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Methionine γ-lyase: Mechanistic deductions from the kinetic pH-effects The role of the ionic state of a substrate in the enzymatic activity

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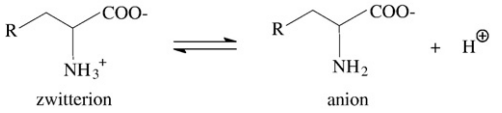
ABSTRACT

We have studied and compared the pH-dependencies of the main kinetic parameters for the α,γ-elimination reactions of methionine γ-lyase (MGL) of *Citrobacter intermedius* with natural substrate, L-methionine, with its phosphinic analogue, and for α,β-elimination reaction with S-methyl-L-cysteine. From the pH-dependency of k_{cat}/K_m for the reaction with L-methionine we have concluded that MGL is selective with respect to the zwitterionic form of its natural substrate. For the reaction of MGL with 1-amino-3-methylthiopropylphosphinic acid the pK_a of the substrate's amino group, equal to 7.55, is not reflected in the pH-profile of k_{cat}/K_m. Consequently, the enzyme does not manifest well-defined selectivity with respect to the zwitterion and anion ionic forms of the substrate. The ascending limbs of pH-dependencies of k_{cat}/K_m for reactions with L-methionine and S-methyl-L-cysteine are controlled by a single pK_a equal to 7.1–7.2, while for the reaction with 1-amino-3-methylthiopropylphosphinic acid two equal pK_as of 6.2 were found in the respective pH-profile. The descending limbs of pH-dependencies of k_{cat}/K_m for the reactions with S-methyl-L-cysteine and racemic 1-amino-3-methylthiopropylphosphinic acid are very similar and are controlled by two acidic groups having average pK_a values of 8.7. On the basis of these results we suggest a mechanism of catalytic action of MGL. According to this mechanism Tyr 113, in its conjugated base form, acts as an acceptor of the proton from the amino group of the substrate upon its binding in the active site. Elimination of the leaving thiol groups during both α,γ- and α,β-elimination reactions is assisted by the acidic groups of Tyr 113 and Tyr 58. Both tyrosyl residues are able to fulfill this catalytic function with different efficiencies depending on the type of elimination reaction. Tyr 113 residue plays the determining role in the α,γ-elimination, and Tyr 58 — in the α,β-elimination process.

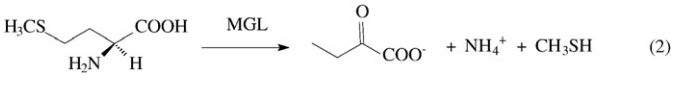
1. Introduction

Pyridoxal-5′-phosphate (PLP)-dependent enzymes are responsible for numerous metabolic transformations of amino acids in nature. In the mechanisms of such reactions an important role is played by the transfers of protons between definite functional groups of substrates and catalytic groups of enzymes in the active sites. In this connection the question of the ionic state in which the amino acid substrate binds with the enzyme becomes a principal one. Although the transfer of a proton from the amino group of the substrate to a certain functional group in the active site was postulated as one of the key stages in enzyme mechanisms [1–3] the specificity of enzymes with respect to a certain ionic state of the substrate was not systematically examined until now. This, probably, is mainly due to the fact that at physiological pH-values amino acids usually exist predominantly in the zwitterion form Eq. (1), and kinetic studies at high pH-values may be complicated by a tendency of enzymes to denaturation which interferes with the interpretation of the results.



(1)

The PLP-dependent methionine γ-lyase (MGL; EC 4.4.1.11) is an enzyme of L-methionine catabolism that catalyzes the decomposition of L-methionine to methanethiol, α-ketobutyrate and ammonia Eq. (2).



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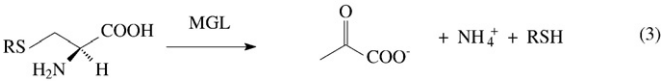

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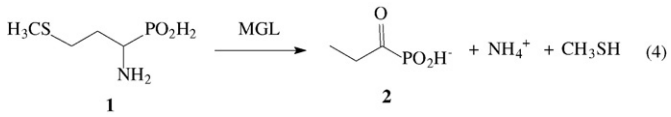
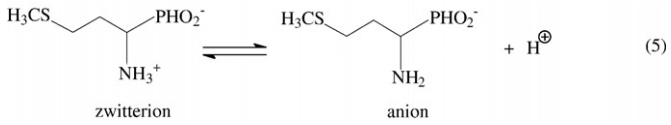
MGL is able also to catalyze α,β-elimination of S-substituted cysteines to respective alkylthiols, pyruvate and ammonia Eq. (3), as well as γ- and β-replacement reactions of L-methionine and L-cysteine derivatives respectively [4,5]. This enzyme has been isolated from a number of sources [6–10]. For some MGLs gene encoding and cloning [11,12] and site-directed mutagenesis studies [13] were reported, and crystal structures have been determined at good resolution [14–16]. However, the pH-dependencies of the main kinetic parameters for the reactions of MGL with characteristic substrates were not studied. Alferov et al. [17] have shown recently that MGL of *Citrobacter intermedius* displays a considerable activity with respect to 1-amino-3-methylthiopropylphosphinic acid **1**, the phosphinic analogue of methionine, catalyzing its α,γ-elimination Eq. (4), which is analogous to the reaction with the natural substrate.



It is known that for phosphinic analogues of amino acids the pK_a values of amino groups are considerably less than that for the normal amino acids [18]. This creates a good opportunity to study the effect of amino group dissociation on the enzymatic activity because a drastic change in the ratio of the ionic forms of the substrate (see Eq. 5) should be accomplished in the range of physiological pH-values.



In the present work we compared the pH-dependencies of k_{cat} and k_{cat}/K_m for the α,γ-elimination reactions of MGL with natural substrate, L-methionine, with its phosphinic analogue 1, and for α,β-elimination reaction with S-methyl-L-cysteine. Some preliminary results for the reaction of phosphinic analogue of methionine were reported in [19]. In the present work we reconsidered these results in more detail having obtained more experimental data in a wider range of pH. We have found that strict enzyme selectivity with respect to the ionic state of the substrate is not a general rule, at least for MGL reactions, and in certain cases both the zwitterion and anion forms of the substrate can be efficiently bound and catalytically transformed by the enzyme.

2. Materials and methods

The recombinant MGL was obtained from *Escherichia coli* BL 21 (DE3) cells containing pET-mgl plasmid with the inserted *megL* gene from *Citrobacter freundii* cells. Growing of the cells and isolation of the enzyme was carried out as described by Manukhov et al. [12]. Activity of the enzyme preparation was assayed by measuring the rate of α-ketobutyrate formation from L-methionine by the method of Friedemann and Haugen [20]. One unit of enzymic activity was determined as the amount of enzyme catalyzing transformation of 1 μmol of L-methionine per min at 30 °C and concentration of L-methionine 40 mM.

Racemic 1-amino-3-methylthiopropylphosphinic (**1**) was synthesized as described earlier [21]. The value of pK_a for the amino group of **1** was determined as described for other phosphinic amino acids in [18].

2.1. Isolation and identification of 1-oxopropylphosphinic acid (**2**)

1-Oxopropylphosphinic acid (**2**), the product of the enzymic reaction, was identified as its 2,4-dinitrophenylhydrazone. The quantitative determination of **2** was carried out in accordance with the general method of Friedemann and Haugen [20]. The calibration curve was constructed using the authentic **2**; for its 2,4-dinitropenylhydrazone $\varepsilon_{540} = 6300\ M^{-1}cm^{-1}$ was found in 1.6 M NaOH.

2.2. Kinetic measurements

To study the dependencies of the main kinetic parameters of MGL catalyzed reactions we used 33 mM potassium phosphate or 0.1 M potassium borate buffer solutions. Ionic strength was adjusted to 0.1 M by adding KCL.

To determine the initial rates of γ-elimination reactions with L-methionine and with analogue **1** reaction mixtures containing definite concentrations of the substrates and enzyme and 0.1 mM PLP in a total volume of 1 ml were placed in 50 ml flasks, the surface area of the solution being equal to 17–18 cm^2. Flasks were stoppered and incubated with reciprocal stirring at 30 °C for definite times. The reactions were stopped by adding 0.1 ml of 30% trichloroacetic acid, the denatured enzyme was separated by centrifugation and the concentrations of keto-products formed were determined.

The rates of β-elimination reaction of MGL with S-methyl-L-cysteine were measured using a coupled assay with lactate dehydrogenase and NADH measured at 340 nm as described for tryptophanase [22].

2.3. Data analysis and definitions used

Kinetic values of k_{cat} and K_m were obtained by fitting the data (initial velocity vs substrate concentration) to the Michaelis–Menten equation using a nonlinear least-square program (ENZFITTER). To fit the pH-dependencies of k_{cat}/K_m and k_{cat} we used the FORTRAN programs of Cleland [23] adapted to run on IBM-compatible personal computers. In the resulting equations the pK_a and pK_b definitions were used for pK_a values reflected in the ascending and descending branches of the dependencies respectively, and the pK_A term was used to denote the pK_a of the amino group of a substrate.

2.4. Molecular modeling

The atomic coordinates of the MGL complexed with L-vinylglycine (Nikulin et al., unpublished) and *Trichomonas vaginalis* MGL in the complex with propargylglycine (PDB 1E5E) were used to model the external aldimines of the enzyme with L-methionine and S-methyl-L-cysteine by the programs "O" and "PRODRG".

3. Results and discussion

Alferov et al. [17] have shown earlier that the γ-substitution reaction between **1** and benzylthiol, catalyzed by MGL, is stereospecific with respect to the (R)-configuration of **1**, corresponding to the configuration of L-methionine. In our preliminary studies [19] we compared the α,γ-elimination reactions of MGL with the racemic **1** and with (R)-enantiomer of **1**, and found that the interaction of the enzyme with non-reacting (S)-**I**, if any, does not seriously interfere with its reaction with the (R)-enantiomer. Bearing this in mind, we used the racemic **1** in further kinetic experiments.

Takakura et al. [24] have demonstrated that MGL displays very high affinity to the reaction product, methanethiol, and called in question the possibility of using the method of initial rates for kinetic studies. They suggested, instead, to carry out the reactions in a closed system, preventing the evaporation of methanethiol