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Analytical Biochemistry 327 (2004) 233–240

ANALYTICAL BIOCHEMISTRY

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Assay method for antitumor L-methionine γ-lyase: comprehensive kinetic analysis of the complex reaction with L-methionine

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Received 14 October 2003

Abstract

L-Methionine γ-lyase (EC 4.4.1.11) is a pyridoxal 5′-phosphate-dependent multifunctional enzyme. Measuring the initial velocity of α-ketobutyrate production by α,γ-elimination of L-methionine catalyzed by L-methionine γ-lyase is not very feasible, because the enzyme simultaneously catalyzes both γ-replacement and α,γ-elimination. To develop an accurate enzyme assay, the comprehensive enzyme kinetics needed to be elucidated by progress curve analysis on the basis of a reaction model for conversion of L-methionine to α-ketobutyrate, methanethiol, and ammonia with pyridoxal 5′-phosphate as a cofactor. Kinetic parameters were determined by linear transformation using an approximation of a Maclaurin series from the whole velocity of α-ketobutyrate production including α,γ-elimination and γ-replacement. The significance of γ-replacement was revealed both theoretically and practically by the kinetic analysis. The enzyme activity was standardized and represented as the V_{max} value taking into consideration γ-replacement in the presence of L-methionine at 37 °C and pH 8.0. The novel method that we proposed is accurate, sensitive, reproducible, and linear over a wide range for the determination of L-methionine γ-lyase activity.

Keywords: L-Methionine γ-lyase; Pyridoxal 5′-phosphate; Enzyme kinetics; Enzyme activity; Progress curve analysis

L-Methionine γ-lyase (METase;[1] EC 4.4.1.11) is a multifunctional enzyme which catalyzes both the α,γ-elimination and γ-replacement of L-methionine and its derivatives and the α,β-elimination and β-replacement of *S*-substituted L-cysteines [1,2]. The enzyme is a member of the γ family of pyridoxal 5′-phosphate (PLP)-dependent enzymes [3]. METases have been isolated, purified, and characterized from several microorganisms such as *Pseudomonas putida* (= *ovalis*) [4–10], *Clostridium sporogenes* [11], *Aeromonas* sp. [12], *Citrobacter intermedius* [13], *Brevibacterium linens* [14], *Trichomonas vaginalis* [15], and *Porphyromonas gingivalis* [16].

Several practical applications of METase have been reported. Fung et al. [17] described a potentiometric enzyme electrode prepared with immobilized METase for the assay of L-methionine. Tanaka et al. [18] demonstrated a method of selective determination of L-methionine and L-cysteine using METase and they [19] also described the synthesis of D-α-aminobutyrate using both METase and D-amino acid aminotransferase. The antitumor efficacy of METase, which is based on the greater L-methionine dependency of cancer cells for growth compared to normal cells [20–23] has been investigated. Upon L-methionine depletion, L-methionine-dependent cancer cells were not able to divide and became arrested in the late-S/G_2 phase of the cell cycle

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[1] *Abbreviations used:* METase, L-methionine γ-lyase; PLP, pyridoxal 5′-phosphate; Tween 80, polyoxyethylene (20) sorbitan monooleate; IPTG, isopropyl-β-D-thiogalactopyranoside; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate; TLC, thin-layer chromatography.

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doi:10.1016/j.ab.2004.01.024

[24–26]. The METase derived from *P. putida* has been extensively used for these applications due to its high stability and activity. The METase genes from *P. putida* were cloned and expressed in *Escherichia coli* by Inoue et al. [27], Hori et al. [28], and Tan et al. [29]. Motoshima et al. [30] determined the crystal structure of METase from *P. putida*. Tan et al. [29] reported the overexpression and purification of recombinant METase with high purity and low endotoxins for preclinical studies on cancer therapy. Yoshioka et al. [31] showed that recombinant METase and 5-fluorouracil exerted synergistic antitumor efficacy against the Lewis Lung carcinoma in vivo.

To utilize METase for these purposes, it is necessary to accurately evaluate the enzyme activity and to analyze the kinetics of the enzyme reaction with L-methionine. The most common method available for expressing the METase activity is the estimation of velocity of α-ketobutyrate production from L-methionine by α,γ-elimination on the basis of Michaelis–Menten kinetics. This method has been conventionally employed to characterize the enzymological properties of METase [4–15]. On the other hand, Tanaka et al. [1] measured the velocity of replacement between L-methionine and various thiol compounds and revealed the considerable importance of γ-replacement. Since METase shows multifunctional catalysis [1,2], α,γ-elimination and γ-replacement should not be considered separately. Less attention has been paid to evaluating the quantification of enzyme activities and to analyzing kinetic studies for the whole reactions with L-methionine. In this article, we initially show that the conventional enzyme assay is unsuitable for accurate estimation of METase activity. To investigate the comprehensive kinetic analysis, we construct a mechanism-based enzyme reaction model, elicit the velocity equation, perform a progress curve analysis, and determine the kinetic parameters for the whole enzyme reactions. Finally, we establish a reliable enzyme assay system which takes both α,γ-elimination and γ-replacement into consideration in the presence of L-methionine as a substrate. Details of this accurate evaluation of METase activity are described.

Materials and methods

Chemicals

L-Methionine, dithiothreitol, polyoxyethylene (20) sorbitan monooleate (Tween 80), isopropyl-β-D-thiogalactopyranoside (IPTG), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) were purchased from Wako Pure Chemical (Osaka, Japan). L-Ethionine, L-homoserine, polypropylene glycol No. 2000, and PLP were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals used in this study were of analytical reagent grade or the highest grade commercially available.

Bacterial strain and plasmid construction

E. coli JM109 was used as the host strain for expression of recombinant METase. The plasmid containing METase gene from *P. putida* ICR3460 was constructed according to a previous report by Inoue et al. [27]. The METase gene except the initiation codon was amplified by PCR with a set of forward (5′-CCCGGTACCACGG CTCCAACAAGCTCCCAG) and reverse (5′-CTCGAG ACGGGTTCAGGCACTCGCCTT) primers and inserted into pMOSblue vector (Amersham Biosciences, Piscataway, NJ). The fragment treated with *Kpn*I-T4 DNA polymerase and *Xba*I including METase gene except the initiation codon was ligated to pTrc99A vector (Amersham Biosciences), resulting in pMGL1101. To change the antibiotic marker from ampicillin to tetracycline, the fragment treated with *Eco*RI, *Eco*T14I, and T4 DNA polymerase from pBR322 was ligated to the large *Dra*I fragment from pMGL1101. This plasmid designated as pMGL1204 was used for recombinant METase expression.

Production of recombinant METase

E. coli JM109 harboring pMGL1204 was incubated in 200 ml seed medium (1.0% sodium succinate, 2.0% yeast extract, 0.02% polypropylene glycol No. 2000, and 10 μg/ml tetracycline hydrochloride, pH 6.0) at 28 °C for 17 h in a 500-ml Erlenmeyer flask with vigorous shaking. The culture broth was transferred to 20 L fermentation medium (1.2% tryptone, 2.4% yeast extract, 4.0% glycerol, 1.25% K_2HPO_4, 0.23% KH_2PO_4, 0.05% polypropylene glycol No. 2000, and 10 μg/ml tetracycline hydrochloride, pH 7.2) in a 30-liter fermentor and incubated at 28 °C. For expression of recombinant METase, IPTG was added at final concentration 0.3 mM after cultivation for 6 h. The bacteria were harvested with a disk-type centrifuge (LAPX 202; Alfa-Laval, Fort Lee, NJ) after fermentation for 24 h. The concentrated cell suspension was diluted with 100 mM sodium phosphate buffer (pH 7.5) containing 0.01% dithiothreitol, 2 mM EDTA, and 1.3 mM PLP to a turbidity of 60 at 650 nm. The cells were disrupted with a Manton Gaulin homogenizer (15 M-8TA; APV-Gaulin, Wilmington, MA).

Purification of recombinant METase

Recombinant METase, which accumulated intracellularly in a soluble form, was purified to homogeneity by heat treatment and a combination of column chromatographies using DEAE–Sepharose FF (pH 7.2, Amersham Biosciences), DEAE–Sepharose FF (pH 8.3), and

Acticlean Etox gels (Sterogen Bioseparation, Arcadia, CA) according to the method of Tan et al. [29]. The purified enzyme gave a single band at 42 kDa on SDS–PAGE by the method of Laemmli [32]. The molecular mass of recombinant METase, measured by gel permeation HPLC on a TSKgel G3000SW column (Tosoh, Tokyo, Japan) eluted with 10 mM sodium phosphate buffer (pH 7.2) containing 120 mM NaCl at a flow rate of 1 ml/min with measurement of absorbance at 220 nm, was approximately 160 kDa. The N-terminal amino acid sequence of the enzyme, which was determined with a protein sequencer (PPSQ21; Shimadzu, Kyoto, Japan), agreed with that deduced from the nucleic acid sequence of the *P. putida* METase gene [27]. The purified enzyme was used for further study and could be stored at −80 °C for several months with no significant loss of activity.

Protein assay

Protein concentration was determined by the method of Watanabe et al. [33] with a protein assay kit (Wako Pure Chemical) using crystalline bovine serum albumin as a standard protein.

Enzyme assay

The standard enzyme assay was performed according to the method of Esaki and Soda [8] with a slight modification. The enzyme solution was prepared by appropriate dilution with 100 mM potassium phosphate buffer (pH 8.0) containing 0.01% dithiothreitol, 1 mM EDTA, 10 μM PLP, and 0.05% Tween 80. One milliliter of the substrate solution consisting of 100 mM potassium phosphate buffer (pH 8.0), 25 mM L-methionine, and 10 μM PLP was preincubated at 37 °C in a glass tube with a sealed cap. The enzymatic reaction was initiated by adding 50 μl of the enzyme solution to the preincubated substrate solution at 37 °C. After incubating the reaction mixture for precisely 10 min in a glass tube with a sealed cap, the enzymatic reaction was terminated by adding 100 μl of 50% trichloroacetic acid. A blank test was carried out in the same procedure except with the reverse addition order of the enzyme solution and 50% trichloroacetic acid. Subsequently, the chemical reaction was initiated by adding both 1.6 ml of 1 M sodium acetic acid buffer (pH 5.0) and 0.6 ml of 0.1% MBTH into 0.8 ml of the above enzymatic reaction mixture. After incubating the mixture at 50 °C for 40 min in a glass tube with a sealed cap and cooling it down to 25 °C, the amount of α-ketobutyrate produced was measured spectrophotometrically ($\varepsilon = 15,740$ ml/mmol/cm, UV-1600; Shimadzu) based on the increase in absorbance at 320 nm (ΔE) from the blank. One unit of METase was defined as the amount of enzyme that produced 1 μmol of α-ketobutyrate per minute at infinite concentration of L-methionine. The specific activity was represented as the enzyme activity in units per milligram protein.

Results and discussion

Identification of reaction products

α-Keto acids produced from various substrates by α, γ-elimination were identified as their 2,4-dinitrophenylhydrazone derivatives by thin-layer chromatography (TLC) with two different solvent systems (1-butanol saturated with 3% ammonia, 1-butanol:water:ethanol (5:1:1, v/v/v)) using silica gel plates ($60F_{254}$; Merck, Darmstadt, Germany) [5,10]. The R_f values of phenylhydrazone derivatives of α-keto acids produced from L-methionine, L-ethionine, and L-homoserine by α,γ-elimination on TLC were identical to that of authentic α-ketobutyrate (1-butanol saturated with 3% ammonia, $R_f = 0.62$; 1-butanol:water:ethanol, $R_f = 0.69$). The METase derived from *P. putida* cleaved L-homoserine and formed α-ketobutyrate, ammonia, and water with α,γ-elimination, in marked contrast to the enzyme derived from *T. vaginalis* which does not act on L-homoserine [15].

Time course of enzyme reaction

The progress curve profiles of α-ketobutyrate production during the enzyme reaction against various substrates with variation in their concentrations are shown in Fig. 1. When the substrate was L-methionine, the amount of α-ketobutyrate produced did not linearly increase during the enzyme reaction at any of the substrate concentrations (Fig. 1A). The nonlinear increase of α-ketobutyrate production is caused by multifunctional catalysis of METase. In general, it is helpful to represent the enzyme activity as the initial increase in velocity of product formation in a zero-order reaction on the basis of Michaelis–Menten kinetics. However, as shown in Fig. 1A, it was not possible to accurately monitor the initial velocities of α-ketobutyrate produced from L-methionine using ordinary experimental apparatus, other than a stopped-flow assay system. We had difficulty in defining METase activity as the initial velocity of α-ketobutyrate production and in determining the kinetic parameters under the present conditions. Moreover we could not obtain reproducible progress curve results using an open system without a sealed cap during the enzyme reaction (data not shown). This was probably due to vaporization of methanethiol. A similar trend for α-ketobutyrate production was also observed using L-ethionine as a substrate (Fig. 1B).

In contrast to L-methionine and L-ethionine, when the substrate was L-homoserine, α-ketobutyrate was linearly increased during the enzyme reaction at any of

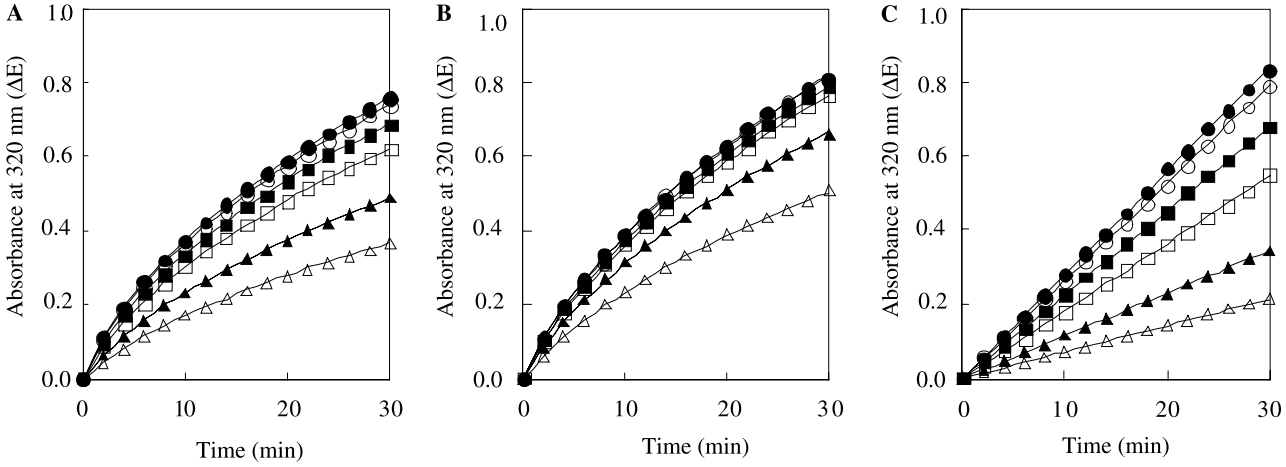

Fig. 1. Progress curves of α-ketobutyrate production in the presence of L-methionine (A), L-ethionine (B), and L-homoserine (C). (A) The substrate concentrations were 50 mM (●), 25 mM (○), 10 mM (■), 5 mM (□), 2 mM (▲), and 1 mM (△). The enzyme concentration was 0.31 μg/ml in the reaction mixture. (B) The substrate concentrations were 50 mM (●), 25 mM (○), 5 mM (■), 3 mM (□), 1 mM (▲), and 0.5 mM (△). The enzyme concentration was 0.45 μg/ml in the reaction mixture. (C) The substrate concentrations were 500 mM (●), 250 mM (○), 100 mM (■), 50 mM (□), 20 mM (▲), and 10 mM (△). The enzyme concentration was 5.4 μg/ml in the reaction mixture. Symbols represent experimental results and curves denote simulations from determined kinetic parameters.

the substrate concentrations (Fig. 1C). The enzyme catalyzed the formation of α-ketobutyrate, ammonia, and water from L-homoserine. We obviously observed that γ-replacement had a certainly important role in the enzyme reaction. The results demonstrated the necessity of comprehensive kinetic analysis of enzyme reaction involving both γ-replacement and α,γ-elimination.

Model construction of enzyme reaction

The mechanism of α,γ-elimination and γ-replacement catalyzed by METase was proposed [1,2,34,35]. This is a generally accepted reaction mechanism for α,γ-elimination and γ-replacement occurring via an external Schiff's base by PLP-dependent enzymes. To facilitate the interpretation of the enzyme reaction mechanism, we present a scheme of the reaction mechanism in Fig. 2, where S, I, and P denote L-methionine and its derivatives, corresponding thiols or selenols, and α-ketobutyrate, respectively, and *k*s are the respective rate constants of reactions. Since α-ketobutyrate, methanethiol, and ammonia are produced stoichiometrically with consumption of L-methionine [5], an essential characteristic of this mechanism is simply that there are three species of the enzyme: a holo enzyme covalently bounded between PLP and ε-amino group of an active-site lysine residue (PLP-E), an external aldimine or tautomeric intermediates formed between PLP and L-methionine and its derivatives (PLP-S), and a β,γ-unsaturated ketimine or tautomeric intermediates including an α,β-unsaturated aldimine (PLP-V).

The King–Altman schematic method [36] was applied to derive the velocity expression according to the reaction model of Fig. 2. The whole velocity of α-ketobutyrate production from L-methionine is described by the equation

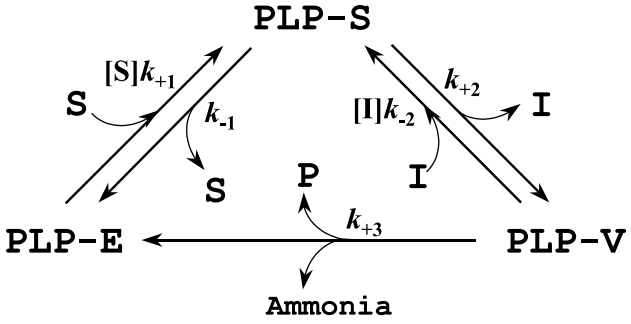

Fig. 2. Schematic representation of α,γ-elimination and γ-replacement by METase in the presence of L-methionine and its derivatives on the basis of the proposed reaction mechanism. S, I, and P are L-methionine and its derivatives, corresponding thiols or selenols, and α-ketobutyrate, respectively, and *k*s are the respective rate constants of reactions. PLP-E, PLP-S, and PLP-V are a holo enzyme covalently bounded between PLP and ε-amino group of an active-site lysine residue, an external aldimine or tautomeric intermediates formed between PLP and L-methionine and its derivatives, and a β,γ-unsaturated ketimine or tautomeric intermediates including an α,β-unsaturated aldimine, respectively, as an essential characteristic of the enzyme reaction.

$$\begin{aligned}\frac{d[P]}{dt} &= k_{+3}[\text{PLP-V}] \\ &= k_{+1}k_{+2}k_{+3}[E]_0[S]/\{k_{+2}k_{+3} + k_{-1}k_{-2}[I] \\ &\quad + k_{-1}k_{+3} + k_{+1}k_{+3}[S] + k_{+1}k_{-2}[I][S] \\ &\quad + k_{+1}k_{+2}[S]\}, \end{aligned} \quad (1)$$

where [P], [S], [I], [PLP-V], and $[E]_0$ represent concentrations of α-ketobutyrate, L-methionine, methanethiol, β,γ-unsaturated ketimine or tautomeric intermediates

including an α,β-unsaturated aldimine, and initial enzyme, respectively, because no inactivation of enzyme was recognized during the incubation under experimental conditions. Eq. (1) can be transformed to Eq. (2) using [I] = [P]:

$$\frac{d[P]}{dt} = \left\{ \frac{k_{+2}k_{+3}}{k_{+2}+k_{+3}}[E]_0([S]_0 - [P]) \right\} \Bigg/ \left\{ ([S]_0 - [P]) \left(1 + \frac{[P]}{\left(\frac{k_{+2}+k_{+3}}{k_{-2}}\right)} \right) + \frac{k_{+3}(k_{-1}+k_{+2})}{k_{+1}(k_{+2}+k_{+3})} \times \left(1 + \frac{[P]}{\left(\frac{(k_{+3}(k_{-1}+k_{+2})}{k_{-1}k_{-2}}\right)} \right) \right\}, \quad (2)$$

where $[S]_0$ is the initial L-methionine concentration. To simplify the expression, Eq. (2) was substituted as follows:

$$\frac{d[P]}{dt} = \frac{V_{max}([S]_0 - [P])}{([S]_0 - [P])\left(1 + \frac{[P]}{K_i'}\right) + K_m\left(1 + \frac{[P]}{K_i}\right)}, \quad (3)$$

where

$$V_{max} = k_{cat}[E]_0, \quad k_{cat} = \frac{k_{+2}k_{+3}}{k_{+2}+k_{+3}},$$
$$K_m = \frac{k_{+3}(k_{-1}+k_{+2})}{k_{+1}(k_{+2}+k_{+3})}, \quad K_i = \frac{k_{+3}(k_{-1}+k_{+2})}{k_{-1}k_{-2}},$$
$$\text{and} \quad K_i' = \frac{k_{+2}+k_{+3}}{k_{-2}},$$

respectively. The whole velocity of α-ketobutyrate production from L-methionine was provided by Eq. (3).

Determination of kinetic parameters by progress curve analysis

Since the initial velocity of α-ketobutyrate production cannot be estimated (Fig. 1A), progress curve analysis is applied. Integration of Eq. (3) gave Eq. (4):

$$\int_0^t V_{max}\,dt = \int_0^{[P]} \frac{([S]_0 - [P])\left(1 + \frac{[P]}{K_i'}\right) + K_m\left(1 + \frac{[P]}{K_i}\right)}{([S]_0 - [P])} d[P]. \quad (4)$$

Eq. (4) was expanded to Eq. (5):

$$V_{max} = \frac{1}{t}\left\{ -\frac{K_mK_i + K_m[S]_0}{K_i} \ln\left(1 - \frac{[P]}{[S]_0}\right) + [P]\left(1 - \frac{K_m}{K_i} + \frac{[P]}{2K_i'}\right) \right\}. \quad (5)$$

Nonlinear regression analysis requires complicated calculations and computer programs. We transformed the linear form using an approximation of the Maclaurin series to circumvent nonlinear regression analysis. Eq. (5) was refined as follows:

$$V_{max} = \frac{1}{t}\left\{ \left(\frac{K_m}{[S]_0} + 1\right)[P] + \left(\frac{K_m}{2[S]_0^2} + \frac{K_m}{2K_i[S]_0} + \frac{1}{2K_i'}\right)[P]^2 \right\}, \quad (6)$$

because

$$\ln\left(1 - \frac{[P]}{[S]_0}\right) \approx -\frac{[P]}{[S]_0} - \frac{[P]^2}{2[S]_0^2}, \quad \text{herein,} \quad \frac{[P]}{[S]_0} < 1.$$

Eq. (6) was rearranged as follows:

$$\frac{t}{[P]} = \frac{1}{V_{max}}\left\{ \left(\frac{K_m}{[S]_0} + 1\right) + \left(\frac{K_m}{2[S]_0^2} + \frac{K_m}{2K_i[S]_0} + \frac{1}{2K_i'}\right)[P] \right\}. \quad (7)$$

If our approach is applicable to this system, the plots of $t/[P]$ against [P] should give straight lines according to Eq. (7). As shown in Fig. 3, the plots derived from the experimental data of Fig. 1A demonstrated a linear relation at all initial substrate concentrations. To determine the kinetic parameters, secondary plots with respect to the ordinate intercepts (α) and the slopes (β) of respective lines in Fig. 3 were performed. Fig. 4 illustrates the replots of α against $1/[S]_0$. The curve is linear and should obey the following equation:

$$\alpha = \frac{1}{V_{max}} + \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]_0}. \quad (8)$$

The V_{max} and K_m values were obtained from the ordinate intercept and the slope of the straight line according to Eq. (8) in Fig. 4. Similarly Fig. 5 depicts the replots of

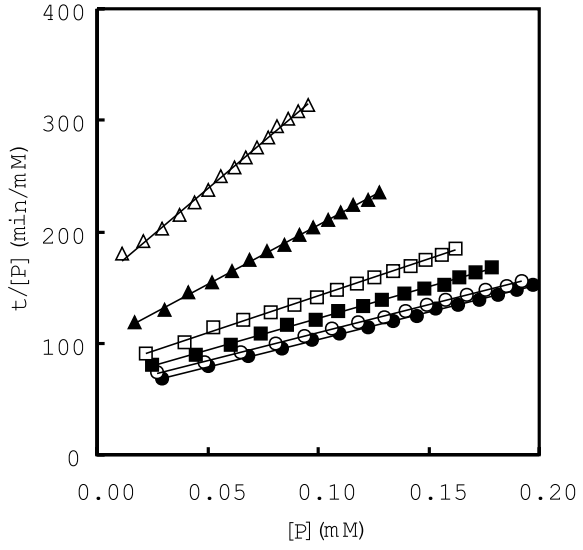

Fig. 3. Plots of $t/[P]$ against [P] transformed by linear regression from velocity equation in the presence of L-methionine. The substrate concentrations were 50 mM (●), 25 mM (○), 10 mM (■), 5 mM (□), 2 mM (▲), and 1 mM (△).

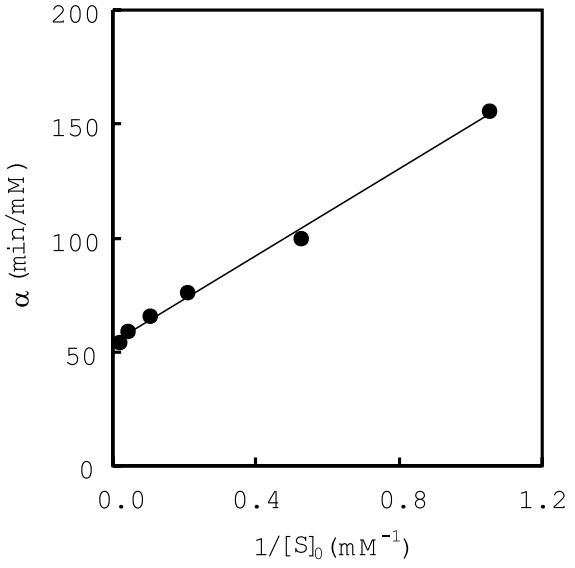

Fig. 4. Secondary plots of α against $1/[S]_0$ transformed by linear regression from velocity equation in the presence of L-methionine. α is the ordinate intercept of the straight line of $t/[P]$ against [P] in Fig. 3.

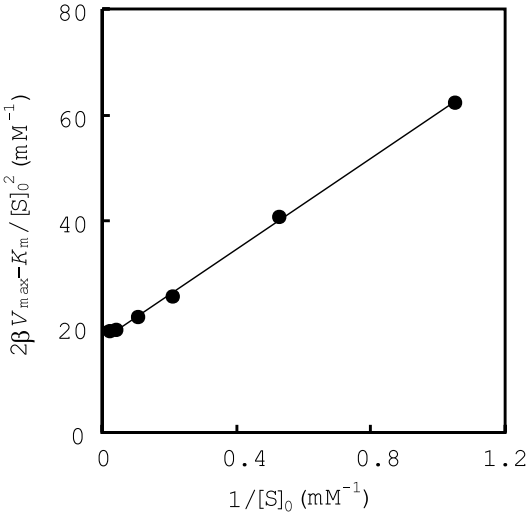

Fig. 5. Secondary plots of $(2\beta V_{max} - K_m/[S]_0^2)$ against $1/[S]_0$ transformed by linear regression from velocity equation in the presence of L-methionine. β is the slope of the straight line of $t/[P]$ against [P] in Fig. 3.

$(2\beta V_{max} - K_m/[S]_0^2)$ against $1/[S]_0$. This curve is also linear and is represented by the following equation:

$$2\beta V_{max} - \frac{K_m}{[S]_0^2} = \frac{1}{K_i'} + \left(\frac{K_m}{K_i}\right)\frac{1}{[S]_0}. \quad (9)$$

The K_i and K_i' values were calculated from the ordinate intercept and the slope of the straight line according to Eq. (9) in Fig. 5. In this way, the kinetic parameters were determined by the progress curve analysis according to the velocity equation on the basis of the enzyme reaction model.

The kinetic parameters for L-ethionine were also determined in the same manner. In the case of L-homoserine as a substrate, we considered k_{-2} in Fig. 2 as zero, because γ-replacement apparently did not occur as shown in Fig. 1. As a result, the kinetic parameters for L-homoserine were obtained in the same manner as those for L-methionine or from ordinary double reciprocal plots. The kinetic parameters that we determined are summarized in Table 1. The significance of γ-replacement was theoretically and practically elucidated. The respective curves in Fig. 1 are simulated from the determined parameters, which well represent the experimental data. The results of kinetic studies demonstrate our approach to be reliable and valid.

Table 1
Kinetic paremeters of recombinant METase determined from progress curve analysis on the basis of enzyme reaction model

Substrate	V_{max} (μmol/min/mg)	K_m (mM)	K_i (mM)	K_i' (mM)
L-Methionine	57	1.7	0.05	0.07
L-Ethionine	34	0.2	0.07	0.09
L-Homoserine	1.4	30	—	—

There has been no previous report on comprehensive kinetic analysis of α,γ-elimination and γ-replacement by METase. Elucidation of complete enzyme kinetics can help to characterize the reaction mechanisms. This approach to kinetic analysis will be useful for investigating the kinetic properties of other versatile PLP-dependent enzymes as well as METase.

Definition of enzyme activity for whole reaction

The key obstacle to the quantification of METase activity comes from the γ-replacement of L-methionine. Both K_i and K_i' values that we determined were markedly low. Therefore, it was difficult to measure the initial velocities of α-ketobutyrate production on zero-order reaction in the presence of L-methionine as was expected, even when the substrate concentration was much higher than the K_m value. We concluded that the initial velocity of α-ketobutyrate production could not be used to accurately represent METase activity.

To overcome this disadvantage, we defined the enzyme activity as the V_{max} value calculated in the presence of L-methionine at 25 mM on the basis of Eq. (6). Determination of METase activity by this method is shown in Fig. 6, in comparison with the conventional initial velocity. The V_{max} values remained constant over a wide range of ΔE while the initial velocities showed lower values and decreased with increasing ΔE. The specific activity of purified recombinant METase represented as the V_{max} value was 57 U/mg under the experimental conditions used.

Our proposed assay system is superior to conventional assay systems from the viewpoint of accuracy and does not require sophisticated equipment, cumbersome prep-

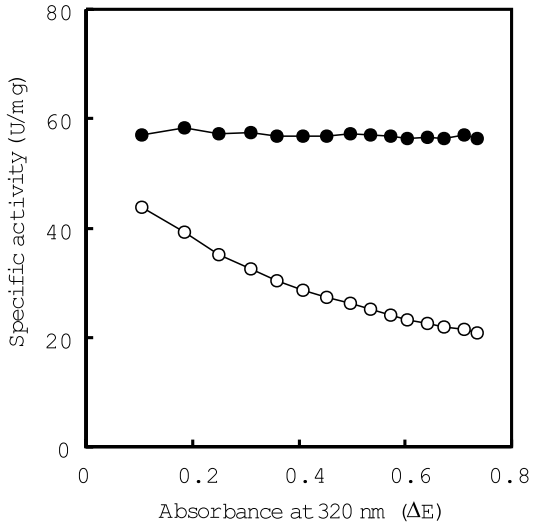

Fig. 6. Comparison of the specific activities of recombinant METase between the V_{max} values (●) and the conventional initial velocities (○) in the presence of 25 mM L-methionine.

aration, or major change of previous assay procedures. It allowed reliable quality and quantity determination of METase. This simple, accurate, and standardized analytical enzyme assay method should serve as a powerful tool for facilitating development of METase applications. Moreover, the novel representation which defines the enzyme activity as the V_{max} value instead of initial velocity can be readily adapted to quantification of other multifunctional PLP-dependent enzymes.

Acknowledgments

The authors are indebted to Mr. Kensaku Akita, Mr. Kobee Kobayashi, and Dr. Shintaro Misaki for their valuable suggestions.

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