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Comparison of peroxidase activities of hemin, cytochrome *c* and microperoxidase-11 in molecular solvents and imidazolium-based ionic liquids

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Abstract

The ability of Fe(III)protoporphyrin(IX) chloride (hemin), microperoxidase-11 (MP-11), and cytochrome *c* (cyt-*c*) to oxidize 2-methoxyphenol (guaiacol) was examined in the room-temperature ionic liquids (IL) 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([bmim][Tf_2N]) and the hexafluorophosphates of 1-butyl- and 1-octyl-3-methylimidazolium, ([bmim][PF_6] and [omim][PF_6]), respectively. All three biocatalysts displayed peroxidase activity when activated by an electron acceptor, *tert*-butyl hydroperoxide for hemin and hydrogen peroxide for MP-11 and cyt-*c*. Hemin required the addition of a coordinating base, pyridine or *N*-methylimidazole (NMI), to produce an active complex. Cyt-*c* did not require exogenous ligands for activity in IL, although their addition increased peroxidase activity. MP-11 could not be solubilized without an exogenous ligand, therefore, whether MP-11 was active in the absence of such ligands was not determined. Pyridine provided higher activities than NMI for the three catalysts. Hemin and MP-11 peroxidase activities were markedly higher in IL compared to molecular solvents of similar polarity, as characterized by probe solvatochromic behavior, while cyt-*c* activity was comparable between both types of solvents. There was no consistent preference by the catalysts for a particular IL. These observations indicate that IL are suitable media for bioelectrocatalysis.

Keywords: Ionic liquids; Biocatalysis; Non-aqueous media; Cytochrome *c*; Oxidoreductase

1. Introduction

The reactivity of hemoproteins is to a large extent determined by the nature of the heme ligands, the immediate electrostatic environment and the presence of water molecules in the heme cavity [1]. Thus, hemoproteins with sulfur (from cysteinyl or methionyl groups) serving as the proximal heme ligand typically perform a broad range of oxygen transfer functions (mono-oxygenases, etc.), while hemoproteins with heterocyclic nitrogen (from histidyl) proximal ligands act as peroxidases [2–4]. Cytochrome *c* (cyt-*c*) is a classic redox-active protein with an imidazolium ligand in the fifth coordination site and a loosely associated methionyl group in the sixth coordination sphere of the heme [5]. Changes to the electrostatic field in the vicinity of the heme brought about by modifications to polypeptide side chain groups not directly ligated to the heme alter the formal potential, E^0 of cyt-*c* [1]. When cyt-*c* is slightly perturbed, such as by

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association with phospholipids or by the presence of organic solvents, the methionyl group is displaced and the redox protein acquires peroxidase activity [6,7]. The presence or absence of heme cavity water can in turn influence whether a hemoprotein will display peroxidase or catalase activity [8]. While many of the individual factors influencing hemoprotein function have been identified, a far greater knowledge is necessary to formulate predictive rules.

The heme cavity of hemoproteins has a relatively low dielectric in comparison to the aqueous medium in which the protein is typically dissolved. Non-aqueous media have been used to investigate the role of media dielectric on the E^0 and activity of heme-containing proteins. Polar, water-miscible organic solvents have been used and, in some of the cases, proteins can be directly dissolved in the medium [9,10]. However, polar organic solvents generally result in substantial loss of enzyme function and the influence of these solvents can lead to changes in E^0 contrary to expectations [7,11]. Non-polar, hydrophobic solvents are often found to be more suitable for retention of enzyme activity, presumably due to their reduced likelihood of stripping essential water from the enzyme [12], not because of their low dielectric. Producing true solutions of hemoproteins in non-polar solvents in order to perform spectroscopic studies requires extensive chemical modification of the protein or envelopment in a surfactant shell, which may obscure subtle medium effects [13,14]. Thus, there are many limitations to using conventional organic solvents to explore the influence of medium dielectric on the properties of hemoproteins.

Room-temperature ionic liquids (IL) are novel solvents with properties distinctly different from those of conventional organic solvents. Certain imidazolium-based IL are water-immisible and hydrophobic, yet have polarities comparable to short-chain primary alcohols [15–18]. IL enhance the activity of transition metal complexes, because of their unique combination of high polarity and low coordination power for the catalyst [19,20]. IL are attractive for non-aqueous electrochemical applications, and thus may also be viable as media for bioelectrochemical synthesis and biosensing. The electrochemical properties of biomimetic heme in IL have been recently reported [21].

To date, only a few enzymes have been examined in IL [22–28]. These initial results suggest that at least some enzymes tolerate IL at least as well as conventional molecular solvents. The present work develops the concept that IL provide a suitable (i.e. non-denaturing, non-inhibitory) environment for non-aqueous bioelectrocatalysis. The fundamental issue addressed is whether cyt-*c* will retain its peroxidase activity in IL, and whether this activity is distinctly different from that which may be observed with analogs of the cyt-*c* active site—namely, microperoxidase-11(MP-11) and Fe(III)protoporphyrin(IX) chloride (hemin). MP-11 is a mini-enzyme containing a histidyl-coordinated heme *c* prosthetic group covalently attached through thioether bonds to an 11-amino-acid peptide derived from cyt-*c* (residues 11–21) by proteolytic digestion. In addition, a comparison is made of the peroxidase activity of these model enzymes in IL with that observed in conventional molecular solvents.

2. Experimental

2.1. Materials

Hemin Cl was from ICN (Costa Mesa, CA), hydrogen peroxide (30% aqueous) was from Fisher Scientific, and all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO). (Note: names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.) Methanol was HPLC grade. The hexafluorophosphates of 1-butyl- and 1-octyl-3-methylimidazolium, [bmim][PF_6] and [omim][PF_6], respectively, were prepared as described previously [25]. 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([bmim][Tf_2N]) was prepared by the metathesis of the corresponding bromide and lithium salts [15]. The water content of the ionic liquids was reduced to $<0.01\%$ (w/w) by heating them at 110 °C under reduced pressure for 24 h. The ionic liquids were stored under dry N_2.

2.2. Peroxidase activity assay

2-Methoxyphenol (guaiacol) oxidation was measured by following color formation at 470 nm using

a Shimadzu 1240 spectrophotometer (also used for collecting absorption spectra) with the cell holder modified with heating tape to maintain the cuvette (1.0 cm pathlength) at 40 °C. A temperature of 40 ± 1 °C was used throughout to facilate the handling and transfer of the viscous ionic liquids. An absorption coefficient of $2.66 \times 10^4\ M^{-1}\ cm^{-1}$ was assumed for the oxidation product [29]. Reactants were not stirred in the cuvette during the reaction. The rate of color formation was multiplied by four to obtain the guaiacol oxidation rate. (While originally thought to be tetraguaiacol, the reaction product is now believed to be 3,3′-dimethoxy-4,4′-biphenoquinone [30,31].) Typical reaction conditions consisted of 25 mM guaiacol, 0.2–2.0 mM *tert*-butyl hydroperoxide (*t*-BuOOH; diluted from decane) or H_2O_2, 125 mM pyridine or *N*-methylimidazole (NMI), and 8–16 μM heme (hemin, MP-11, or horse heart cyt-*c*). Reactants were mixed in a stirred vial at 40 °C for 4 min prior to the addition of peroxide to initiate the reaction. The reaction medium was transferred to the cuvette and absorbance readings were recorded at 10 s intervals for 2 min, starting 1 min from the addition of peroxide. Concentrations of heme catalyst and peroxide were adjusted so as to capture the linear portion of the reaction (0.1–0.6 a.u.).

Solutions of hemin, MP-11 or cyt-*c* were prepared on the day of the experiment and protected from light prior to use. Hemin Cl (1.53 mM) was dissolved in methanol (except where noted), while MP-11 (0.8 mM) was dissolved in dimethyl sulfoxide (DMSO) containing 0.3 M 18-crown-6 ether and 0.7 M pyridine or NMI [32]. Cyt-*c* (0.4 mM) was dissolved in methanol containing 0.16 M 2-(hydroxymethyl)-18-crown-6 ether [33]. The MP-11 and cyt-*c* samples were agitated briefly in a Branson 2200 bath sonicator and then centrifuged (Fisher micro-centrifuge model 235B) for 1 min to remove undissolved material. The soluble fraction concentration was determined by dilution into aqueous buffer and measurement of the Soret band (404 nm for MP-11, including 125 mM NMI, and 408 nm for cyt-*c*) absorbance.

2.3. Spectra and ligand binding analysis

UV–VIS spectra were obtained using the spectrophotometer modifed as described previously. Quartz cuvettes (1 cm pathlength) were used, with cuvettes and cell holder maintained at 40 ± 1 °C.

Scatchard analysis [34] for determining the dissociation constants (K_d) of NMI and pyridine with hemin in [bmim][Tf_2N] were conducted at 40 °C by recording the change in absorbance at 634 nm. Small, incremental additions (10–60 μl) of ligand (NMI or pyridine) were made to 3.0 ml of a 15.3 μM hemin solution. The cuvette was stirred for 5 min at 40 °C, then equilibrated for an additional 5 min in the cell holder before reading the absorbance. Linear regression of the change in absorbance versus the change in absorbance divided by the ligand concentration yielded K_d (slope of the line). The free ligand concentration was assumed to be equal to the total ligand concentration. Reported data are the average of three separate determinations.

3. Results

3.1. Peroxidase steady-state kinetic model

Conventional treatment of the peroxidase reaction invokes a three-step mechanism involving the formation of two intermediates catalyst states, compound I and II, as follows:

$$\text{peroxidase} + \text{ROOH} \xrightarrow{k_1} \text{compound I} + \text{ROH} \quad (1)$$

$$\text{compound I} + \text{AH} \xrightarrow{k_2} \text{compound II} + \text{A}^{\bullet} \quad (2)$$

$$\text{compound II} + \text{AH} \xrightarrow{k_3} \text{peroxidase} + \text{A}^{\bullet} + \text{H}_2\text{O} \quad (3)$$

In the present case, the hydrogen acceptor (ROOH) is *tert*-butyl hydrogen peroxide (*t*-BuOOH) or hydrogen peroxide, and the hydrogen donor is guaiacol. Given the irreversibility of the reaction, the rate in terms of donor disappearance (v) can be formulated as [35]:

$$v = \frac{[\text{peroxidase}]}{1/(k_1[\text{ROOH}]) + 1/(k_3[\text{AH}])} \quad (4)$$

With high concentrations of donor, the assumption that k_3 [AH] ≫ k_1[ROOH] reduces the rate equation to a simple second-order expression (Eq. (5)):

$$v = k_1[\text{ROOH}][\text{peroxidase}] \quad (5)$$

3.2. Hemin kinetics and ligand binding

The peroxidase activity and ligand-binding behavior of hemin was examined in IL. Guaiacol oxidation rates were found to vary linearly with *t*-BuOOH (0.25–2.0 mM; $r^2 = 0.999$) and hemin (4–16 μM; $r^2 = 0.996$), confirming the validity of Eq. (5) for the estimation of k_1 (Fig. 1). No reaction was observed in the absence of hemin or *t*-BuOOH. The presence of a heterocyclic nucleophile, such as NMI or pyridine was required for the activity. Ligation of hemin by these nucleophiles was observed spectrophotometrically. Hemin in [bmim][Tf_2N] had a broad Soret band at 400 nm and a distinct charge transfer (CT) band at 634 nm (Fig. 2). Addition of NMI or pyridine shifted and sharpened the Soret and Q bands

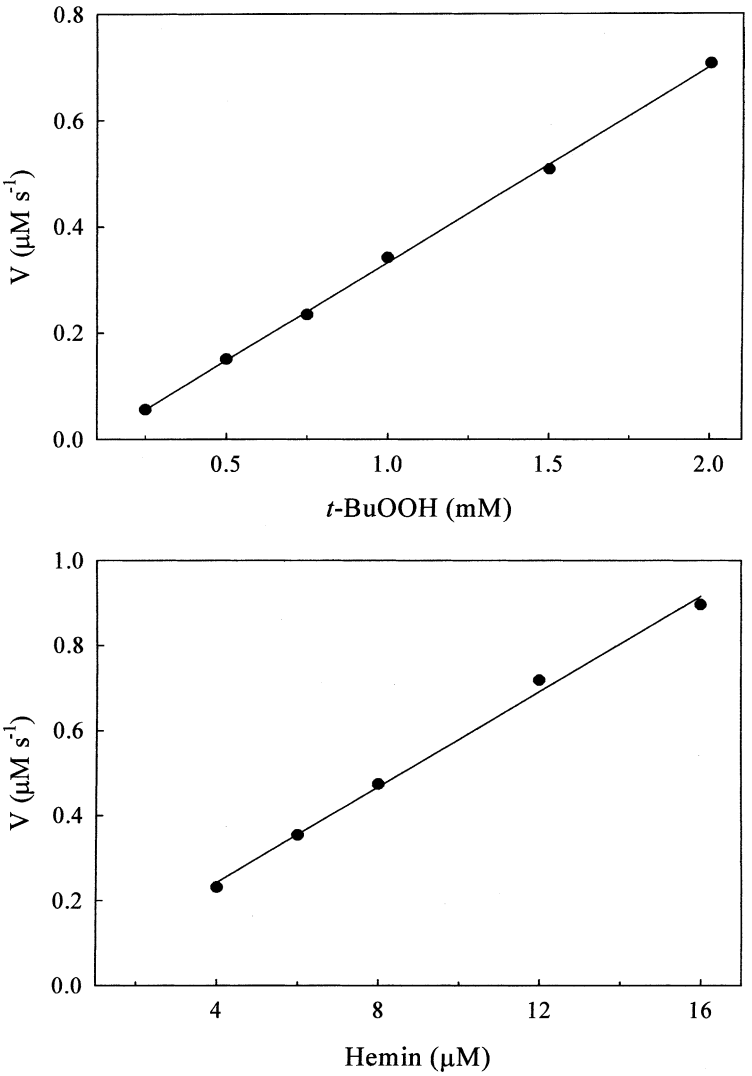

Fig. 1. Relationship between hemin guaiacol oxidation activity and peroxide concentration (upper panel) and hemin concentration (lower panel) in [bmim][Tf_2N]. For examining the influence of *t*-BuOOH, the hemin concentration was kept constant at 7.65 μM. The *t*-BuOOH concentration was 1.0 mM when examining the effect of hemin concentration on activity. In all the cases, 125 mM NMI was present and the temperature was maintained at 40 °C.

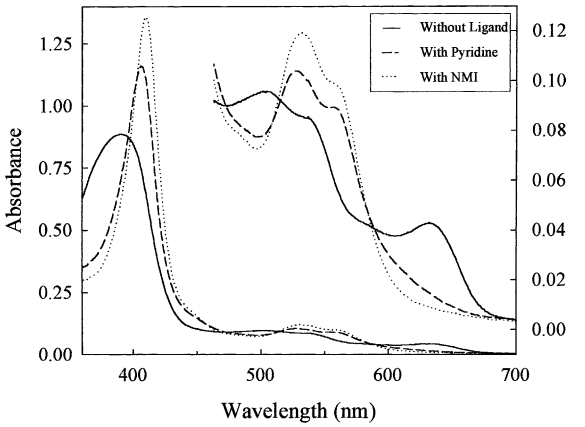

Fig. 2. Ligand effects on hemin spectra in [bmim][Tf_2N]. The hemin concentration was 15.3 μM (1% (v/v) methanol carried over with the dilution from the stock hemin solution). NMI and pyridine were 125 mM when present and the cell compartment was maintained at 40 °C.

(500–575 nm), while diminishing the CT band. By following the change in CT intensity with additions of NMI or pyridine, estimates were obtained of the dissociation constants (K_d) 0.37 and 47 mM, respectively, indicating that NMI has a ca. 100-fold greater affinity than pyridine for hemin in [bmim][Tf_2N] (Fig. 3).

The correlation between ligand association and the level of hemin peroxidase activity is demonstrated by comparing changes in activity with the extent of ligand binding (Fig. 4). The basic binding equation (Eq. (6)) expresses the extent of ligand association (θ) as a function of free ligand concentration, [L]:

$$\theta = \frac{[L]}{K_d + [L]} \quad (6)$$

At low concentrations of NMI (Fig. 4, upper panel), peroxidase activity increased proportionally with the increase in NMI binding. At very high concentrations of NMI, peroxidase acitivity began to decline (Fig. 4, inset), but there were no accompanying changes in the hemin spectrum. The decrease in activity at high NMI concentrations may be attributed to NMI binding to the sixth coordination site of the porphyrin iron. There was also a close correlation between pyridine binding and hemin peroxidase activity (Fig. 4, lower panel), albeit at much higher concentrations than that observed with NMI. It is worthy noting that the peroxidase activity of hemin was seven-fold greater with pyridine than with NMI when using the optimal concentrations of the two ligands. Thus, although ligand binding is a requirement for activity, there is no positive correlation between ligand binding strength and hemin peroxidase activity in [bmim][Tf_2N].

Hemin k_1-values depended to a small extent on the type of ionic liquid, with activity decreasing in the order [bmim][Tf_2N] > [bmim][PF_6] > [omim][PF_6] (Table 1). Hemin in [bmim][PF_6] had a spectrum similar to that found with [bmim][Tf_2N], including a CT band that disappeared with the addition of NMI (spectra not shown). This indicates that the PF_6^- anion does not appear to coordinate with hemin. In general, these findings suggest that the nature of the ionic liquid's cation or anion may have little impact on hemin peroxidase activity, at least for the limited range of IL examined in this work.

The activity of hemin in ionic liquids was ca. 10–40-fold higher than that observed with methanol (Table 1). Methanol required the inclusion of NMI or pyridine for peroxidase activity, as did the IL. NMI and pyridine were able to ligate hemin in methanol as effectively as in [bmim][Tf_2N], as judged by changes in CT band intensity (not shown). To test whether methanol may act as an inhibitor of the guaiacol reaction with hemin in IL, hemin was solubilized initially in DMSO (instead of methanol). The guaiacol oxidation activity of the DMSO-prepared hemin was then examined in [bmim][Tf_2N] with varying concentrations of methanol added. Methanol concentrations of 0.5–1.0% (v/v) diminished the activity slightly (10–15%), while higher concentrations of methanol progressively decreased activity further

Table 1
Hemin peroxidase activity in various solvents

Solvent	With NMI[a]	With pyridine[a]
[bmim][Tf_2N]	35 ± 5	150 ± 10
[bmim][PF_6]	27 ± 4	136 ± 10
[omim][PF_6]	18 ± 2	71 ± 2
Methanol	1.6 ± 0.2	3.4 ± 0.9
DMSO	~0.1	Trace

Reaction conditions: 0.25–2.0 mM *t*-BuOOH for the ionic liquids and 25 mM *t*-BuOOH for the molecular solvents, 7.65 μM hemin, 25 mM guaiacol, and 125 mM NMI or pyridine, at 40 °C ($n = 3$).
[a] $k_1 \times 10^{-3}$ ($M^{-1}\,s^{-1}$).

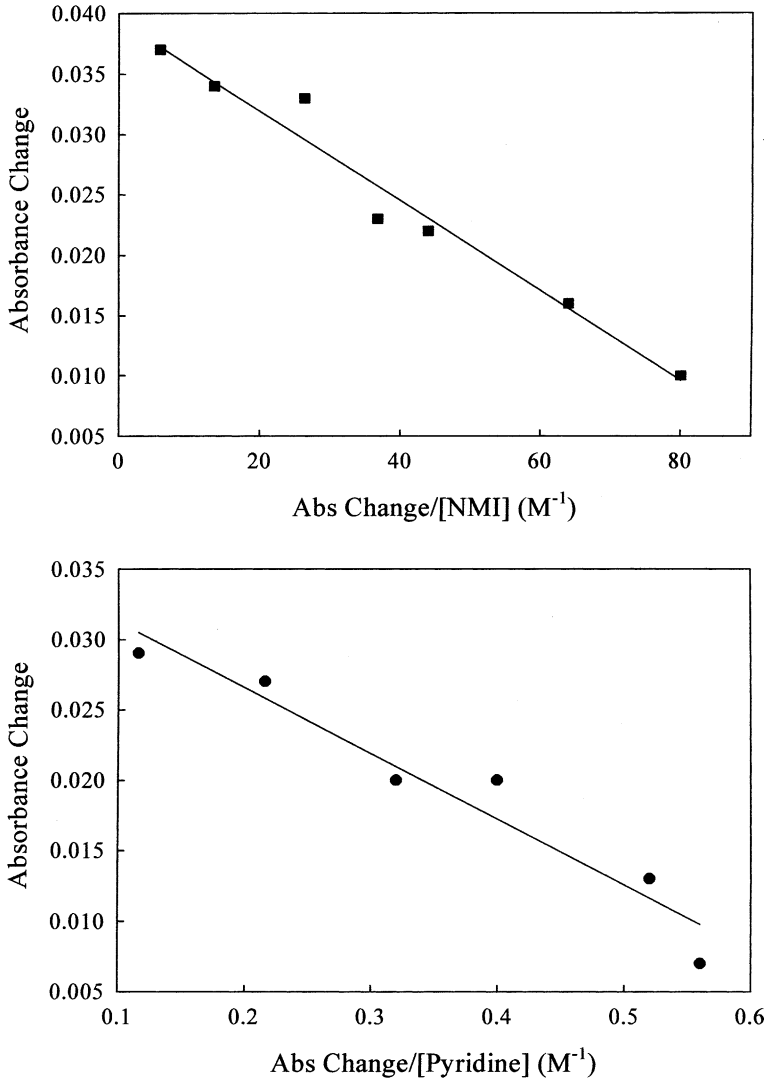

Fig. 3. Scatchard analysis of NMI (upper panel) and pyridine (lower panel) binding to hemin in [bmim][Tf_2N]. The data are derived from three separate determinations of changes in CT band intensity with varying ligand concentration. The hemin concentration was 15.3 μM and the cell compartment was maintained at 40 °C.

(data not shown), suggesting that methanol may be an inhibitor or reactant. DMSO proved to be far worse than methanol as a solvent for hemin peroxidase activity (Table 1), although there is no expectation that DMSO would serve as a substrate in the peroxidase reaction. IL are, thus, far better media than the molecular solvents methanol or DMSO for hemin peroxidase activity.

3.3. Cytochrome c spectra and peroxidase kinetics

The dissolution of cyt-*c* in non-aqueous media can be facilitated by complexation with crown ethers, which are believed to associate with the charged surface groups of the protein [36]. While cyt-*c* failed to be directly solubilized in IL containing 2-hydroxymethyl-18-crown-6 ether, dilution of cyt-*c*

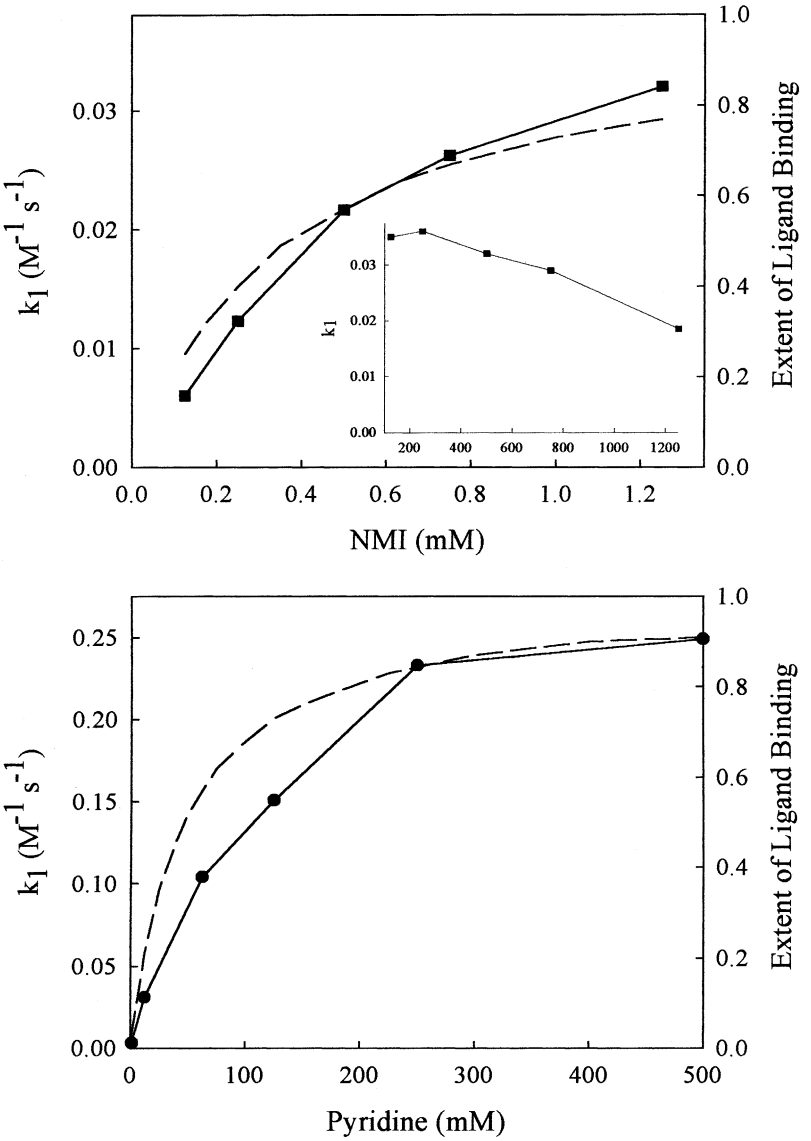

Fig. 4. Comparison of NMI (upper panel) and pyridine (lower panel) concentrations on k_1(solid lines), determined by guaiacol oxidation, and the extent of hemin ligation (broken lines), calculated using Eq. (6) and the K_d-values determined from Fig. 3; influence of high NMI concentrations on k_1 ($M^{-1}\,s^{-1}$; abscissa units are mM, upper panel insert).

prepared in methanol/crown ether into IL produced solutions of cyt-*c*. Cyt-*c* in IL were not indefinitely stable as aggregation was noted after several hours following dilution into the IL. It is worthy noting that while 2-hydroxymethyl-18-crown-6 ether was effective in solubilizing cyt-*c* in all the IL examined herein, IL designed specifically with alkyl ether groups for solubilizing proteins failed to do so with cyt-*c* [37].

The UV–VIS spectra of cyt-*c* solubilized in IL did not appreciably differ from those of cyt-*c* in methanol/crown ether, with sharp Soret peaks at 408 nm and lacking an apparent CT band at 695 nm (spectra not shown). The CT band present in native

cyt-*c* in aqueous media is very weak and temperature sensitive [38], which may account for why it was not observed. Addition of NMI or pyridine to cyt-*c* samples in IL did not alter the spectra. These observations indicate that the heme pocket of cyt-*c* remains largely intact with the protein dissolved in IL, but the sixth axial ligand of the heme may no longer be occupied by a methionyl group.

With *t*-BuOOH as the oxidant, only a trace amount of peroxidase activity could be observed with cyt-*c* in IL. However, H_2O_2 produced a pronounced guaiacol oxidation activity with cyt-*c*. The preference of peroxidases for H_2O_2 over that of alkyl hydroperoxides in aqueous media is well known [3,39]. When using H_2O_2 greater care must be taken to keep its concentration low, as high concentrations can lead to inhibition and destruction of the catalyst [40,41]. Thus, the steady-state oxidation of guaiacol with H_2O_2 usually was sustained for a shorter period in either IL or molecular solvents than observed with hemin and *t*-BuOOH. Because k_1-values fell with increasing H_2O_2 concentration, the initial peroxide concentration was 0.2 mM for these experiments (with ca. 16 μM cyt-*c*), except where noted.

Unlike hemin, cyt-*c* required no augmentation with ligands to display peroxidase activity in IL (Table 2). However, the addition of either NMI or pyridine to the IL substantially increased the activity of cyt-*c*, with pyridine producing the greater effect (as found with hemin). This observation suggests that NMI and pyridine are influencing the catalytic activity of cyt-*c* in IL, but it is unclear whether this is through interactions within the heme pocket or elsewhere on the protein. Addition of 2,4,6-collidine (2,4,6-trimethylpyridine), which is known not to coordinate with heme [42] and to cyt-*c* in IL did not alter cyt-*c* peroxidase activity (data not shown), indicating that the effect of NMI and pyridine is due to complexation with the heme rather than as general acid–base catalysts. The type of IL had a small impact on activity, with [bmim][Tf_2N] providing the highest activity, again as observed with hemin, indicating that there are no solvent specific cation or anion interactions with the protein. Cyt-*c* in methanol without added ligand had an activity level comparable to that observed in [bmim][Tf_2N] with ligand (Table 2). The peroxidase activity of cyt-*c*/crown ether complexes in methanol has been noted previously [33]. Unexpectedly, inclusion of pyridine in the methanol reaction medium did not stimulate activity. When NMI was added to methanol, the cyt-*c* was not soluble or active. Cyt-*c* displayed very low activity in DMSO, with or without pyridine present. Thus, the catalytic behavior of cyt-*c* is distinctly different from hemin in IL and molecular solvents.

Table 2
Cytochrome *c* peroxidase activity in various solvents

Solvent	Without ligand[a]	With NMI[a]	With pyridine[a]
[bmim][Tf_2N]	11 ± 1	28 ± 1	82 ± 8
[bmim][PF_6]	4.1 ± 0.1	36 ± 1	92 ± 4
[omim][PF_6]	1.7 ± 0.3[b]	28 ± 2	46 ± 5
Methanol	74 ± 7	NS[c]	63 ± 5
DMSO	1.2 ± 0.2[b]	NS	1.8 ± 0.2[b]

Reaction conditions: ~16 μM cyt-*c*, 25 mM guaiacol, 0.2 mM H_2O_2, and 125 mM NMI or pyridine, at 40 °C ($n = 3$).
[a] $k_1 \times 10^{-3}$ ($M^{-1}\,s^{-1}$).
[b] 2.0 mM H_2O_2.
[c] Cyt-*c* not soluble.

3.4. Microperoxidase spectra and peroxidase kinetics

The spectra of MP-11 solubilized in [bmim][Tf_2N] with either NMI or pyridine as ligands were indistinguishable from that of the MP-11 solublized in aqueous buffer, having sharp Soret peaks at 403–405 nm and lacking CT bands. The oligopeptide chain of MP-11 includes the histidinyl group that serves as the axial imidazole donor to the heme in cyt-*c*. It is unknown whether this imidazole ligand is displaced by NMI and pyridine in IL or molecular solvents. With the assistance of crown ether and ligand, MP-11 forms a solution in [bmim][Tf_2N] that is not visibly different from cyt-*c*.

The peroxidase activity of MP-11 resembled hemin in some respects and cyt-*c* in others. In either IL or molecular solvents, MP-11 required H_2O_2 as the oxidant rather than *t*-BuOOH to show any appreciable peroxidase activity. This behavior is similar to that of cyt-*c*. In methanol, MP-11 activity is substantially higher with pyridine as the ligand compared to NMI, behavior similar to that of hemin (cf. Tables 1 and 3). Recall that cyt-*c* activity in methanol was not stimulated by ligand (Table 2). MP-11 activity in IL also was higher with pyridine as the ligand, which is what was observed with both hemin and cyt-*c* in this solvent (note that the absolute values of k_1 in Table 1

Table 3
Microperoxidase-11 activity in various solvents

Solvent	With NMI[a]	With pyridine[a]
[bmim][Tf_2N]	37 ± 1	230 ± 20
[bmim][PF_6]	41 ± 2	330 ± 10
Methanol	6.8 ± 0.5	30 ± 3
DMSO	1.5 ± 0.2	Trace

Reaction conditions: 4–16 μM MP-11, 25 mM guaiacol, 0.2–2.0 mM H_2O_2, and 125 mM NMI or pyridine, at 40 °C ($n = 3$).
[a] $k_1 \times 10^{-3}$ ($M^{-1}\,s^{-1}$).

cannot be directly compared with the values given in Tables 2 and 3 as the former were determined using *t*-BuOOH and the latter with H_2O_2). Addition of methanol to [bmim][Tf_2N] at concentrations of 0.5 and 1.0% (v/v) decreased the activity by 15 and 20%, respectively, while addition of water (1.0% (v/v)) did not alter MP-11 activity. A comparison of MP-11 in various solvents indicates that, as with hemin, IL allowed significantly higher activities than molecular solvents (Table 3).

4. Discussion

In the present study, we have examined the peroxidase activity of cyt-*c* and its analogs, MP-11 and hemin, in IL. That all three catalysts do indeed display such activity indicates the need to insure that the peroxidase activity observed with cyt-*c* is not attributable to an exposed heme *c* prosthetic group of an unfolded protein. Several lines of evidence indicate that this is not the case. (i) The visible spectrum of cyt-*c* solublized in IL indicates that the heme *c* group retains its fifth and possibly sixth axial ligands, although whether the heme *c* group of native cyt-*c* retains its low spin state in IL has not been determined. (ii) Cyt-*c* displays peroxidase activity in IL without addition of heterocyclic bases while hemin does not. (iii) Cyt-*c* requires H_2O_2 as an oxidant, while hemin displays substantial activity with either H_2O_2 or *t*-BuOOH. Distinguishing between the activities of cyt-*c* and MP-11 in IL was less clear as both required H_2O_2 and preferred pyridine over NMI as an added coordinating group, although the second order rate constant (k_1) was higher for MP-11 than for cyt-*c* (cf. Tables 2 and 3). The catalytic behavior of cyt-*c* in methanol was readily distinguished from that of MP-11 in methanol as the former did not manifest an enhanced activity in the presence of pyridine. Native cyt-*c* in aqueous media accommodates the binding of ligands, such as imidazole and pyridine in its heme pocket [43], so the association of such ligands with the cyt-*c* heme group in IL is not indicative of a drastic conformational change. In contrast, solubilization of cyt-*c* in methanol (with crown ether) leads to an opening of the heme crevice and a shift of the sixth axial ligand from Met80 to a lysine side chain, producing a conformational change similar to that observed with cyt-*c* in alkaline aqueous media [33,44]. Similar effects have been observed with other water-miscible molecular solvents [7,45]. Further spectroscopic evidence is needed to determine whether the conformation of the heme cavity is different in IL from that of cyt-*c* in aqueous or polar medium, hydrogen-bonding molecular solvents. The findings, presented herein, suggest that hydrophobic, non-coordinating IL provide an environment less disruptive to the structure of native cyt-*c* than molecular solvents of similar polarity.

The non-coordinating nature of IL may further accelerate the peroxidase reaction by stabilizing the highly charged iron–peroxo or –oxo intermediates (compound I) generated in the rate-limiting step of the reaction. The high-valent species formed in peroxidases, catalases and cytochromes are also found in simpler model compounds. For instance, low-temperature spectroscopic studies established that iron–porphyrin complexes in toluene form PFe(III)OOC$(CH_3)_3$, where P is tetramesitylporphyrin or tetra-*p*-tolylporphyrin and, in the presence of NMI, PFe(IV)=O as intermediates in the decomposition of *t*-BuOOH [46]. Isotopic and spectroscopic studies indicated that microperoxidase-8, an eight-amino acid relative of the MP-11 used in the present work, forms a high valent iron–oxo species during its catalytic cycle [47–49]. A rate-enhancing effect on the formation of high valent iron–oxo intermediates would be expected for the weakly coordinating anions PF_6^- and $(CF_3SO_2)_2N^-$ of IL [50]. The positive effects of IL when using organometallic catalysts have been demonstrated for a broad range of reactions including epoxidations, hydrogenations, hydroformulations, polymerizations and Heck couplings [20,50–52]. The substantial rate enhancement by IL in comparison to conventional molecular solvents on the peroxidase

reaction conducted by hemin and MP-11 is in accord with these earlier findings.

The medium polarity or dielectric is expected to influence the redox behavior of cyt-*c* [1,11]. This has been explicitly demonstrated for cyt-*c*'s reversible Fe(III)/Fe(II) couple in aqueous mixtures with water-miscible organic (molecular) solvents [7]. The presumption that medium polarity will also impact high-valent iron–oxo heme complex redox properties stands largely untested as these species are more difficult to probe by conventional electrochemical techniques. Studies of model metaloporphyrins suggest that solvent polarity may influence the radical localization site within the porphyrin ring, potentially altering reactivity [53,54]. A number of recent studies have examined the polarity of ionic liquids, relying on measures of solvent strength (degree of solvent-probe interaction) to infer polarity and estimate relative dielectric constants ($\in$), although the relationship between these physical properties is complex [15–18]. A consensus estimate of polarity places [bmim][Tf_2N], [bmim][PF_6] and [omim][PF_6] in the range of the short-chain primary alcohols ($\in$ is 33 for methanol and 25 for ethanol), which coincides roughly with estimates of the effective dielectic of the cyt-*c* heme pocket ($\in$ is $\sim$20) [1]. Therefore, given equivalent heme ligation states, the peroxidase activity, presumably reflecting the stability of high-valent heme species (compounds I and II), in IL and methanol should be comparable, which is not the case for cyt-*c*, MP-11 or hemin. Solvent dielectric, thus, cannot be as important as solvent coordinating ability and nucleophility, as described previously, and specific ligand effects (mentioned later) in determining peroxidase activity.

The type of IL used has been found to impact the activity of enzymes. Lozano et al. [26] have attributed differences in *Candida antarctica* lipase B activity in various IL to the effect of solvent polarity (i.e. higher IL polarity led to greater transesterification rates). Laszlo and Compton [25] observed that α-chymotrypsin transesterification rates were substantially higher in [omim][PF_6] than in [bmim][PF_6]. Carmichael and Seddon [16] found the polarity of selected IL to increase as [bmim][PF_6] < [bmim][Tf_2N] < [omim][PF_6], while Muldoon et al. [18] portrayed the order of increasing polarity to be just the opposite, [omim][PF_6] < [bmim][Tf_2N] < [bmim][PF_6], but in either of the cases the differences are small. Thus, attributing correlations between enzyme activity and IL type to solvent polarity, therefore, may be inappropriate for lipases and proteases. The relationship between water activity and IL type may be more of an influencing factor for these enzymes [55,56]. However, peroxidase activity by cyt-*c*, MP-11, or hemin was found to be uninfluenced by water. Hemin activity increased in the order [omim][PF_6] < [bmim][PF_6] < [bmim][Tf_2N] (Table 1), which may be ascribed to differences in solvent viscosity (682, 450, and 69 cP, respectively [57]) rather than solvent polarity or water activity. This trend was not maintained with cyt-*c* (Table 2) or MP-11 (Table 3), suggesting that unrealized factors are involved.

Apart from solvent effects, ligand coordination to heme is expected to have a large impact on peroxidase activity. Tohjo et al. [58] observed that binding of methanol or pyridine to hemin in aqueous buffer brought about dramatic increases in peroxidase activity. Activation of hemin activity was associated with conversion of water-complexed μ-oxo-heme dimers to ligand complexed monomers. A similar correlation was found in this work between ligand binding (NMI or pyridine) and hemin peroxidase activity in IL (Fig. 4), although the activity of hemin in IL appears to be several orders of magnitude lower than in water [58]. Ligand binding to hemin–surfactant complexes were found to stimulate peroxidase activity in benzene [59]. NMI forms bis-imidazole adducts with model Fe(III)–porphyrin complexes in methylene dichloride, while bulkier ligands produced dimers and mono-imidazole adducts [60]. Trans-axial ligands (including the triflate anion and methanol) were found to modulate styrene epoxidation rates of Fe(III)–porphyrin complexes in methylene dichloride [61]. Traylor et al. [42] determined a K_d-value of 0.2 mM for NMI binding to the methyl ester of hemin in methanol, which is very similar to the affinity of NMI for hemin in [bmim][Tf_2N] (0.25 mM). With hemin modified to include a covalently bound imidazole group, producing a simple model of microperoxidase, externally added NMI was able to bind to the complex [42]. Micro-peroxidase is known to bind exogenous ligands in aqueous and non-aqueous media [32,43,62]. Thus, the findings that NMI and pyridine modify the activities of hemin, MP-11 and cyt-*c* in IL are consistent with

observations made with these catalysts in molecular solvents.

In summary, cyt-*c*, MP-11 and hemin demonstrate substantial peroxidase activity in IL. Exogenous ligands coordinate to the Fe(III) protoporphyrin moiety, modifying the catalyst's activity. The activities of hemin and MP-11 were significantly higher in IL than in methanol or DMSO, reflecting the non-coordinating, weak nucleophilicities of IL compared to similarly polar molecular solvents. These findings are consistent with earlier observations that IL may be suitable media for biocatalysis.

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