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An Ionically Tagged Water-Soluble Artificial Enzyme Promotes the Dephosphorylation Reaction with Nitroimidazole: Enhanced Ionic Liquid Effect and Mechanism

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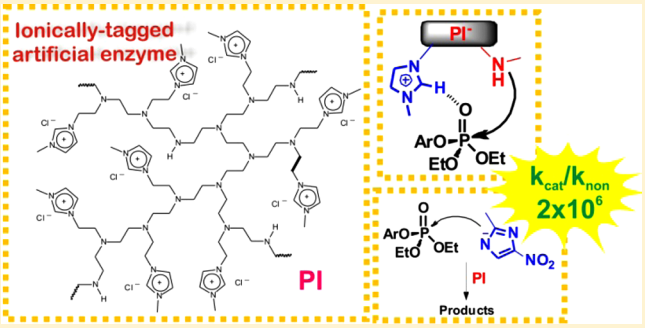
Supporting Information

ABSTRACT: In this paper, we describe a novel synthesized ionically tagged water-soluble artificial enzyme (PI) that can efficiently cleave phosphate esters, with enhanced an ionic liquid effect through cooperative effects for the substrate activation and further nucleophilic reaction. The dephosphorylation reaction with PI was evaluated in the presence and absence of 2-methyl-4(5)-nitroimidazole, showing impressive rate enhancements of up to 2 × 10^6-fold, ascribed to the imidazolide species known as excellent nucleophiles, and formed favorably at lower pH values in the presence of PI.



Dephosphorylation processes make up a particular class of vital biological reactions, intrinsically related to phosphorylated structures such as DNA and RNA. These processes are indeed extremely unfavorable, therefore requiring enzymes.[1] There is also a great interest in developing novel bioinspired catalytic systems that may help with both the understanding of these enzymatic processes and the development of artificial enzymes.[2] Macromolecules are in this sense ideal backbones for this purpose because they encompass active sites, as well as neighboring domains that can synergistically assist the reaction. They can also assemble, leading to nanoreactors that can concentrate reactants and thus accelerate the reactions. When new catalytic systems are being designed, optimal reactive groups are required. In this context, much emphasis is given to imidazole/imidazolium moieties that are present in many enzymatic active sites because of their versatility. They may efficiently act as general acid−base pairs and nucleophilic catalysts.[3,4] The presence of imidazolium moieties also favors the so-called ionic liquid effect with a typical enhancement of both yields and selectivities as a consequence of ion pairing and the formation of supramolecular aggregates.[5] Although the origin of the ionic liquid effect is hotly debated in the scientific literature, some compelling evidence points to cooperative cation−anion stabilizing effects.[6] In principle, ion pairs of imidazolium-based cations as neighboring polar domains could assist the reaction more efficiently as a consequence of solubility effects and the ionic liquid effect. Imidazolium-based derivatives are in this sense thought to be ideal because of their known high thermal and chemical stabilities (plus their nearly universal solubility), which may certainly help to bring all reactants to the same phase, or to the artificial synthetic enzyme domains, therefore facilitating the reaction.

Herein, we studied the dephosphorylation reaction promoted by a novel synthesized ionically tagged (imidazolium-based) water-soluble artificial enzyme [PI (Scheme 1)], expected to act as a nanoreactor with an enhanced ionic liquid effect. The model substrate evaluated was the triester 2,4-dinitrophenyl phosphate (DEDNPP). The incorporation of imidazolium cations should potentiate the ionic liquid effect in the artificial enzyme toward dephosphorylation reactions, and to the best of our knowledge, no ionically tagged synthetic enzyme has been described.

We also studied the reaction of 2-methyl-4(5)-nitroimidazole (MNI) with DEDNPP in the presence of PI in an aqueous medium, which cannot be studied without PI because MNI is a water-insoluble nucleophile. These nitrated imidazole derivatives are of great interest because they constitute many pharmaceuticals[7,8] and little is known about their action, especially against phosphate esters, that could point to possible carcinogenic activities. In fact, dephosphorylation processes are known to be related to many dangerous diseases.

The PI structure was first characterized by $^1H-^{15}N$ HMBC experiments (see the Supporting Information), typically used for the characterization of the artificial enzyme synthesized from commercially available polyethylene imines,[9−11] and spectra show typical hydrogens from the imidazolium-based ionic liquid structures (9.30 and 9.11 ppm for C2-H, 8−7 ppm

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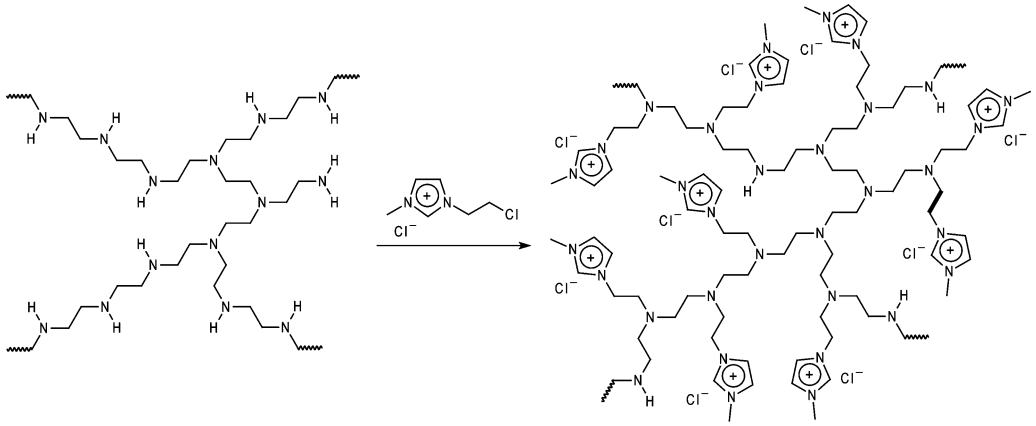
Scheme 1. Synthesis of the Water-Soluble Ionically Tagged Artificial Enzyme (PI)



for C4-H and C5-H, 5.01 ppm for C7-H, and 4.05 and 4.08 ppm for methyl C6-H) correlated with different types of nitrogen atoms at 167.0 and 174.1 ppm (^{15}N) from the imidazolium ring. As expected, the presence of varied signals for similar ^{1}H and ^{15}N atoms is the net result of the differences in the chemical environments of the polymeric structure of PI. In addition, the signal at 25 ppm observed in the ^{15}N analysis, which is correlated with ^{1}H at 2.80 ppm, reveals that tertiary amines are predominant in the PI structure.

Potentiometric titration was conducted to characterize PI as well as the PI/MNI system, regarding the existing pK_a values. These provide important information for inferring the catalytic activity of specific species, because the pH effect in the reactions studied (*vide infra*) will be evaluated. Fitting the titration profiles (see Figure S2 of the Supporting Information) with the program BEST7, for PI, yielded four pK_a values: pK_{a1} = 5.41, pK_{a2} = 7.50, pK_{a3} = 8.99, and pK_{a4} = 9.69 (errors for all pK_a values of ±0.03) [ascribed to the amine sites (NH_2^+)]. Previous reports[12] agree with the assumption of multiple pK_a values for a macromolecule with several units of similar acid–base moieties, attributed to the neighboring groups that affect the equilibrium, e.g., stabilizing the deprotonated species. The pK_a of an amine group can therefore be shifted depending on whether it bears a neutral or protonated amine group, just as observed in natural enzymes. The values are additionally consistent with other complex amine-based macromolecules[13] such as lysozyme.[14] In the case of the MNI/PI system, the same four pK_a values for PI were determined with an additional pK_a of 10.48 ± 0.01, attributed to MNI (formation of imidazolide species), which agrees with those of other nitroimidazole derivatives.[15]

The reaction of DEDNPP with PI in the presence and absence of MNI was followed at different pH values, and the pH–rate profiles obtained are shown in Figure 1 along with data for the spontaneous reaction of DEDNPP in water.[3] Results clearly show that the dephosphorylation of DEDNPP is accelerated with PI and the PI/MNI system upon comparison to the spontaneous reactions. It is also noted that k_{obs} increases with pH for the reactions studied, suggesting that reactive species are formed at higher pH values. In the case of solely PI, this can be attributed to the amine groups, potential nucleophilic sites, which are knowingly reactive at higher pH, i.e., neutral. For the PI/MNI system, the imidazole group from MNI can additionally act as nucleophilic sites, with a reactivity that depends on the pH, evidencing the importance of the

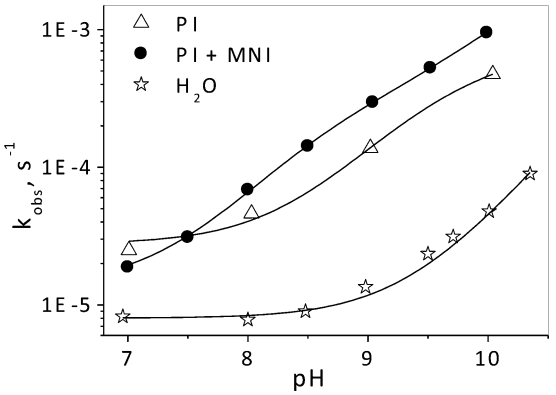


Figure 1. pH–rate profile for the reaction of DEDNPP with PI (3.14 mg/mL) in the absence (△) and presence (●) of 0.02 M MNI. Spontaneous hydrolysis of DEDNPP is shown for comparison (☆) at 25 °C.

previous titration study. The data in Figure 1 were fitted with eq 1

$$k_{obs} = k_0 + k_{OH}[OH] + k_{PI^+}\chi_{PI^+} + k_{PI^-}\chi_{PI^-} + k_{MNI}\chi_{MNI} \quad (1)$$

which in all cases considers the reaction of DEDNPP with water (k_0) and hydroxide (k_{OH}). With only PI, the last terms of the equation were not considered, related to the MNI species. Although PI has many possible pK_a values for the amine moieties (*vide supra*), only one pK_a was required to fit the data, and two species were relevant in this reaction: a partially protonated species (e.g., bipolar) with some neutral amine groups with neighboring protonated groups (molar fraction χ_{PI^+}, k_{PI^+}) and a totally deprotonated species with neutral amine groups (χ_{PI^-}, k_{PI^-}). In the presence of MNI, an additional species was considered, regarding the deprotonated imidazolide group (χ_{MNI^-}, k_{MNI^-}), known to form at pH ∼12 for nitroimidazoles.[15] All these possible pathways are illustrated in Scheme 2.

Table 1 presents the kinetic parameters obtained from the fitting data in Figure 1, where values of k_0 and k_{OH} are from previous studies.[3] For the reaction of DEDNPP with only PI, results show that the neutral species (PI^-) is the most reactive, as expected, leading to ∼80-fold rate enhancements compared to that of the spontaneous reaction. Considering that PI has many of its nucleophilic sites hindered, acting more as an aggregated ionic liquid, this reactivity is impressive. The kinetic

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Scheme 2. Pathways for the Possible Reactions of DEDNPP with PI and PI/MNI

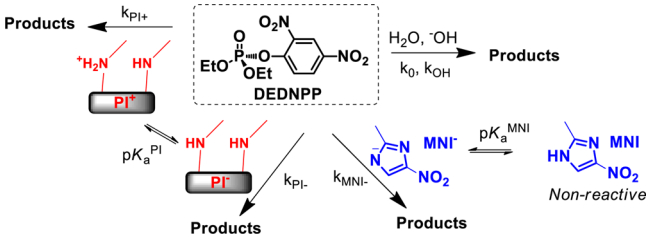


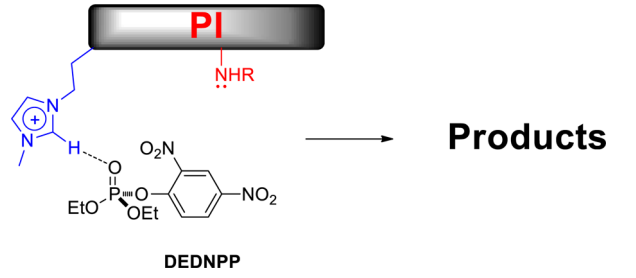
Table 1. Kinetic Parameters Obtained for the Reactions of DEDNPP

H_2O, $-OH$: $k_0 = 8.0 \times 10^{-6}$ s^{-1}; $k_{OH} = 0.42$ M^{-1} s^{-1}		
constant	PI	PI/MNI
k_{PI^+} (s^{-1})	1.88×10^{-5}	3.14×10^{-5}
k_{PI^-} (s^{-1})	5.97×10^{-4}	5.65×10^{-4}
k_{MNI^-} (s^{-1})	–	5.4×10^{-3}
pK_a^{PI}	9.69	
pK_a^{MNI}	–	10.56

pK_a determined for PI is consistent with the titration data, showing that above this critical value, the amine moieties of PI are neutral, crucial for inferring nucleophilic reactivity. As mentioned, macromolecules with multiple protonation sites with the same functionality (e.g., amine) are known to have several pK_a, values because neighboring groups affect deprotonation of subsequent sites.[12] Multiple equilibria are, however, not crucial in the kinetics evaluated, and an overall equilibrium can be reasonably considered (PI^+ to PI^-). The PI^+ species has indeed little contribution but is necessary for fitting the data, evidencing that at lower pH values (<9.5) an overall partially neutral species of PI is reactive in the reaction evaluated. The concentration of PI was varied, showing a linear profile with k_{obs} (see Figure S3 of the Supporting Information), characteristic of a nucleophilic attack. The obtained second-order constant for PI was 0.11 g^{-1} mL^{-1} s^{-1}, extremely high for macromolecule-mediated dephosphorylation reactions. For example, the powerful α-nucleophilic polyhydroxamate is effective in cleaving DEDNPP[12] with a k_N of 0.017 g^{-1} mL^{-1} s^{-1}, evidencing the high reactivity of PI. For the reaction with the PI/MNI system, similar constants for PI were found, and for MNI, rate enhancements were even more impressive [~700-fold, ascribed to the anionic imidazolide species (MNI^-)]. It should be noticed that the reaction with solely MNI cannot be evaluated because the substrate is water-insoluble in the absence of PI (even at low concentrations and varying pH values, with different surfactants, etc.). Likewise, MNI is highly reactive at a considerably low concentration (0.02 M), and the most commonly compared parameter in nucleophilic reactions is the second-order rate constant, which is 0.27 M^{-1} s^{-1} (among the highest reported with DEDNPP). Even imidazole, which lacks the nitro group that readily decreases reactivity, presents a k_N of 0.177 M^{-1} s^{-1}.[3] The kinetic pK_a determined for MNI is also consistent with the titration study. Finally, the neutral species of MNI did not show significant reactivity.

With regard to the mechanism, the presence of imidazolium rings in the PI structure is possibly helping in activating the substrate toward the dephosphorylation reaction. A remarkable acceleration of the transesterification reaction of some phosphate ester derivatives in the presence of imidazolium-based ionic liquids has been demonstrated.[16] Dupont[17] and others[18] have also proposed C═O activation in the presence of imidazolium cations through the interaction of C═O and H-C2 between the acidic hydrogen at position C2 of the imidazolium ring and the basic oxygen of the C═O group. On the basis of the data obtained herein and the literature evidence, an activation mode could be proposed for DEDNPP (Scheme 3). In this sense, both parts of the synthetic enzyme play a role

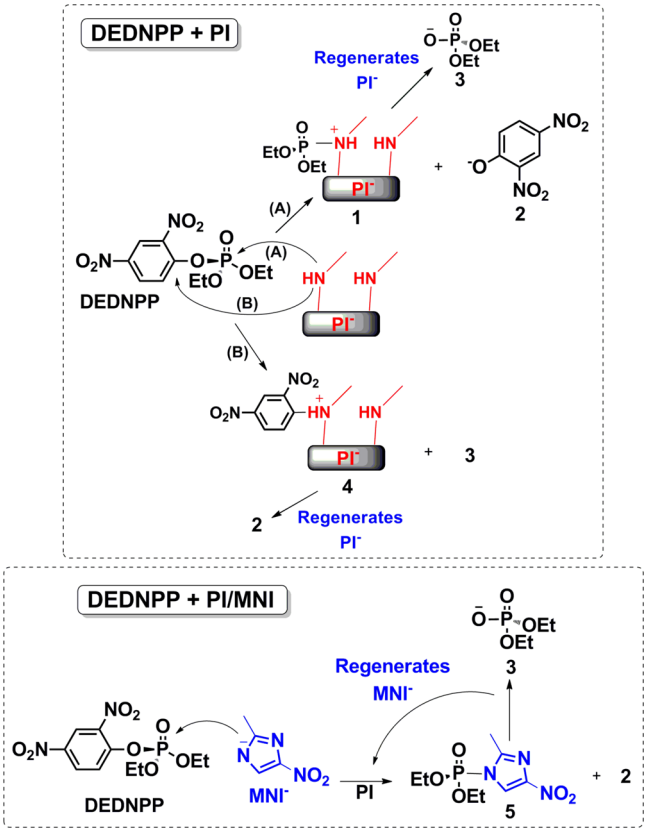
Scheme 3. Substrate Activation by the Imidazolium Cation of PI



in the reaction acceleration. That is, the amine groups (basic sites) and the imidazolium rings (acidic sites) are displaying a cooperative effect for the dephosphorylation reaction.

Scheme 4 presents an overall mechanism proposed for the reactions studied here. Thus, DEDNPP can be attracted to the PI neighboring domains by the polar regions of the

Scheme 4. Proposed Mechanism for the Studied Reactions[a]



[a]Note the substrate activation by the imidazolium cation has been omitted for the sake of clarity.

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imidazolium cation, therefore being activated to react with the amine sites. Nucleophilic attack by the amine sites occurs via the following two paths: (A) on the phosphoryl group leading to a phosphorylated intermediate (**1**) and (B) on the aromatic carbon affording intermediate **4**. These intermediates are formed by a concerted step[3,19] and can readily decompose, regenerating PI and thus comprising a catalytic nanoreactor with cooperative effects through the ionic liquid effect.

Analogous mechanisms of DEDNPP with amines have been reported[3,19−23] but without any ionic liquid effect exploited so far. In the study presented here, we propose the attack on the phosphorus atom is predominant because the initial formation of phenolic product **2** should mostly come from path A, in accordance with the literature.[19] Thermodynamic parameters were also obtained (Eyring plot given in Figure S4 of the Supporting Information) for the reaction of DEDNPP with PI, giving a $\Delta S^{\ddagger}$ of −24.87 cal K^{-1} mol^{-1}, a $\Delta H^{\ddagger}$ of 15.5 kcal mol^{-1}, and a $\Delta G^{\ddagger}$ of 22.91 kcal mol^{-1} (25 °C), which agree with those of the proposed nucleophilic pathway.[3]

In the case of the reaction of DEDNPP with PI/MNI, which should also be attracted to the PI domains, an additional nucleophilic reaction should occur between the anionic nitrogen of imidazolide (MNI) and the phosphoryl group, leading to intermediate **5**, which is known to be very unstable.[3] Imidazolide is not usually studied because its formation is associated with a very high pK_a (>14) for solely imidazole.[15] Herein, the withdrawing nitro group lowers this pK_a (10.5, *vide supra*), allowing the formation of the highly reactive species MNI^-. This is also favored by the solubility and stabilizing effects caused by the presence of imidazolium-based neighboring domains in the synthetic enzyme structure. The neutral species of MNI is not reactive, because the available nitrogen is known to be a weak nucleophile (with a pK_a of <3).[15] Finally, an attack on the aromatic carbon may be discarded, because previous studies showed that this path is highly unfavorable with imidazole derivatives.[3]

Overall, results showed that PI may efficiently cleave phosphate esters, such as DEDNPP, behaving as a catalytic nanoreactor with an enhanced ionic liquid effect through cooperative effects for the substrate activation and further nucleophilic reaction. PI proved to be capable of dissolving MNI in an aqueous medium, and this PI/MNI system catalyzed the cleavage of DEDNPP with rate enhancements of up to 2×10^6-fold, comparing second-order constant k_N with that of the spontaneous reaction. The high reactivity of MNI is mostly ascribed to the imidazolide species, known to act as excellent nucleophiles, and formed favorably at lower pH values in the presence of PI (i.e., milder conditions), in contrast to other imidazole-based systems (pH >13). As previously mentioned, nitroimidazoles comprise many pharmaceuticals, and the results presented here evidence that precautions are necessary, because these compounds are potential nucleophiles that can attack our biological phosphate esters (e.g., DNA and RNA), causing defects, for example, and leading to tumoral processes. Lastly, these results have great potential for the design of water-soluble artificial enzymes with an enhanced ionic liquid effect and also detoxifying agents, because organophosphorus compounds constitute many chemical weapons and pesticides that need to be monitored and eliminated (i.e., detoxification).[24]

EXPERIMENTAL SECTION

Materials. MNI was obtained commercially, and DEDNPP was prepared as described previously.[3] For PI synthesis, commercially available polyethylene imine (branched, $M_w \sim 800$ by GPC, $M_n \sim 600$ by GPC) was dried by azeotropic water removal. The anhydrous PEI (5.00 g) was dissolved in anhydrous CH_2Cl_2 (10 mL) in a sealed Schlenk tube; 1.40 g of 1-(2-chloroethyl)-3-methylimidazolium chloride was added followed by 2,6-lutidine (10 g). The mixture was heated at 100 °C for 2 h. After this time period, the Schlenk tube was unsealed and the mixture washed with CH_2Cl_2 followed by AcOEt, affording PI in nearly quantitative yield.

NMR. All NMR measurements were taken on a spectrometer (11.75 T) operating at 500.13 MHz for ^1H and at 50.68 MHz for ^{15}N. Typically, a 2D $^1H-^{15}N$ HMBC pulse sequence from the Bruker User Library was used. All experiments were performed using 40 mg of PI in 600 μL of CD_3OD in a NMR tube containing a sealed capillary tube charged with nitromethane used as the external reference to set the scale (4.80 ppm for ^1H and 381.7 ppm for ^{15}N).[25]

Potentiometric Titration. Potentiometric titration was conducted in a 100.0 mL thermostated cell at 25 °C. The solutions were acidified with HCl (0.11 M) and titrated with small increments of KOH (9.95×10^{-2} M, CO_2 free). The pH was monitored by a pHmeter. BEST7 was used to determine the equilibrium constants.[26]

Kinetics. Reactions that were followed spectrophotometrically were started by adding 10.0 μL from a stock solution of the substrate DEDNPP (7.5×10^{-3} M in acetonitrile) in 3.0 mL of the reaction solution, under pseudo-first-order kinetics. Solutions were buffered with 0.01 M $KHCO_3$ (pH 7.0) and K_2HPO_4 (7.5−10.0). Reactions were followed by the appearance of 2,4-dinitrophenol (**2**) at 400 nm with a thermostated cell holder maintained at 25 °C. Observed first-order rate constants (k_{obs}) were calculated from nonlinear plots versus time by using the Levenberg algorithm using Origin8.

ASSOCIATED CONTENT

Supporting Information

NMR spectra, potentiometric titration curves, and kinetic data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b00750.

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Notes

The authors declare no competing financial interest.

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