < 0.92$) and the slopes do not follow the order **IL-3**> **IL-1** > **IL-2**.

The analysis of the absorption properties of these ILs, with the help of eq 3, shows that the observed decrease of the FDH fluorescence is not only due to the light absorption by the ILs but that additional events should occur.

Quenching Properties of the ILs. The quenching properties of **ILs-1−3** and of imidazole were first evaluated with a tryptophan solution as a model of accessible tryptophanyl residues (Scheme 3, way b). Imidazole was chosen as a nonalkylated and noncharged model of imidazolium-based ILs. The decrease of the fluorescence due to the light absorption (inner filter) is evaluated with the eq 3 (Supporting Information, SI 4). The decrease of fluorescence in the presence of imidazole verified eq 3 ($R^2 > 0.97$) showing that the imidazole does not quench tryptophan fluorescence. The decrease of the fluorescence of tryptophan by imidazole is consequently only due to the inner filter effect. On the contrary, **ILs-1−3** are strong quenchers of the tryptophan fluorescence. The K_{SV}^{IL} values calculated for the quenching of the tryptophan solution are equal to 30 M^{-1}, 35 M^{-1}, and 41 M^{-1} for **IL-1**, **IL-2**, and **IL-3**, respectively (Table 2). The quenching properties of these ILs are suspected to be due to the interaction of the indole ring with the positively charged and alkyl substituted imidazolium ring.[20] The most important conclusion from these experiments is that the decrease of a protein fluorescence observed in the presence of ILs could not only be simply attributed to the protein unfolding but also to the quenching of the accessible fluorescent residues of the protein by the ILs ions.

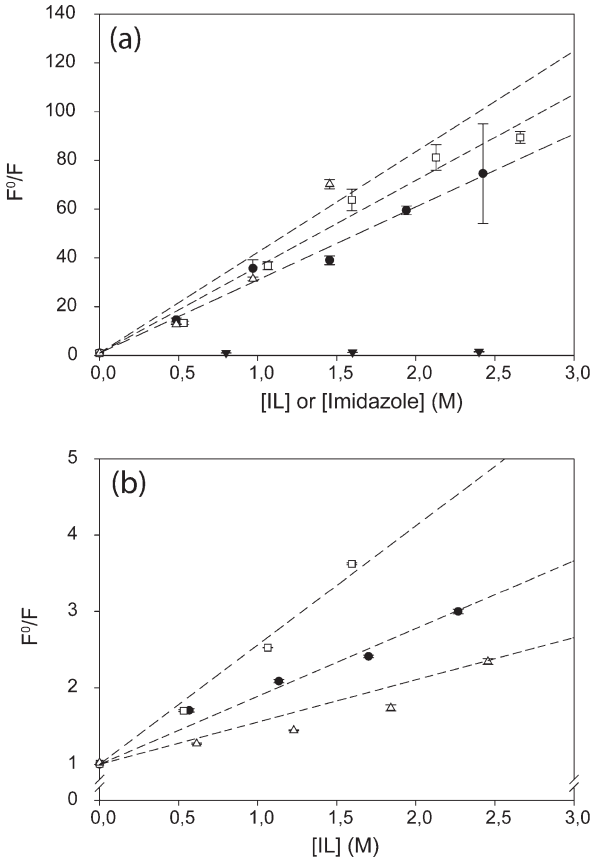

Figure 3. Stern−Volmer plots of the quenching of FDH fluorescence (a) and of tryptophanyl residues (b) by **IL-1** (●), **IL-2** (□), or **IL-3** (△). F and F_0 are the fluorescence intensities with and without a quencher, respectively. Data are obtained under the MILc.

The quenching of the overall FDH fluorescence by **ILs-1−3** is investigated below the MILc and analyzed by the Stern−Volmer plot (Figure 3). The K_{SV}^{IL} values calculated from the Stern−Volmer plot at low IL concentration are comprised between 0.1 M^{-1} and 1.6 M^{-1} (Table 2). This suggests that at low IL concentration, the fluorescent residues are less accessible to the solvent compared to a soluble fluorophore (with a higher K_{SV}^{IL} value like tryptophan) and that the protein is not unfolded. Nevertheless, this interpretation is excessive because the decrease of the fluorescence intensity in the presence of ILs could be due to quenching and/or to protein unfolding. Expecting ILs act as charged collisional quenchers, the increase of their concentration will increase their quenching efficiency, but they could also simultaneously unfold the FDH. Obviously, it is impossible to separate easily the two mechanisms with steady-state fluorescence. An even more hazardous analysis of protein quenching by IL is to work at concentrations above the MILc. The K_{SV}^{IL} values are estimated to be between 91 M^{-1} and 176 M^{-1}, at high ILs

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concentrations, which could erroneously suggest that the fluorophores became fully accessible to the ionic liquid, e.g., that the FDH is totally unfolded. As stated earlier, above the MILc, the determined K_{SV}^{IL} values are strongly subjected to high experimental errors due to the low signal/noise ratio, and these values are not relevant. Therefore, despite a correct subtraction of the background, reliable measurements of the intrinsic protein fluorescence could only be performed at IL concentrations below the MILc, demonstrating the importance of the determination of this parameter.

Then, we should admit that the determination of the K_{SV}^{IL} value does not help to understand whether a particular enzyme unfolds or not in the presence of a specific IL. The increase of the IL concentration takes part in the attenuation of fluorescence, but if the protein does not unfold, the term f(Fluo) in eq 3 remains constant, and its contribution to the K_{SV}^{IL} will not affect the linear behavior of the Stern−Volmer plot.

Apparently, the only evidence of the unfolding of a protein in an ionic liquid will be a sharp increase of the $F^0_{em,f}/F_{em,f}$ values once a critical ionic liquid concentration is reached. This supposes that the enzyme unfolds at a critical IL concentration revealing buried fluorescent residues. Unfortunately, this is unlikely to occur because unfolding is more related to a multistep process rather than a concentration-dependent critical one. Additionally, as most of the proteins contains more than one single fluorescent residue, their respective contribution to the Stern−Volmer plot is nearly impossible to solve by simple means (e.g., steady-state fluorescence).

Now, it appears evident that the study of the conformation of an enzyme in an IL could not be simply related to the observation of the quenching of its intrinsic fluorescence by the IL alone.

Quenching of FDH Fluorescence by Dynamic and Static Quenchers. As the measurement of Ksv^{IL} is not a suitable parameter to determine the influence of the **ILs-1−3** on the FDH conformation, the quenching of the FDH fluorescence by iodide or acrylamide has been conducted in **ILs-1−3,** at concentrations below the MILc (Scheme 3, way c). Two kinds of quencher are used to explore the two general quenching processes. The first one, iodide, is a charged dynamic quencher that interacts with the fluorophores located near the enzyme surface. When a protein unfolds, the fluorescent residues are more exposed to the solvent, and the quenching efficiency increases.[49,51] Another way to study the protein unfolding is to use a neutral quencher, also called static quencher (e.g., acrylamide), which can penetrate into the protein core and form a reversible complex with the fluorescent residues.[49,52] Two models are used to interpret the quenching efficiency. The first model is a two-step diffusion-controlled model where the quenching efficiency is essentially due to the quenching of the fluorophores, which are near the protein surface as it is the case for iodide.[53] The second model is the penetration model where the quenching efficiency is associated with the propensity of the quencher to penetrate within the structural cavities, which appear when a protein unfolds and form dark complexes.[52] Both models lead to the same general observation: the quenching efficiency, reflected by the K_{SV}^{Q} values, increases when the protein unfolds.[49,54]

The FDH is a rather complex enzyme regarding the number of fluorophores it contains (16 tyrosines and 5 tryptophanes). Therefore, we have conducted quenching experiments by iodide or acrylamide after excitation at 280 nm to evaluate the overall FDH conformation and at 295 nm in order to acquire specific data concerning the tryptophanyl residues. First, the fluorescence

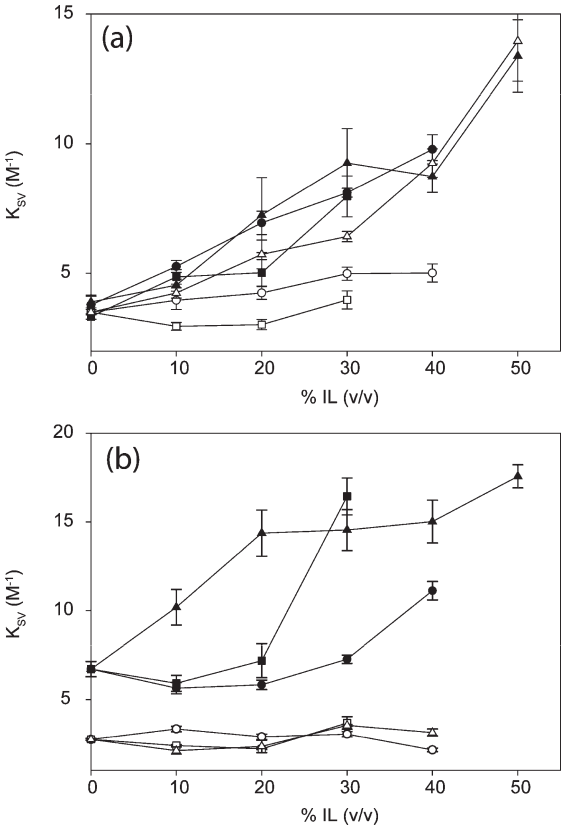

Figure 4. Evolution of the Stern−Volmer constant (K_{SV}^{Q}) of the quenching of FDH (filled symbol) and of tryptophanyl fluorescence (empty symbol) by (a) iodide or (b) acrylamide as the concentration of **IL-1** (● and ○), **IL-2** (■ and □), or **IL-3** (▲ and △) increases.

of the ILs tested in this work is quenched by iodide or acrylamide after excitation at 280 and 295 nm. The K_{SV}^{Q} calculated for the quenching of the ILs fluorescence at 335 nm, by iodide or acrylamide, could only be measured for **IL-1** because **IL-2** and **IL-3** do not remit significant fluorescence at this wavelength (Figure 1). The decrease of the K_{SV}^{Q} value with the IL concentration is attributed to the increase of the viscosity of the medium.[49,53] (Data not shown.) Therefore, the limited quenching of the ILs by iodide or acrylamide will not affect the quenching of the FDH fluorescence.

Quenching by Iodide. The Stern−Volmer constant of the quenching of the overall FDH fluorescence by iodide is determined in the presence of different ILs concentrations (Figure 4). When the FDH fluorescence is quenched by iodide, the K_{SV}^{Q} values calculated after excitation at 280 nm increase with the IL concentration and are found to be in the order of M^{-1} (Figure 4a). As the ILs fluorescence is not quenched by iodide, the variation of the K_{SV}^{Iodide} value is necessarily due to the variation of the FDH fluorescence.

Slight structural variations of the FDH occur as **ILs-1−3** concentrations increase because the quenching by iodide is correlated to the fluctuations of the enzyme conformation.[49,52] The K_{SV}^{Iodide} values are multiplied by 2.96, 2.41, and 4.35 in the presence of 40% (v/v) of **IL-1**, 30% (v/v) of **IL-2**, and 60% (v/v) of **IL-3** respectively, compared to K_{SV}^{Iodide} determined in aqueous solution (Figure 4a).

The Stern−Volmer constants are also determined after excitation at 295 nm in order to estimate the tryptophanyl

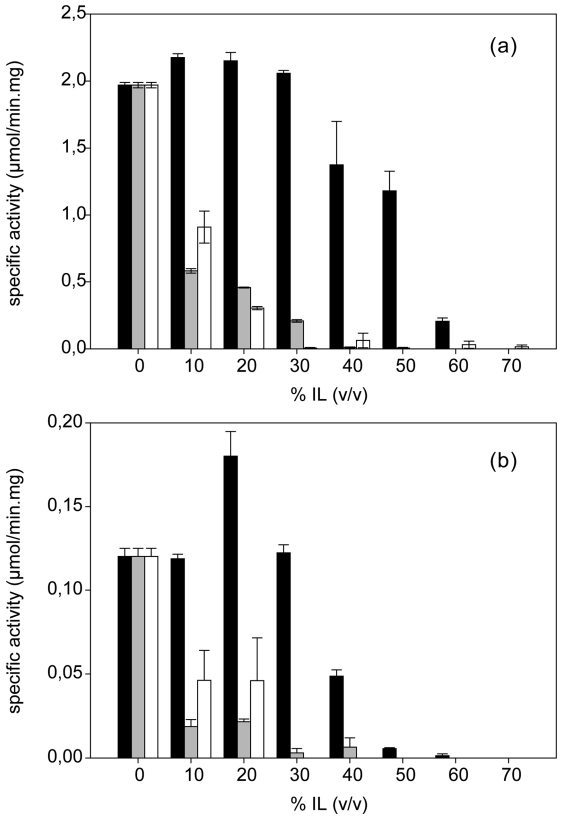

Figure 5. Activity of the FDH in presence of 0−70% (v/v) of **ILs-1**−**3** measured by following (a) the NADH apparition at 340 nm or (b) the formazan formation at 560 nm. The black, gray, and white bars represent the activities of the FDH in **IL-1**, **IL-2**, and **IL-3**, respectively.

accessibility to iodide when the **ILs-1**−**3** concentration increases up to the MILc. At 295 nm, the K_{SV}^{Iodide} value in the presence of **IL-1** and **IL-2** does not significantly vary from 0% (3.49 M^{-1}) to 40% of **IL-1** (5.01 M^{-1}) or 30% of **IL-2** (3.97 M^{-1}). In these two ILs, the tryptophanyl environment is not strongly affected by the fluctuation of the overall enzyme conformation. This is probably because, in the case of the FDH, the five tryptophanyl are not deeply buried inside the protein core, and iodide is able to quench most of their fluorescence in the absence of **IL-1**−**2**.

In the presence of **IL-3**, the quenching efficiency of iodide is much higher than in **IL-1** or **IL-2**. The K_{SV}^{Iodide} value increases with **IL-3** concentration and reaches nearly 14 M^{-1} in 50% (v/v) of this IL. This shows that tryptophanyl residues, if accessible to iodide in the absence of any IL, could be quenched more efficiently in the presence of **IL-3**. This IL is a stronger denaturing IL than **IL-1** or **IL-2** and should unfold the FDH with a different mechanism.

Quenching by Acrylamide. Second, the overall FDH fluorescence is quenched by acrylamide after excitation at 280 nm, at IL concentrations below the MILc. The behavior of the $K_{SV}^{Acrylamide}$ value differs for the three ILs (Figure 4b). The $K_{SV}^{Acrylamide}$ values slightly decreases below 30% (v/v) of **IL-1** and 20% (v/v) of **IL-2**. This could be attributed to a closer conformation of the FDH preventing the acrylamide penetration inside the protein core or, more probably, to the influence of the viscosity on the $K_{SV}^{Acrylamide}$ value. Above these two IL concentrations, the $K_{SV}^{Acrylamide}$ values sharply increase by a factor of 1.65 and of 2.44 for **IL-1** and **IL-2**, respectively. This could be attributed to a partial protein unfolding, which allows the acrylamide to penetrate inside the protein core to form complexes with the newly accessible tyrosinyl or tryptophanyl residues. The increase of the $K_{SV}^{Acrylamide}$ value is correlated to the inactivation of the FDH above 20% (v/v) of **IL-2** (Figure 5). This also corresponds to the concentration at which the FDH activity begins to decrease (above 30% (v/v) of **IL-1**) (Figure 5). In the presence of **IL-3**, the $K_{SV}^{Acrylamide}$ value is multiplied by a factor of 2.14 at 20% (v/v) of this IL and remains constant above this concentration. Higher **IL-3** concentrations do not increase the $K_{SV}^{Acrylamide}$ value meaning that the resulting conformation allows the access of this quencher to most of the FDH fluorophores by the penetrating quencher. Interestingly, if the specific quenching of tryptophanyl fluorescence by acrylamide is followed at 295 nm, the $K_{SV}^{Acrylamide}$ value does not increase regardless of the IL structure or the IL concentration used (∼2.7−3 M^{-1}). It appears that tryptophanyl residues are accessible to acrylamide even in aqueous solution as they were accessible to iodide. Nevertheless, in the presence of **IL-3**, the accessibility of iodide to tryptophanyl residues increases with the IL concentration, while the accessibility of acrylamide does not. This is probably due to the ionic nature of iodide in which interactions with the FDH are limited by electrostatic repulsion. The unfolding of the FDH by **IL-3** should allow a higher access of iodide to tryptophanyl residues as proved by the increase of the K_{SV}^{Iodide} value.

The differences between the quenching by iodide and by acrylamide can be explained by the nature of the quencher. Iodide quenches the fluorescence of residues exposed to the solvent, and its quenching efficiency is correlated with the fluctuations of the enzyme conformation. It is also submitted to electrostatic repulsion by the FDH residues. Acrylamide is not a dynamic quencher like iodide;[49] it should quench the fluorescence according to the penetration model.[52] The maximum quenching efficiency is reached when the FDH is sufficiently unfolded to allow the access of the acrylamide to most of the fluorophores. However, this does not necessarily require a complete unfolding of the protein. The $K_{SV}^{Acrylamide}$ value is reached above 20% (v/v) of **IL-3** and at 30% of **IL-2** but cannot be measured for **IL-1** due to its lower MILc (Figure 4b). It is interesting to notice that, in the case of the FDH, the study of tryptophanyl fluorescence by a static quencher supports the idea that the FDH does not unfold, while the study of the overall fluorescence and the use of a dynamic quencher shows that different mechanisms of unfolding occur according to the IL studied.

The effect of **ILs-1**−**3** on the FDH conformation is compared to the effect of a classical denaturant like urea (Supporting Information, SI 6). At a concentration of 1.6 M, the K_{SV}^{Iodide} determined in the presence of urea is 1.5 times higher than in buffer, while in the presence of ILs, it is multiplied by ∼2.5 depending on the IL. With higher concentrations (4.16 M), the K_{SV}^{Iodide} is multiplied by 2.4 with urea and by a 4.3 with **IL-3**. Similar results are obtained with acrylamide as a quencher (Supporting Informatin, SI 6). All together, these results show that these water-miscible ILs are stronger denaturing agents than urea.

Structure−Activity Relationship in ILs. The FDH activity was measured in the presence of 0−70% (v/v) of **ILs-1**−**3** in order to determine if a relationship between the K_{SV}^{Q} and the FDH activity could be observed. The activity was measured in the presence of 0−70% (v/v) of ILs (Figure 5) by two methods: by measuring the NADH formation at 340 nm (Figure 5a) or by

measuring the formation of formazan at 560 nm (Figure 5b). Both methods gave similar results. In the presence of **IL-1**, the FDH activity begins to decrease above 30% (v/v), and the enzyme is totally inactivated at 70% (v/v) of this IL. The enzyme activity is even slightly increased in the presence of 10−20% of **IL-1** as we previously observed.[16,55] The deleterious effect on the activity is more pronounced in the presence of **IL-2** and **IL-3**. In these two ILs, the activity decreases above 10% (v/v) of IL and is negligible above 30% (v/v) of **IL-2** and above 20% (v/v) of **IL-3** (Figure 5).

As shown earlier, the tryptophan accessibility by acrylamide or iodide in **IL-1** or **IL-2** does not change as the IL concentration increases. The decrease of activity cannot be linked to a higher accessibility of these residues. In **IL-1**, the activity does not decrease with the IL concentration, while in 30% (v/v) of **IL-2**, the FDH is inactivated, and the tryptophanyl accessibility is unchanged. As previously stated, the tryptophanyl residues are located near the FDH surface and are consequently less sensitive to the conformation fluctuations. The higher accessibility of tryptophanyl residues by iodide in the presence of **IL-3** could be nevertheless related to the decrease of activity in this IL. This suggests that **IL-3** is a stronger denaturing agent than **IL-1** and **IL-2** and that it unfolds the FDH with a different mechanism.

The relationship between the overall FDH fluorescence and the FDH activity reveals more interesting features. In the presence of 10−30% of **IL-1**, some of the FDH fluorophores become more accessible to the solvent (and to iodide within) as the K_{SV}^{Iodide} value increases. The FDH remains sufficiently compact to prevent acrylamide to form additional dark complexes. Nevertheless, this affects the enzyme activity. At higher **IL-1** concentrations (40% (v/v) and higher), the $K_{SV}^{Acrylamide}$ value significantly increases meaning that more fluorophores are accessible to the static quencher. This is accompanied by a decrease of the activity. Therefore, we could relate the FDH activity in **IL-1** to the accessibility of the fluorophores (tyrosinyl and tryptophanyl residues) to a static quencher. The **IL-2** also led to the modification of the FDH conformation as proved by the increase of the K_{SV}^{Iodide} value with the IL concentration and the concomitant loss of activity. This suggests that the unfolding by **IL-2** leads to an enzyme conformation different from that observed in the presence of **IL-1** and, therefore, to a different mechanism. **IL-2** is composed of a [BMIm] cation, while **IL-1** contains a [MMIm] cation. Therefore, an inactivation mechanism involving hydrophobic interactions could be envisaged.[8] Additionally, the $K_{SV}^{Acrylamide}$ value (determined at 280 nm) is constant from 0 to 20% (v/v) of **IL-2** and sharply increases at higher concentrations (>30%) of **IL-2**, whereas the enzyme is inactivated from 10% of **IL-2.** This suggests that the first step of inactivation does not change the tryptophanyl environment.

Finally, the activity sharply decreases between 10 and 30% of **IL-3** when the $K_{SV}^{Acrylamide}$ value increases. At higher **IL-3** concentrations, both the $K_{SV}^{Acrylamide}$ value and the activity remain constant (inactive enzyme), meaning that, with **IL-3**, the protein unfolds at low concentrations, allowing the acrylamide to interact with the fluorescent residues (tyrosinyl and tryptophanyl). As the $K_{SV}^{Acrylamide}$ value does not increase significantly, at concentration >30% (v/v) of **IL-3**, most of the fluorophores should be accessible to the acrylamide. The **IL-3** is composed of a phosphonate anion, which is smaller than the dimethylphosphate anion of **IL-1**. Therefore, it could be considered as more chaotropic and more easily destabilizes the protein structures.[16−19,55] Similar conclusions concerning the solubilization of biomacromolecules (e.g., cellulose fibres) by phosphonates anions were recently obtained by another group.[56]

CONCLUSIONS

In the present report, we have described how steady-state fluorescence spectroscopy could be used without ambiguity to study protein conformation in the presence of water-miscible ILs despite their strong UV-absorbance. First, we defined the MILc as the maximum IL concentration at which the FDH fluorescence signal is relevant for structural studies. It depends on the ionic liquid used, on its purity, and on the protein concentration, and it should be determined for each enzyme−IL pair. Ideally, if the protein is sufficiently concentrated and does not precipitate or aggregate, the MILc could reach high concentration. We also evidenced that the decrease of the FDH fluorescence could be due to absorption of the excitation and emission lights but also to the quenching of enzyme fluorescence by the IL itself. Therefore, the exact reason of the decrease of the protein fluorescence appears to be unknown with steady-state fluorescence alone. We have proposed to use additional quenchers (e.g., iodide and acrylamide) in steady-state fluorescence experiments to solve this problem.

We have applied this methodology to a rather complex enzyme, the FDH from *Candida boidinii*. The attenuation of the FDH fluorescence by dynamic (e.g., iodide) or static (e.g., acrylamide) quenchers has been performed in three water-miscible imidazolium-based ILs under the MILc. The determination of the Stern−Volmer constants (K_{SV}^{Q}), of the total fluorophores as well as of the tryptophanyl residues, is necessary to understand the effect of each IL onto the FDH conformation. The quenching of the fluorescence of the tryptophanyl residues is not necessarily the best method to evaluate a protein structure in ILs. In the case of the FDH, these residues are located at the protein surface and are less sensitive to conformational variations. This points out the necessity of performing experiments with both quenchers in order to explain the protein behavior. Therefore, by studying the overall FDH fluorescence, in the presence of dynamic or static quenchers, we found that **IL-3** induces stronger structural changes than **IL-1** and **IL-2**, probably due to the presence of phosphonate ions. Moreover, it appears that the FDH in **IL-1** and **IL-2** presents similar accessibility to acrylamide and iodide but a different activity profile meaning that the unfolding mechanism is different.

This method was found to be suitable for many laboratories without an excessive amount of enzyme or expensive devices. It can help to understand how an IL unfolds, or not, a protein containing many fluorophores. It could also lead to the re-evaluation of previous reports dealing with intrinsic enzyme fluorescence in ILs.

ASSOCIATED CONTENT

Supporting Information. Lehrer representation of the quenching of the FDH fluorescence by iodide in PBS buffer; structure of the FDH from *Candida boidinii*; correlation between the emission light intensity of the FDH and the concentration of the ILs; quenching of the FDH fluorescence by imidazole; quenching of a tryptophan solution by the ILs or imidazole; and evolution of the K_{SV} value due to the quenching of the FDH fluorescence by acrylamide or iodide in the presence of different

concentration of urea. This material is available free of charge via the Internet at http://pubs.acs.org.

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