Analytical Biochemistry 507 (2016) 21–26

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A continuous spectrophotometric assay and nonlinear kinetic analysis of methionine γ-lyase catalysis

Timothy C. Foo [a], Andrew C. Terentis [a, *], Kallidaikurichi V. Venkatachalam [b, **]

[a] Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, FL 33431, USA
[b] Department of Biochemistry, College of Medical Sciences, Nova Southeastern University, Fort Lauderdale, FL 33328, USA

ARTICLE INFO

Article history:
Received 27 January 2016
Received in revised form
8 April 2016
Accepted 13 May 2016
Available online 24 May 2016

Keywords:
Methionine γ-lyase
Pyridoxal-5′-phosphate
Porphyromonas gingivalis
Enzyme catalysis
UV–Vis spectrophotometry

ABSTRACT

In this article, we present a new, easy-to-implement assay for methionine γ-lyase (MGL)-catalyzed γ-elimination reactions of L-methionine and its analogues that produce α-ketobutyrate (α-KB) as product. The assay employs ultraviolet–visible (UV–Vis) spectrophotometry to continuously monitor the rate of formation of α-KB by its absorbance at 315 nm. We also employ a nonlinear data analysis method that obviates the need for an "initial slope" determination, which can introduce errors when the progress curves are nonlinear. The spectrophotometric assay is validated through product analysis by ^{1}H NMR (nuclear magnetic resonance), which showed that under the conditions of study L-methionine (L-met) and L-methionine sulfone (L-met sulfone) substrates were converted to α-KB product with greater than 99% yield. Using this assay method, we determined for the first time the Michaelis–Menten parameters for a recombinant form of MGL from *Porphyromonas gingivalis*, obtaining respective k_{cat} and K_m values of 328 ± 8 min^{-1} and 1.2 ± 0.1 mM for L-met γ-elimination and 2048 ± 59 min^{-1} and 38 ± 2 mM for L-met sulfone γ-elimination reactions. We envisage that this assay method will be useful for determining the activity of MGL γ-elimination reactions that produce α-KB as the end product.

The pyridoxal 5′-phosphate (PLP) dependent enzyme, methionine γ-lyase (MGL; EC 4.4.1.11), catalyzes the γ-elimination of L-methionine (L-met) forming α-ketobutyrate (α-KB), ammonium, and methanethiol (Scheme 1). MGL also catalyzes γ-replacement reactions of L-met with thiol compounds to form S-substituted L-met analogues as well as α-β-elimination and β-replacement of L-cysteine and various S-substituted L-cysteine analogues [1,2]. MGL is a globular protein that functions as a homotetramer, with each identical subunit (~43 kDa) containing a PLP cofactor [3]. MGL has been isolated from various sources such as pathogenic bacteria (e.g., *Clostridium tetani* [4], *Porphyromonas gingivalis* [5]) and pathogenic protozoa (e.g., *Entamoeba histolytica* [6], *Trichomonas vaginalis* [7]). Extensive *in vitro* biochemical and biophysical studies have been published on three recombinant MGL isoforms from *Pseudomonas putida* (= *Pseudomonas ovalis*) [8–10], *Citrobacter freundii* [3,11,12], and *T. vaginalis* [13,14].

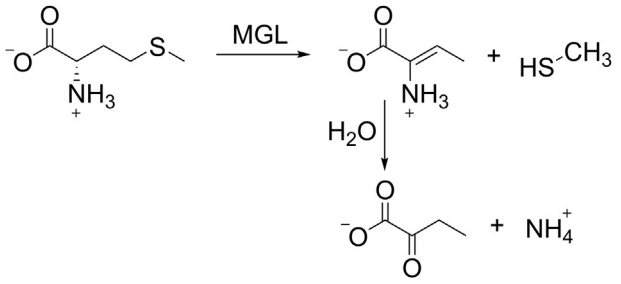
Besides its physiological roles in organisms, MGL is also a therapeutic target for pathogenic diseases such as trichomoniasis, gingivitis, amoebiasis, and cancer [15]. An MGL substrate analogue, trifluoromethionine (TFM), is a prodrug that has emerged as a lead compound for a novel class of potent antibiotics [16,17]. Cancerous cells have a higher requirement for L-met; thus, exogenous MGL was used to reduce plasma L-met concentrations to inhibit tumor growth in several animal models [18–22]. Because bacterial-derived MGLs have a short half-life in serum, a human cystathionine-γ-lyase mutant was recently engineered to exhibit MGL activity with a longer serum half-life [23]. In addition, cancer cells transfected with the *P. gingivalis* gene exhibited severe cell aggregation and death [24]. Thus, there are broad prospects for MGL's applications in medicine. However, there still lacks a thorough understanding of the mechanisms of the reactions catalyzed by MGL, which can be obtained from detailed enzyme kinetic studies. Such studies require a reliable and simple enzyme assay system and method of data analysis, both of which have been lacking for MGL.

Most of the MGL enzymatic activity assays employed to date have involved derivatizing the product to a chromophoric species,

Abbreviations used: PLP, pyridoxal 5′-phosphate; MGL, methionine γ-lyase; L-met, L-methionine; α-KB, α-ketobutyrate; TFM, trifluoromethionine; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; DNPH, dinitrophenylhydrazine; LDH, lactate dehydrogenase; L-met sulfone, L-methionine sulfone; UV–Vis, ultraviolet–visible; NMR, nuclear magnetic resonance; DTT, dithiothreitol.

* Corresponding author.
** Corresponding author.
E-mail addresses: terentis@fau.edu (A.C. Terentis), venk@nova.edu (K.V. Venkatachalam).

http://dx.doi.org/10.1016/j.ab.2016.05.010
0003-2697/



Scheme 1. The γ-elimination reaction of L-met catalyzed by MGL and subsequent hydrolysis of 2-aminocrotonate produces α-KB with methanethiol and ammonium side products.

which is then quantified by spectrophotometry. For example, one commonly employed assay uses 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) to react with the carbonyl group of the product to form an azine chromophore (ε_{320nm} = 15.7 mM^{-1} cm^{-1}) [25]. A similar assay uses dinitrophenylhydrazine (DNPH, Brady's reagent) as the derivatizing agent [26]. Assays based on product derivatization are discontinuous, batch-processing methods that are time-consuming and limited in their ability to provide the large number of data points required for a precise kinetic analysis. Product derivatization is also associated with the potential risk of unwanted side reactions and product modifications. For example, both MBTH and DNPH are known to react with the aldehyde group of free PLP that is usually added to MGL assay mixtures [25], whereas the molar absorptivity of the azine chromophore is sensitive to pH, both of which could lead to errors in product quantitation.

Another MGL assay is a continuous secondary enzyme assay that uses lactate dehydrogenase (LDH) to reduce α-KB to α-hydroxybutyrate and oxidize NADH to NAD^+. The LDH must be present in significantly large excess to prevent its reaction from being the rate-limiting reaction. Therefore, the LDH assay might not be feasible for efficient MGL substrates or substrates that yield products that inactivate LDH [14,27].

A further complicating factor for MGL kinetic studies is that it can exhibit nonlinear enzyme kinetics due to product inhibition. Such is the case for the reversible γ-displacement reaction of L-met (Scheme 2) [28]. The nonlinearity of reaction progress curves severely limits the accuracy of standard steady-state, initial velocity approaches that are based on the assumption of irreversible product release. In these cases, nonlinear modeling of whole progress curves is required to obtain accurate kinetic parameters.

In this article, we report the first continuous (real-time) MGL activity assay that directly measures α-KB formation by spectrophotometry without the need for derivatization. This assay is both more expedient and more accurate than previously reported batch assay approaches because it enables a large number of time points to be collected for the reaction progress curves. Furthermore, we introduce a nonlinear progress curve analysis that avoids the errors associated with the initial slopes or data linearization methods. The first Michaelis–Menten parameters for MGL from *P. gingivalis* strain W83 are reported for L-met and L-methionine sulfone (L-met sulfone) substrates.

Materials and methods

Chemicals

L-Methionine, L-methionine sulfone, sodium α-ketobutyrate, pyridoxal 5′-phosphate hydrate, and all other chemicals used in this study were purchased from Sigma–Aldrich and were of analytical reagent grade or higher.

Expression and purification of MGL

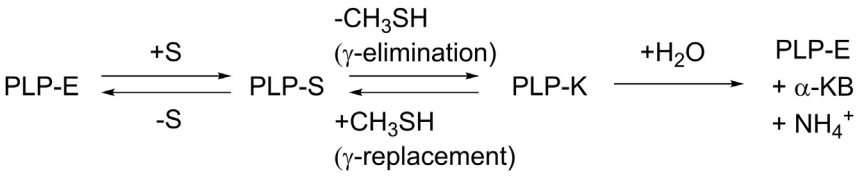
Full details for the cloning, expression, and purification of MGL from *P. gingivalis* were published previously [29]. For this work, the purified protein product exhibited a 280/425-nm absorbance ratio of approximately 6. The sample was stored at −80 °C in storage buffer containing 10% glycerol.

Holoenzyme preparation and quantitation

The purified MGL was taken from −80 °C storage and treated with PLP (100 μM) overnight at 4 °C to form the fully reconstituted holoenzyme. The MGL/PLP mixture was then filtered through a size exclusion column (M_w > 5000 Da, Sephadex G-25) and eluted with 100 mM sodium phosphate buffer containing 150 mM KCl (pH 7.5) (here referred to as phosphate buffer). The MGL fraction was collected, stored on ice, and used for experiments. The final 280/425-nm ratio for the reconstituted enzyme was typically within the range 3.8–4.7. The molar absorptivity of MGL at 280 nm is estimated to be ε_{280nm} = 28 mM^{-1} cm^{-1} [30]. The molar absorptivity at 425 nm is estimated to be 7 mM^{-1} cm^{-1} based on the molar absorptivity of *C. freundii* MGL [31] and that of the PLP–valine Schiff base [32]. Therefore, the observed 280/425-nm ratio of approximately 4 is as expected for the holoenzyme (i.e., 4 PLP cofactor units per protein tetramer). For the enzyme assay, the enzyme concentration was determined by its absorbance at 425 nm. Therefore, the kinetic parameters (k_{cat} and K_m) determined in this work are expressed on a per-PLP (i.e., per monomeric subunit) basis.

Enzyme assay

For all enzyme kinetic measurements, the enzyme reaction was conducted at 37 °C and consisted of phosphate buffer, 1.1 μM MGL, and varying concentrations of L-met (250 μM–40 mM) or L-met sulfone (2–88 mM). The substrate stock (200 mM prepared in phosphate buffer) was diluted into a micro quartz cuvette (1-cm path length, Starna Cells, 28-Q-10) with phosphate buffer to the desired concentration yielding 495 μl. Afterward, 5 μl of MGL stock solution was placed on a Teflon stopper that was used to seal the cuvette. The Teflon-stoppered cuvette was then placed inside the spectrophotometer and heated to 37 °C. On thermal equilibration,



Scheme 2. Simplified MGL reaction scheme highlighting the role of reversible γ-displacement, which may lead to product inhibition and nonlinear enzyme kinetics. Abbreviations: PLP-E, enzyme–PLP internal aldimine; S, L-met substrate; PLP-S, external aldimine; PLP-K, unsaturated ketimine enzyme intermediate [11,28].