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A fluorescence assay for tetradecyltrimethylammonium mono-oxygenase activity that catalyzes the cleavage of the C–N bond with the production of trimethylamine

A.S. Liffourrena, P.S. Boeris, M.A. Salvano, G.I. Lucchesi *

Departamento de Biología Molecular, Facultad de Ciencias Exactas, Fisicoquímicas y Naturales, Universidad Nacional de Río Cuarto, 5.800 Río Cuarto, Córdoba, Argentina

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ABSTRACT

This article describes a simple fluorescence method for the determination of tetradecyltrimethylammonium mono-oxygenase (TTAB mono-oxygenase) activity involving N-dealkylation of tetradecyltrimethylammonium bromide with concomitant production of trimethylamine (TMA). Activity was determined by measuring the formation of TMA using the morin reagent and aluminum (Al). Morin reacts with Al to form a fluorescent complex, Al–morin. In the presence of TMA, Al is tightly associated with TMA and cannot be sequestered by morin, thus providing evidence for formation of the Al–TMA complex. The concentration of TMA is estimated by calibration graphs constructed by plotting the fluorescence intensity of the Al–morin complex versus TMA concentration. The fluorescence intensities of the Al–morin complexes quenched by TMA are linearly dependent on both the time of the TTAB mono-oxygenase reaction and the amount of protein used in the reaction. The kinetic behavior is characterized by $K_{0.5} = 4.26 \times 10^{-4}$ M, and the apparent Hill coefficient (n_{app}) = 2.24. These values are both comparable to those determined by GC–MS ($K_{0.5} = 4.41 \times 10^{-4}$ M and $n_{app} = 2.35$). The advantages of this assay include rapid and efficient implementation and potential employment for routine accurate determinations of TTAB mono-oxygenase activity over a wide range of substrate concentrations.

Quaternary ammonium-based surfactants (QACs) are commercially available chemicals commonly used as biocides or disinfectants in a variety of products, including cosmetics, antiseptic solutions, textile finishes, house-cleaning products, and fabric softeners [1–4]. QACs are amphoteric surfactants, generally containing one quaternary nitrogen associated with at least one hydrophobic moiety. Cetrimide USP is technically tetradecyltrimethylammonium bromide, while the generic term Cetrimide refers to a mixture of *N*-alkyltrimethyl ammonium bromides, in which the *n*-alkyl group is between 8 and 18 carbons long [3]. It has been shown that biodegradation of QACs occurs through various pathways. One such important pathway is N-dealkylation, which involves the action of a mono-oxygenase with the production of trimethylamine (TMA) and a corresponding alkyl residue [5–7]. We have recently demonstrated that in *Pseudomonas putida* A ATCC 12633, breakdown of tetradecyltrimethylammonium bromide (TTAB) to an alkyl residue plus TMA was oxygen dependent and occurred in the presence of NADH or NAD(P)H, supporting the notion that mono-oxygenase activity was responsible for the first step in the degradation of this QAC [8]. Current methods for assaying mono-oxygenase activity are widely performed using gas chromatography (GC) in conjunction with mass spectrometry (MS) [5,7,8]. This method has also been adapted for product determination in assays of trimethylamine oxide reductase activity, as well as for determination of the capacity of bacteria to reduce trimethylamine oxide to TMA in taxonomic screening [9]. The major advantage of this technique is higher sensitivity and specificity. However, this method also has inherent problems related to the difficulty in handling low-molecular-mass amines due to high water solubility and volatility, the need for expensive materials and instruments, and the fact that it does not lend itself to rapid studies in which numerous samples must be tested [9–13]. To overcome these limitations, we have developed a simple fluorescence method for determination of TTAB mono-oxygenase activity involving oxidative N-dealkylation of TTAB by measuring the formation of TMA with the use of the morin reagent and the addition of aluminum (Al). Morin (2′,3,4′,5,7-pentahydroxyflavone) is a pentaprotic acid that forms a highly fluorescent complex with Al, with a fluorescence detection limit of 2 nM [14] and an affinity constant of $\log K = 6.5$ [15]. In the presence of compounds that sequester Al, the fluorescent Al–morin complex is not formed [16,17]. The current method relies on the fact that TMA binds Al, thereby greatly reducing the fluorescence of the Al–morin complex. The concentration of TMA can be calculated by measuring the decrease in fluorescence intensity of the Al–morin complex in the presence of TMA.

* Corresponding author. Fax: +54 358 4676232.
E-mail address: glucchesi@exa.unrc.edu.ar (G.I. Lucchesi).

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We have characterized the method and the kinetics of TTAB mono-oxygenase activity using this assay and compared the results with previously reported data from GC–MS.

Materials and methods

Equipment and reagents

All fluorescence measurements were conducted with a Fluoromax-3 spectrophotometer (Jobin Yvon, Inc., Edison, NY, USA). Conventional 1-cm polystyrene cells were used. For GC–MS measurements, a Hewlett Packard Model 5890 with mass detector HP 5972 (Palo Alto, CA, USA) was used.

Morin reagent was obtained from Sigma Chemical (St. Louis, MO, USA) and was used without further purification. TMA hydrochloride, $AlCl_3$, and other reagents were obtained from Aldrich Chemical (St. Louis, MO, USA).

Standard stock solutions of TMA hydrochloride (1 mM) were prepared in double-distilled water (dd-H_2O) and stored at −10 °C. To prepare solutions in the 0.1 to 2 μM range, dilutions were performed daily with dd-H_2O. A stock solution of morin (1 mM) was prepared daily in absolute ethanol, and adequate fresh solutions were prepared by appropriate dilution with dd-H_2O. Standard stock solutions of $AlCl_3$ (1 mM) were prepared by diluting a commercial standard into dd-H_2O. When necessary, the $AlCl_3$ solution was prepared by appropriate dilution in the following buffered solutions: acetic acid–sodium acetate buffer (10 mM, pH 3–5), phosphate buffer (10 mM, pH 7.4), and Tris–HCl buffer (10 mM, pH 9).

Preparation of crude extracts

Cells of *P. putida* A ATCC 12633 grown on 50 mg L^{-1} (0.15 mM) TTAB at stationary phase (D_{660} = 0.4) were harvested by centrifugation at 10,000*g* for 10 min at 4 °C, washed with 10 mM phosphate buffer (pH 7.4), and resuspended in the same buffer. The cells were disrupted by sonication in an ice bath using a Vibra cell ultrasonic processor at 20,000 Hz, 10 times for 10 s each time. After being centrifuged at 20,000*g* for 30 min, the clear supernatant was removed and used as a crude enzyme.

TTAB mono-oxygenase activity assay

TTAB mono-oxygenase activity was assayed at 30 °C in a 1-ml reaction mixture containing 0.5 mM TTAB plus 0.5 mM NAD(P)H, 14 mM phosphate buffer (pH 7.4), and 0.3 mg ml^{-1} protein [8]. After 30 min of incubation at 30 °C, the reaction was stopped by addition of 0.5 ml of 37.5% trichloroacetate (TCA). After centrifugation at 12,000*g* for 10 min, aliquots of 1 and 100 μl of the supernatant were used to detect the product TMA by GC–MS or fluorescence, respectively, as described below. Boiled cell extracts were used as a blank. Unit enzyme activity was expressed as nmol TMA min^{-1} mg $prot^{-1}$.

TMA determination by GC–MS

TMA was estimated by GC–MS with standards of authentic TMA hydrochloride, as described previously [8]. The GC was equipped with an HP-1 column (crosslinked methyl silicone gum, 25 m × 0.32 mm × 0.17 μm film thickness). The flow rate of the helium carrier gas was 30 ml min^{-1}. The detector and the injector temperatures were 280 and 250 °C, respectively; the injection volume was 1 μl; and the column temperature was 50 °C.

TMA determination by fluorescence

TMA was determined using the morin reagent as the fluorochrome with addition of $AlCl_3$. In a final 3-ml volume of 10 mM acetic acid–sodium acetate buffer (pH 3.5), 100-μl aliquots of the supernatant were added to a polystyrene cuvette and mixed well with 50 μl of 3.6 mM $AlCl_3$. After a 30-min incubation, 900 μl of 1 mM morin reagent was added. The fluorescence of the Al–morin complex was measured with a Fluoromax-3 spectrophotometer using excitation and emission wavelengths of 440 and 494 nm, respectively. The concentration of TMA was calculated by calibration graphs constructed previously by plotting the fluorescence intensity of Al–morin complex versus TMA concentration.

Other methods

Protein concentrations were measured by the Bradford method [18] using bovine serum albumin (Sigma Chemical) used as a standard.

Results and discussion

Assay characterization: the interaction between TMA, Al, and morin

It has been previously demonstrated that Al reacts with morin to form a fluorescent complex, Al–morin, with a fluorescence detection limit of 2×10^{-9} M [14] and an affinity constant of log K = 6.5 [15]. Al–morin complex is not formed in the presence of compounds that sequester Al [16,17]. Thus, EDTA (Al–EDTA binding affinity constant log K = 16.5 [19]) quenched all fluorescence (99% reduction) from the Al–morin complex in aqueous solutions [15,20]. Citrate, with an Al binding affinity constant log K = 7.8 [19], is less effective at quenching Al–morin fluorescence (37% reduction) [16]. Since Al is a Lewis acid, we considered that the Al would associate tightly with a suitable Lewis base such as TMA. In this manner, the aluminum would not be sequestered by morin, providing evidence for the formation of the Al–TMA complex. In practice, the presence of TMA decreased the fluorescence intensity of the Al–morin complex. The solubility and ionic nature of aluminum are strongly dependent on pH [21,22]. Further, the pH of the solution influences the degree of ionization of the acidic hydroxyl groups present in morin and, as a result, directly influences the nature of the complexation event [23]. Furthermore, experiments were performed at various pH values in order to evaluate the fluorescence intensity of the Al–morin complex in the presence of TMA. Fig. 1 indicates that at all pH values studied, the fluorescence intensity of the Al–morin complex decreases linearly with increased TMA concentration, indicating that Al and TMA interact directly with each other. The same result was obtained when TMA was added after morin (data not shown), clearly indicating that the binding affinity of the Al–TMA complex is higher than that of the Al–morin complex. At pH 3.5, the slope of the curve increased, indicating that this quantification is most accurate, because small differences in TMA concentrations involve relatively high variations of fluorescence signal. The linear regression equation was $F = -10.65\ [\mathrm{TMA}] + 7.22$. The correlation coefficient (R^2) of 0.9985 indicated a high degree of linearity for this method. The fluorescence intensity produced by 0.35 μM $AlCl_3$, independent of the assay pH values, was quenched to the minimum detectable level when the concentration of TMA reached 0.7 μM. This implies that the molar ratio of TMA to Al in the complex was 2 to 1 (Fig. 1). We did not utilize a morin concentration above 0.3 mM because at those concentrations, morin exists mainly as dimers, and the fluorescence intensity is quenched [24]. The detection limit of TMA, calculated as 3 times the standard deviation of the blank divided by the absolute value of the slope, was 0.006 μM. The limit of quantification, calculated as 10 times the standard deviation of the blank divided by the absolute value of the slope, was 0.02 μM. As a point of comparison, the TMA detection limit

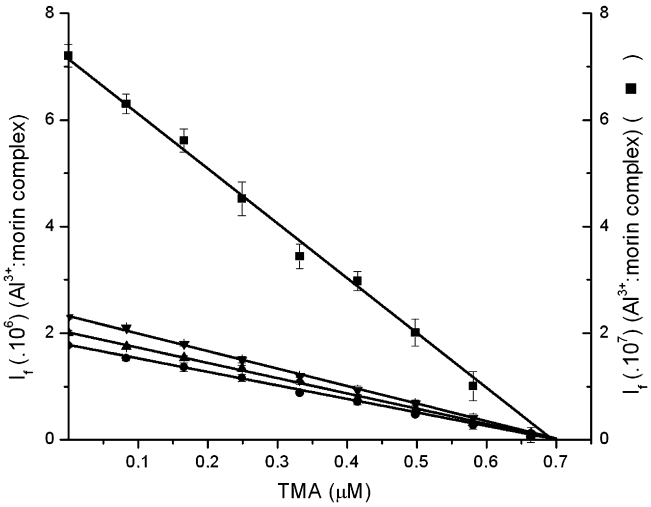

Fig. 1. Effect of TMA concentration on the formation of fluorescent Al–morin complexes. The fluorescence assays were carried out in the presence of various concentrations of TMA in 10 mM acetic acid–sodium acetate buffer at pH 3.5 (■) and pH 5 (▼), 10 mM phosphate buffer at pH 7.4 (▲), or 10 mM Tris–HCl buffer at pH 9 (●). Data correspond to a representative experiment of three independently performed runs.

obtained with the GC–MS assay was 0.008 μM. Additionally, the sensitivities of both methods were similar, yielding limits of detection in the nanomolar range.

Many carboxylates (e.g., citrate, oxalate) displace Al from morin, indicating potential interference with the method. Relative to EDTA [15,20], citrate is less effective in quenching Al–morin fluorescence (99% vs 37% reduction, respectively) [16]. Experiments were conducted in order to evaluate the influence of citrate or oxalate on the fluorescence intensity of the Al–morin complex. Based on the data in Fig. 2, citrate did not significantly modify the Al–morin fluorescence intensity when assayed at pH 3.5. At this pH, the Al–morin fluorescence intensity was quenched by TMA. The same results were obtained with oxalate, and no detectable modifications in the fluorescence signal were observed with succinate or α-ketoglutarate (data not shown).

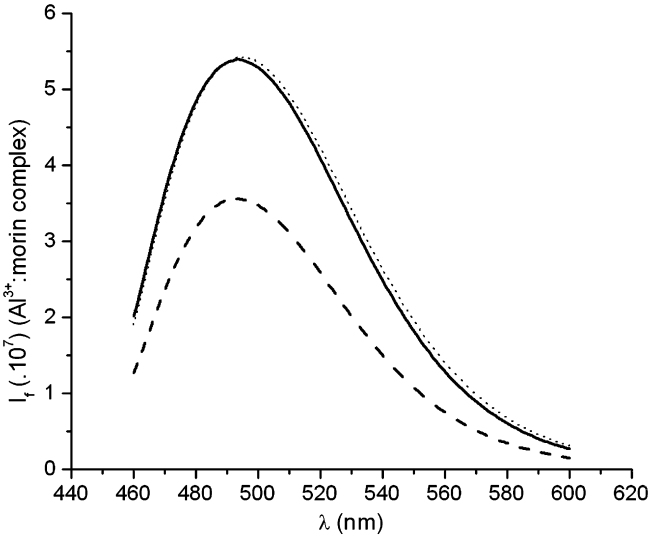

Fig. 2. Fluorescence spectra upon complexation of Al–morin by citrate or TMA at pH 3.5. Emission spectra (λ_{ex}=440 nm): Al-morin, solid line; Al–morin–citrate, dashed line; and Al–morin–TMA, dotted line. Conditions: 0.3 mM morin, 0.35 μM Al, 0.35 μM TMA, and 0.35 μM citrate.

In order to better understand this assay, we sought to determine whether this method could be used with other tertiary amines. We selected dimethylaminoethanol, dimethylglycine, triethanolamine, dimethylpropylamine, and dimethylisopropylamine. In all cases, significant decreases in fluorescence emission from the Al–morin complex due to the tertiary amines and Al interactions were obtained. Slope values obtained in calibration graphs of fluorescence versus concentration from each tertiary amine were comparable to those estimated with TMA (Fig. 1), and the molar ratio tertiary amine–Al complex was 2 to 1 (data not shown).

Also, to determine the selectivity of the assay, secondary amines, primary amines, ammonia, and quaternary ammonium compounds were selected as potential sequestering agents of Al. It was determined that the fluorescence intensity of the Al–morin complex was not quenched in the presence of ammonia, dimethylamine, choline, betaine, tetramethylamine, or tetradecyltrime-

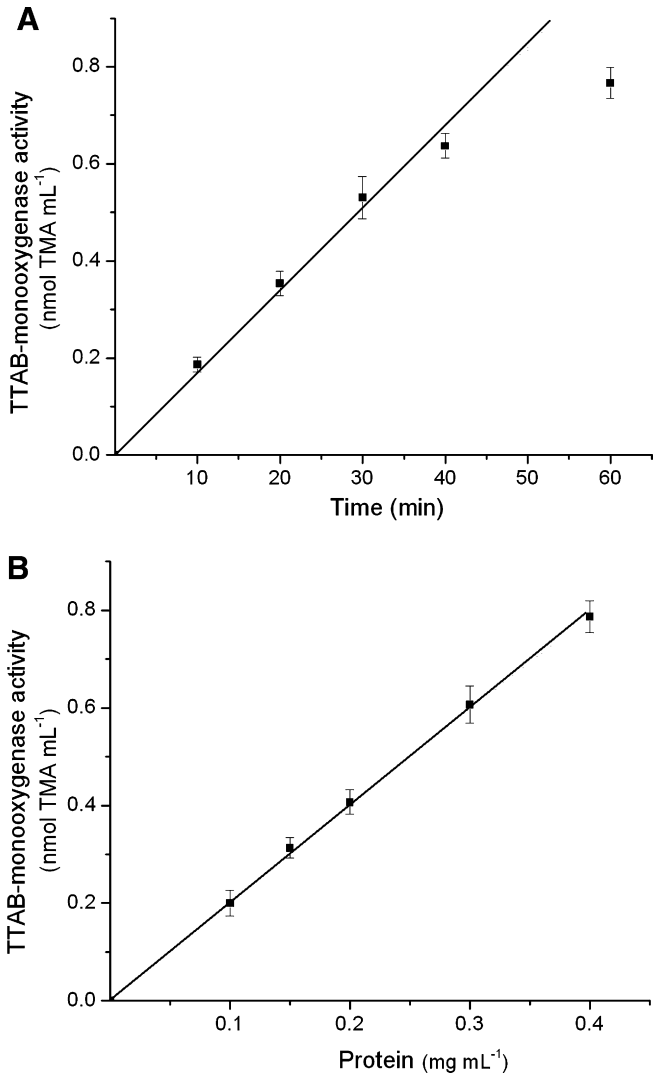

Fig. 3. Time- and enzyme concentration-dependent TTAB mono-oxygenase activity. (A) *P. putida* A ATCC 12633 TTAB mono-oxygenase was assayed with 0.3 mg ml^{-1} protein as described under Materials and methods. The times of each reaction varied. After the reaction was stopped with TCA, TMA concentration was determined in 10 mM acetic acid–sodium acetate buffer (pH 3.5) by a fluorescence assay. (B) TTAB mono-oxygenase activity was assayed as in (A) over 30 min; protein concentrations are indicated. All experiments were repeated three times, and the standard deviations are shown.

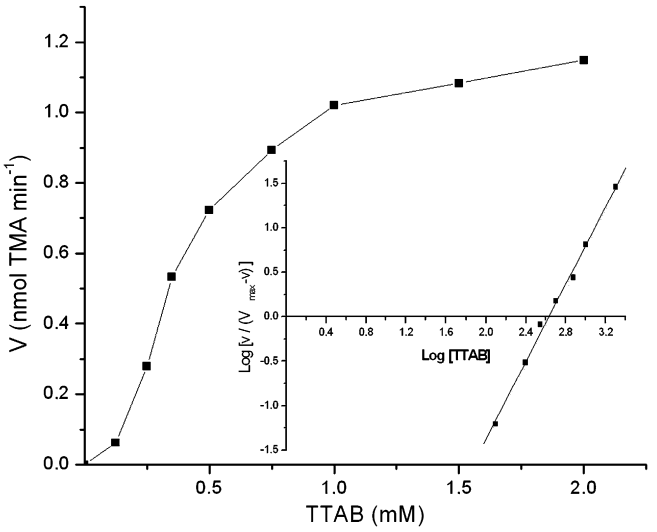

Fig. 4. Saturation curve of *P. putida* A ATCC 12633 TTAB mono-oxygenase by TTAB. The reaction mixture contained 14 mM phosphate buffer (pH 7.4), 0.5 mM NAD(P)H, and substrate as indicated. The reaction was initiated by addition of the enzyme (0.3 mg ml^{-1} protein), and the mixture was then incubated for 30 min. After the reaction was stopped with TCA, TMA concentrations were determined at pH 3.5 by the fluorescence assay. Data correspond to a representative experiment of three independently performed experiments, with enzyme extracts from three different preparations. Inset shows the Hill plot of the same data.

thylammonium, indicating that the method is specific for tertiary amines.

TTAB mono-oxygenase activity

It has previously been shown that in *P. putida* A ATCC 12633, TTAB breakdown may occur through N-dealkylation, which involves the action of a mono-oxygenase and the production of TMA and an alkyl residue [8]. Assays of this mono-oxygenase activity are widely performed using a GC–MS method [5,7,8]. Although this method is appropriate, it is time-consuming and tedious, particularly in studies in which numerous samples need to be tested. In order to determine TTAB mono-oxygenase enzymatic activity with a fluorescence assay, the product TMA was estimated at pH 3.5 by the addition of Al solution and the morin reagent, as described under Materials and methods. In the assay, we selected pH 3.5 in order to detect changes in TMA resulting from TTAB mono-oxygenase activity, because it was previously determined that at this pH value, small differences in TMA concentrations involve relatively high variations of fluorescence signal (Fig. 1). Additionally, the assay is not susceptible to quenching of Al–morin fluorescence by compounds that sequester Al, such as citrate or oxalate (Fig. 2). The fluorescence intensity of the Al–morin complex quenched by TMA was linearly dependent on both the time of the TTAB mono-oxygenase reaction and the amount of protein used in the reaction, indicating that the amount of fluorescence quenched is proportional to the activity of the TTAB mono-oxygenase (Fig. 3A and B). To validate the fluorescence assay for measurement of TTAB mono-oxygenase activity, a comparison was made between data generated by this technique and those obtained by the standard GC–MS method. Both of these quantitative methods were in good accord, as the enzyme activity was 0.066 ± 0.005 and 0.060 ± 0.003 nmol min^{-1} mg prot^{-1} (n = 4) when activity was measured as the formation of TMA by fluorescence or by GC–MS, respectively.

Since the fluorescence intensity of the Al–morin complex was not quenched in the presence of quaternary ammonium compounds, the ability of the fluorescence assay to detect the activity of mono-oxygenase was demonstrated by performing the assay in the presence of TTAB (Fig. 4). The fluorescence assay accurately predicted the specificity of this compound and yielded $K_{0.5}$ and n_{app} (apparent Hill coefficient) values of 4.26×10^{-4} M and 2.24, respectively. For comparison, when TTAB mono-oxygenase activity was characterized using the GC–MS method, a $K_{0.5} = 4.41 \times 10^{-4}$ M and $n_{app} = 2.35$ were obtained ([25] and this paper), which are comparable to the values determined by the novel fluorescence assay. Hence, this method would be advantageously used to study the purification and biochemical characterization of TTAB mono-oxygenase activity.

Conclusions

In this work we have demonstrated a fluorometric assay for the determination of TTAB mono-oxygenase activity, involving N-dealkylation of TTAB, with production of TMA. The activity was determined by measuring the formation of TMA using the addition of Al and the fluorochrome morin. The method proposed is based on the fluorescence quenching of the Al–morin complex by the formed TMA. Using this method, the formation of TMA from TTAB mono-oxygenase activity could be determined over a wide range of substrate concentrations yielding a $K_{0.5}$ value that is comparable to that determined by GC–MS. Importantly, this assay is simple to carry out, has reduced analysis time relative to standard procedures, and can be used for routine determinations of TTAB mono-oxygenase activity and related kinetic parameters.

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