Tetrahedron Letters, Vol. 33, No. 29, pp. 4191-4194, 1992
Printed in Great Britain

0040-4039/92 $5.00 + .00
Pergamon Press Ltd

Ester Substrate PNIQ as a Novel Fluorescent Probe for the Study of Acylation and Deacylation Steps in Ester Hydrolysis Catalyzed by Lipophilic Imidazole•Zn^{2+} Complexes in Micelles

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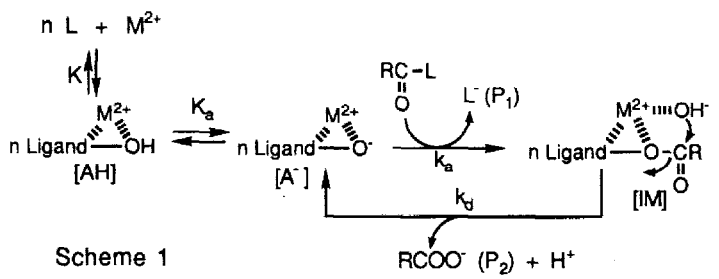
Key Words: Ester hydrolysis, Metalloenzyme model, Fluorescent probe

Absrtact: *p-Nitrophenyl isoquinoline-3-carboxylate (**PNIQ**) has been found to be a versatile ester substrate for the study of catalytic activities of lipophilic imidazole•Zn^{2+} complexes in cationic micelles, i.e. **PNIQ** allows to monitor the rates of both acylation of a catalyst-complex by a uv method and of deacylation of the acylated intermediate to regenerate the catalyst-complex by a fluorescent method.*

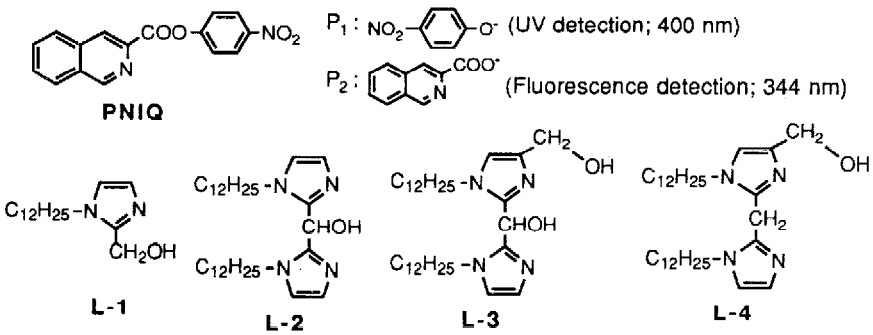
Fluorescent probes have been extensively used for the study of microenvironments of macromolecules or self-organized molecular assemblies such as proteins, membranes, micelles, and/or the related systems,[1] by virtue of their sensitivity to a change of microenvironment. Such a probe can in principle be used to monitor a reaction process by fluorescent spectroscopy.

We have been continuing our studies on the micellar models of hydrolytic metalloenzymes.[2-4] A typical model is a lipophilic imidazole-ligand•metal ion complex as the catalyst mixed with surfactant micelles, and such an imidazole-ligand is active when it has a metal-ion chelating hydroxyl group. The mechanism of catalysis of such a model was reported previously as in Scheme 1,[2,4] in which a metal-ion chelated hydroxylate anion [A^-] attacks the ester carbonyl group and the acylated intermediate [IM] is subsequently deacylated by a metal ion-chelated hydroxide anion.



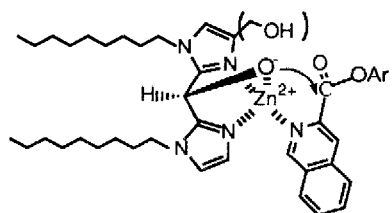
Scheme 1

In such a model study, the rate of acylation (k_a) is commonly determined by monitoring the release of leaving group (L^- or LH) spectrophotometrically based on uv-visible absorption under a condition of an excess ligand over the substrate, but the determination of the rate of deacylation (k_d) is not easy because the leaving carboxylate group is usually colorless in uv-visible region. Although it is known that the rate of deacylation can be determined indirectly by a burst-kinetics[2,5] by using an excess substrate over the ligand so as to make the k_d step being the rate-determining, it is obvious that the determination of it by a direct method is desirable. We have now found that *p*-nitrophenyl isoquinoline-3-carboxylate (**PNIQ**) is a versatile ester substrate which enables to determine the rates of both acylation and deacylation steps.



The ligands tested were lipophilic mono-imidazole **L-1** and bis-imidazoles **L-2**~ **L-4** having one or two hydroxyl group(s). The rate of acylation (k_a) of a ligand was determined by monitoring the release of *p*-nitrophenol (P_1) by uv detection (400 nm), and the rate of deacylation (k_d) was determined by monitoring the increase of fluorescent intensity (325 nm excitation and 344 nm emission) due to the product complex of isoquinoline-3-carboxylate (P_2) with Zn^{2+}, respectively. Here it should be mentioned that the fluorescence intensity of **PNIQ** or a mixture of **PNIQ** and Zn^{2+} is negligibly small.

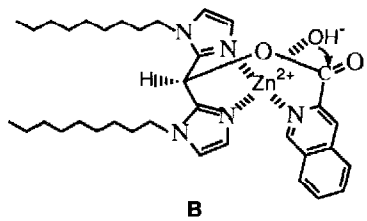
The kinetics were conducted under the conditions of an excess ligand (1×10^{-4} M) and Zn^{2+} (1×10^{-3} M) over **PNIQ** (1×10^{-5} M) in buffered hexadecyltrimethylammonium bromide (HTAB, 1×10^{-2} M) micelles at pH 7.5. The pseudo-first-order rate constants ($k_{obsd} = k_a$) for the release of *p*-nitrophenol were $k_{obsd} \times 10^2 / s^{-1}$ = 2.5 (**L-1**), 40 (**L-2**), 19 (**L-3**), 5.8 (**L-4**), and 0.02 (in the absence of a ligand, Zn^{2+} only), respectively. A higher reactivity of **L-2** or **L-3** may be explained as previously proposed for the hydrolysis of *p*-nitrophenyl picolinate[4b] as due to the formation of a ternary complex **A** in which the secondary hydroxyl group is selectively activated by a coordination of tetrahedral geometry with Zn^{2+}.



The pseudo-first-order rate constants ($k_{obsd} = k_d$) for the release of isoquinoline-3-carboxylate•Zn^{2+} complex were $k_{obsd} \times 10^4 / s^{-1}$ = 1.5 (**L-1**), 210 (**L-2**), 28 (**L-3**), 26 (**L-4**), and 0.4 (in the absence of ligand, Zn^{2+} only), respectively. The fact that the rate constants for the release of *p*-nitrophenol are much larger (more than 100 fold for **L-1** and **L-3**) than those of isoquinoline 3-carboxylate•Zn^{2+} clearly indicates that the hydrolysis

proceeds at least in two steps, i.e. an acylation and a deacylation as in Scheme 1. It is also noticed that those ligands of **L-2**, **L-3**, and **L-4** are good catalysts showing a reasonably high turnover number as compared to the related models.[2]

It is interesting to note that the deacylation rate of **L-2**•Zn^{2+} complex is particularly large, much larger than that of a mono-imidazole **L-1** ligand. It is conceivable that the deacylation occurs through an acylated intermediate **B** in which Zn^{2+} is tightly bound with three nitrogens and a hydroxide anion bound on the fourth coordination site of tetrahedral Zn^{2+} would favorably attack the carbonyl group of the acylated intermediate in a pseudo-intramolecular manner.



B

The acylation site is ambiguous in the cases of **L-3** and **L-4** whether it occurs on the primary or the secondary hydroxyl group. Whatever happens, the deacylation step may be somehow inhibited by a competition between such a hydroxyl group and the free hydroxide anion.

The pH dependency of the deacylation rates for the ligand **L-2** is shown in Fig. 1. As indicated, the $\log k_{obsd}$ values increased linearly with increasing pH with a unit slope up to a saturation level.

The pH of the breaking point is 7.9 which must be the pKa of an acid. The candidate of such an acid is likely to be a Zn^{2+} bound hydrated water as reported in the literature.[6,7] The pKa of 7.9 is surprisingly almost the same as that of the secondary hydroxyl group (pKa 8.1)[2] determined previously for the acylation step, i.e. that in **A**.

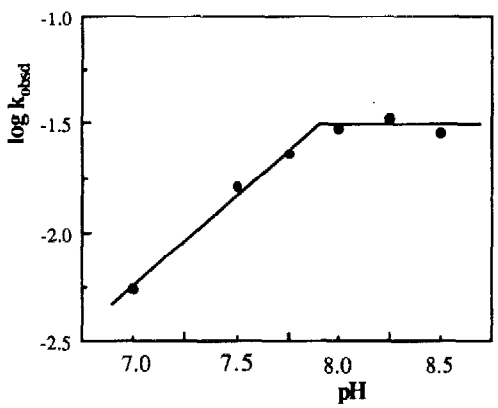


Fig. 1 pH-Rate profile for the deacylation step: [HTAB]=10^{-2} M, [**L-2**]=10^{-4} M, [Zn^{2+}]= 0.5×10^{-3} M, and [**PNIQ**]=10^{-5} M at 25 °C.

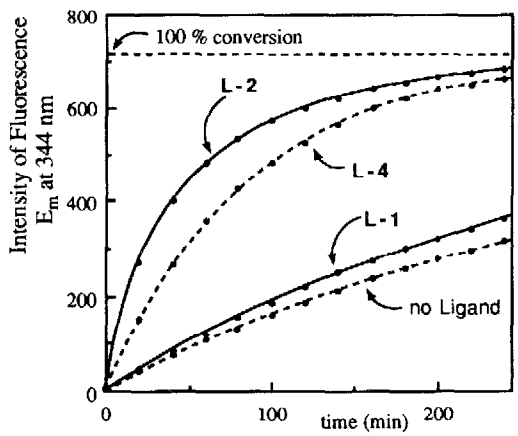


Fig. 2 Time course of the change of intensity of the fluorescence of IQ-Zn^{2+} complex in the reaction of **PNIQ** with **L-1**, **L-2** or **L-4** and Zn^{2+}; [HTAB]= 10^{-2} M, [Ligand]=10^{-6} M, and [Zn^{2+}]=10^{-3} M, [**PNIQ**]=10^{-5} M at pH 7.5 and 25 °C.

As expected from the above k_d values, a smooth turnover of the catalyst up to the 100 % conversion of the reaction was confirmed for **L-2** and **L-4** ligands under a condition of an excess substrate over the catalyst complex as shown in Fig. 2. Here again is shown that the activity of mono-imidazole ligand **L-1** in the deacylation is much smaller than those of bis-imidazole ligands.

In summary, the use of **PNIQ** as a substrate has disclosed the hitherto uncertain features of catalytic activities of several lipophilic imidazole•Zn^{2+} complexes in ester hydrolysis in surfactant micelles, in particular as for the deacylation step, and thus **PNIQ** must be a great help for the design of more active ligands.

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(Received in Japan 7 April 1992)