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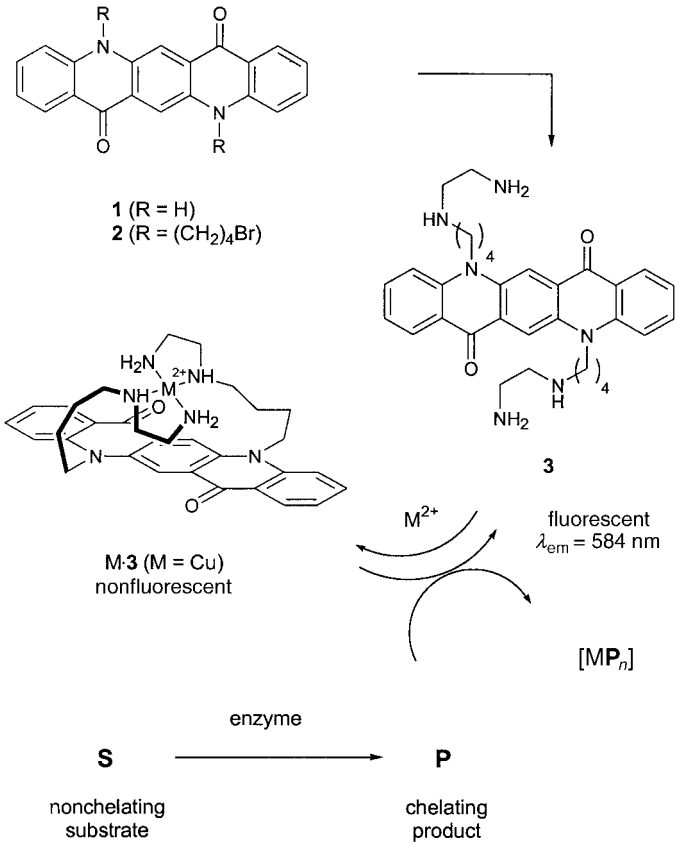
An Enzyme Assay Using pM**

Gérard Klein and Jean-Louis Reymond*

The development of new catalytic methods, in particular enzymatic processes, is increasingly being followed with combinatorial and evolutionary methods, whereby sensitive assays for catalysis play an essential role.[1–3] Most enzyme assays are based on chromogenic or fluorogenic substrates.[1, 4, 5] In many cases, however, it is desirable to measure the reaction of a well-defined substrate of interest, and not that of a different fluorogenic or chromogenic derivative. Such direct detection can be achieved with instrumental methods, such as HPLC and mass spectrometry,[6] by using product-selective fluorescent sensors,[7] or with indirect colorimetric assays based on susbstrate- or product-binding proteins, such as cat-ELISAs (catalytic enzyme-linked immunosorbent assays)[8] or the QUEST method ("querying for enzymes using the three-hybrid system").[9] More simply, one can also measure spectrophotometrically a physico-chemical parameter that responds to the progress of the reaction, such as measuring the temperature by IR thermography,[10] or, for reactions releasing acids or bases, the pH value of the reaction medium with pH indicators.[11] Herein we report a new enzyme assay based on following the evolution of pM ($-\lg[M]$, where M is free metal ions) as a function of reaction time. The change in pM is recorded using an orange fluorescent metal-sensor derived from quinacridone.

The orange fluorescent sensor **3**, which is obtained from quinacridone (**1**) via dibromide **2**, responds to the presence of small concentrations of Cu^{2+} by an almost quantitative quenching of its fluorescence ($\lambda_{em(max)} = 558$ nm). The effect can be explained by formation of a macrocyclic chelate $M \cdot \mathbf{3}$, in which energy transfer quenching occurs due to the proximity of the quenching metal ion to the fluorophore (Scheme 1).[12] The complex $M \cdot \mathbf{3}$ contains a 17-membered ring. The chelate effect induced by such a large ring is expected to be weak. Indeed the macrocycle is readily broken by addition of excess metal ion, leading to the formation of an $M_2 \cdot \mathbf{3}$ complex. This suggests that other weak metal chelators,



Scheme 1. Principle of the fluorescence enzyme assay using pM.

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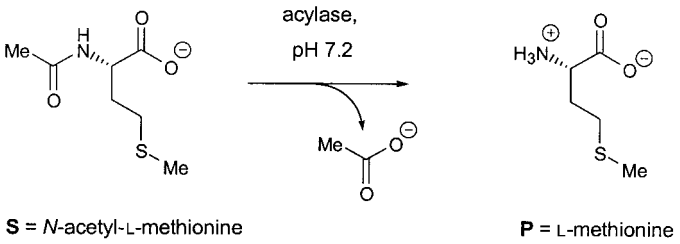
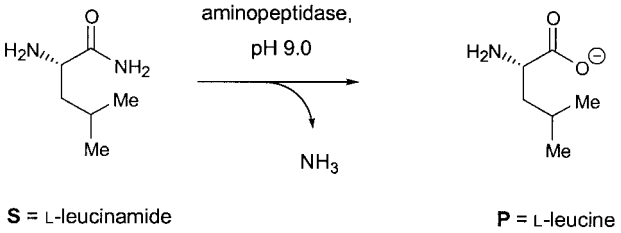
such as simple bidentate ligands, should be able to efficiently compete with **3** for metal coordination and induce a fluorescence increase in proportion to their concentration. Such an effect should allow an enzyme-catalyzed transformation of a noncoordinating substrate **S** into a weakly chelating product **P** to be monitored (Scheme 1).

Two enzyme reactions that produce free amino acids as metal chelating products (**P**) from nonchelating amide precursors (**S**) were chosen to test our sensor concept; these were the hydrolysis of *N*-acetyl-L-methionine to L-methionine by acylase I,[13] and the hydrolysis of L-leucinamide to L-leucine by aminopeptidase (Scheme 2).[14] First, simulated





Scheme 2. Enzyme reactions assayed with a fluorescent pM sensor.

profiles were carried out for conversion of the amide substrates into the corresponding amino acid products in the presence of ligand **3** with Cu^{2+} or Ni^{2+} as reporter metal ions. In all cases the nonfluorescent solution of **3** containing the metal ions responded to the conversion of substrate into product by a strong increase in fluorescence (Figure 1), as expected from complexation of the metal ions by the reaction product.

The actual enzyme assays were then investigated. The reaction of acylase I was recorded at pH 7.2 in the presence of ligand **3** and Cu^{2+}. The reaction of aminopeptidase was recorded at pH 9.0 with both Cu^{2+} and Ni^{2+} as reporter metal ions, since both metals induce quenching by complexation with ligand **3** at that pH. In all cases a time-dependent fluorescence increase was observed, which shows that the sensor was operative in real time (Figures 2 and 3). Indeed the apparent reaction rate increased in proportion to enzyme concentration; this indicates that the enzyme reaction itself was the rate-limiting process in the assay.

The assays were also carried out by mixing aliquots of the enzyme reactions in aqueous buffer with the sensor, consisting of metal and ligand, at fixed time intervals to measure product formation. This protocol allowed the addition of the organic solvent necessary to solubilize the sensor only after the

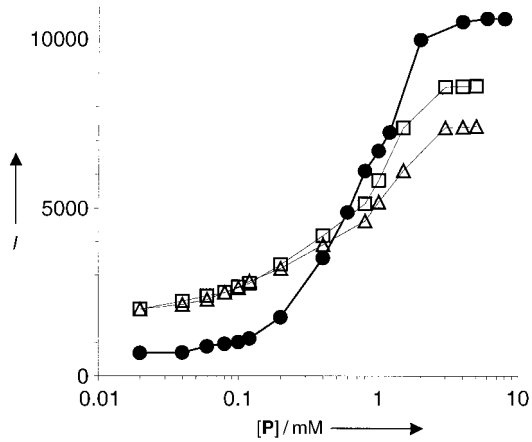


Figure 1. Fluorescence response of pM sensor **3** (1 μM) in the presence of metal ions upon simulated conversion of the following substrates **S** into products **P**: (●) *N*-acetyl-L-methionine (10 mM) into L-methionine, with Cu^{2+} (2 μM) in Bis-Tris (pH 7.2, 5 mM) and water/DMSO (60/40); (□) L-leucinamide (5 mM) into L-leucine, with Cu^{2+} (2 μM) in Tris (pH 9.0, 5 mM), and water/DMF (60/40); (△) same conditions as (□) but with Ni^{2+} (4 μM) replacing Cu^{2+}. All measurements taken at 26 °C with $\lambda_{em} = 580 \pm 50$ nm ($\lambda_{ex} = 485 \pm 20$ nm). Bis-Tris = bis(hydroxyethyl)tris(hydroxymethyl)aminomethane, Tris = tris(hydroxymethyl)aminomethane.

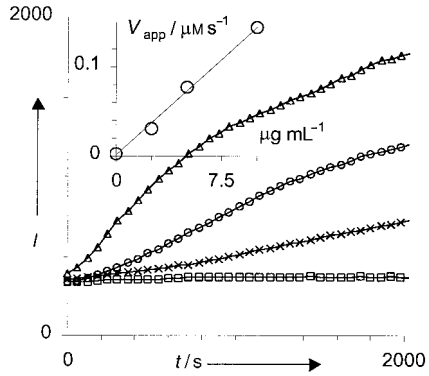


Figure 2. Fluorescence assay for acylase at pH 7.2. Hydrolysis of *N*-acetyl-L-methionine to L-methionine by acylase I is followed with ligand **3** in the presence of Cu^{2+}. Main plot: Time course of fluorescence increase I at $\lambda_{em} = 580 \pm 50$ nm ($\lambda_{ex} = 485 \pm 20$ nm). Enzyme concentrations: △ 10, ○ 5, × 2.5, and □ 0 μg mL^{-1}. Insert: replot of the apparent maximal rate in μM s^{-1} (the steepest linear portion of each curve after conversion of the fluorescence data into product concentration according to the calibration curves in Figure 1) against enzyme concentration in μg mL^{-1}. See Experimental Section for further details.

enzyme reaction had taken place. Under these conditions, acylase I and aminopeptidase were approximately twofold and tenfold more active, respectively, than in the real-time assay. The difference in activity is most likely accounted for by the presence of cosolvent in the real-time assay, since it corresponds to the published cosolvent effect in the case of acylase I.[13] In any event the aliquoting test, which can be conveniently applied as an endpoint measurement, can generally be used to avoid interferences between the cosolvent/pM-sensor system and the enzymes being assayed. Since the assay produces a fluorescent signal simply upon addition of the pM-sensor solution, assay throughput can easily reach several thousand samples per day by manual handling in standard 96-well microtiter plates. The assay is also suitable for ultra-high-throughput screening (UHTS, defined as

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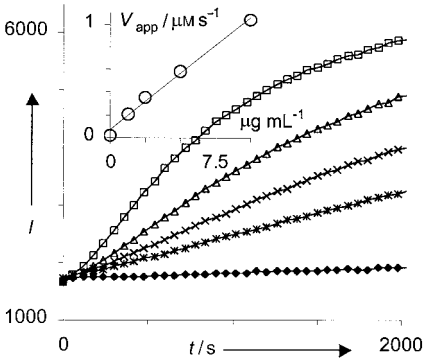

Figure 3. Fluorescence assay for aminopeptidase at pH 9.0. Hydrolysis of L-leucine amide to L-leucine by aminopeptidase is followed with ligand **3** in the presence of Cu^{2+}. Main plot: Time course of fluorescence increase I at $\lambda_{em} = 580 \pm 50$ nm ($\lambda_{ex} = 485 \pm 20$ nm). Enzyme concentrations: □ 10, △ 5, × 2.5, ∗ 1.25, and ◆ 0 μg mL^{-1}. Insert: replot of the apparent maximal rate in μM s^{-1} (steepest linear portion of each curve after conversion of fluorescence data into product concentration according to the calibration curves in Figure 1) against enzyme concentration in μg mL^{-1}. See Experimental Section for further details. Similar results are obtained with Ni^{2+} as the reporter metal ion.

> 100 000 samples per day) if used in high-density microtiter plates (1536-wells) with the corresponding robotic liquid-handling system and plate reader.[15]

The above experiments demonstrate a fluorescence assay for enzymes that liberate weak metal ligands, such as amino acids, as their reaction products. The assay is made possible by the absence of a strong chelate effect in the tetradentate ligand **3**, which allows these reaction products to efficiently compete for metal coordination in a useful concentration range. Ligand **3** can be considered as a pM sensor by its ability to differentiate free metal ions from weakly chelated metal ions. The fact that product sensing occurs indirectly through the pM value makes the assay applicable to a broad variety of substrates, as is the case for assays using pH indicators. Our pM assay detects structural changes otherwise difficult to measure, such as the hydrolysis of a carboxylic amide ($CONH_2$) to the corresponding acid (COOH). The kinetics of ligand exchange at copper and nickel are sufficiently fast for the fluorescent pM sensor to respond in real time to the reaction progress. Its usefulness is illustrated by the fact that the examples presented are the first fluorecence assays for acylase and aminopeptidase.

Experimental Section

Reagents and buffers were prepared in deionized milliQ-filtered water. Ligand **3** was diluted from a 1 mM stock solution of the trifluoroacetate salt in acetonitrile/water (1/1). *N*-acetyl methionine and L-methionine, were diluted from 0.1 M aqueous stock solutions buffered at pH 7.2 and pH 9.0, respectively; these pH values correspond to the reported optimal values for activity of the enzymes. Acylase I (from pig kidney, Fluka 01821) was diluted from a 1 mg mL^{-1} stock solution in 5 mM Bis-Tris (bis(hydroxyethyl)tris(hydroxymethyl)aminomethane) at pH 7.2. Cytosolic leucine aminopeptidase (from pig kidney, Fluka 61860) was diluted from a 1 mg mL^{-1} stock solution in 5 mM Tris (tris(hydroxymethyl)aminomethane) at pH 9.0.

Assays (0.1 mL) were followed at 26 °C in individual wells of round-bottom polypropylene 96-well microtiter plates (Costar) using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems; filters: $\lambda_{ex} = 485 \pm 20$ nm, $\lambda_{em} = 580 \pm 50$ nm, gain = 65). Enzyme assays were initiated by adding enzyme to the following solutions: acylase I: Bis-Tris (pH 7.2, 5 mM) in water/DMSO (60/40) containing ligand **3** (1 μM), $CuCl_2$ (2 μM), and *N*-acetyl-L-methionine (10 mM); aminopeptidase: Tris (pH 9, 5 mM) in water/DMF (60/40) containing ligand **3** (1 μM), $CuCl_2$ (2 μM) or $NiCl_2$ (4 μM), L-leucinamide hydrochloride (5 mM), and $MgSO_4$ (0.1 mM).

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