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Kinetic and Spectral Parameters of Interaction of *Citrobacter freundii* Methionine γ-Lyase with Amino Acids

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Abstract—Kinetic parameters of *Citrobacter freundii* methionine γ-lyase were determined with substrates in γ-elimination reactions as well as the inhibition of the enzyme in the γ-elimination of L-methionine by amino acids with different structure. The data indicate an important contribution of the sulfur atom and methylene groups to the efficiency of binding of substrates and inhibitors. The rate constants of the enzyme-catalyzed exchange of C-α- and C-β-protons with deuterium were determined, as well as the kinetic isotope effect of the deuterium label in the C-α-position of inhibitors on the rate of exchange of their β-protons. Neither stereoselectivity in the β-proton exchange nor noticeable α-isotope effect on the exchange rates of β-protons was found. The ionic and tautomeric composition of the external Schiff base of methionine γ-lyase was determined. Spectral characteristics (absorption and circular dichroism spectra) of complexes with substrates and inhibitors were determined. The spectral and kinetic data indicate that deamination of aminocrotonate should be the rate-determining stage of the enzymatic reaction.

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Key words: *Citrobacter freundii* methionine γ-lyase, pyridoxal 5′-phosphate, substrates, inhibitors, proton exchange, spectral characteristics

Methionine γ-lyase (MGL, EC 4.4.1.11) is a pyridoxal 5′-phosphate-dependent enzyme catalyzing reactions of γ-elimination of L-methionine and β-elimination of L-cysteine and its S-substituted derivatives, as well as reactions of γ- and β-substitution of L-methionine, L-cysteine, and their analogs [1]. The presence of the enzyme in pathogenic bacteria and unicellular eukaryotes [2] and its absence in human and animal cells seem promising for creation of new anti-pathogenic drugs with MGL as the biochemical target. The substrate analog 3-fluoro-L-methionine effectively inhibited the growth of *Trichomonas vaginalis* [3], and in the work [4] suppression by myrsinoic acid of the growth of some periodontal parasites was associated with inhibition of methionine γ-lyase. Spatial structures have been determined for enzymes from *Pseudomonas putida* [5], *Entamoeba histolytica* [6], and *Citrobacter freundii* [7]. The homotetramer MGL with molecular weight of ~170 kDa contains four coenzyme molecules and consists of two so-called catalytic dimers in each of which the catalytic site of the subunit is formed by amino acid residues from both subunits. The chemical mechanism of the enzymatic reaction of γ-elimination supposed in 1990 is presented in the Scheme [8]. The reaction is determined by the involvement of basic group (or groups) at the stages of abstraction of C-α- and C-β-protons from the substrate and acidic catalysis at the stage of methylmercaptan elimination. It is suggested [9] that stages of the C-α-proton abstraction and its transfer to the C4′-atom of the coenzyme and the C-β-proton abstraction are realized by the side amino group of the active site lysine residue that forms in the holoenzyme the so-called internal aldimine with the aldehyde group

Abbreviations: MGL, methionine γ-lyase.
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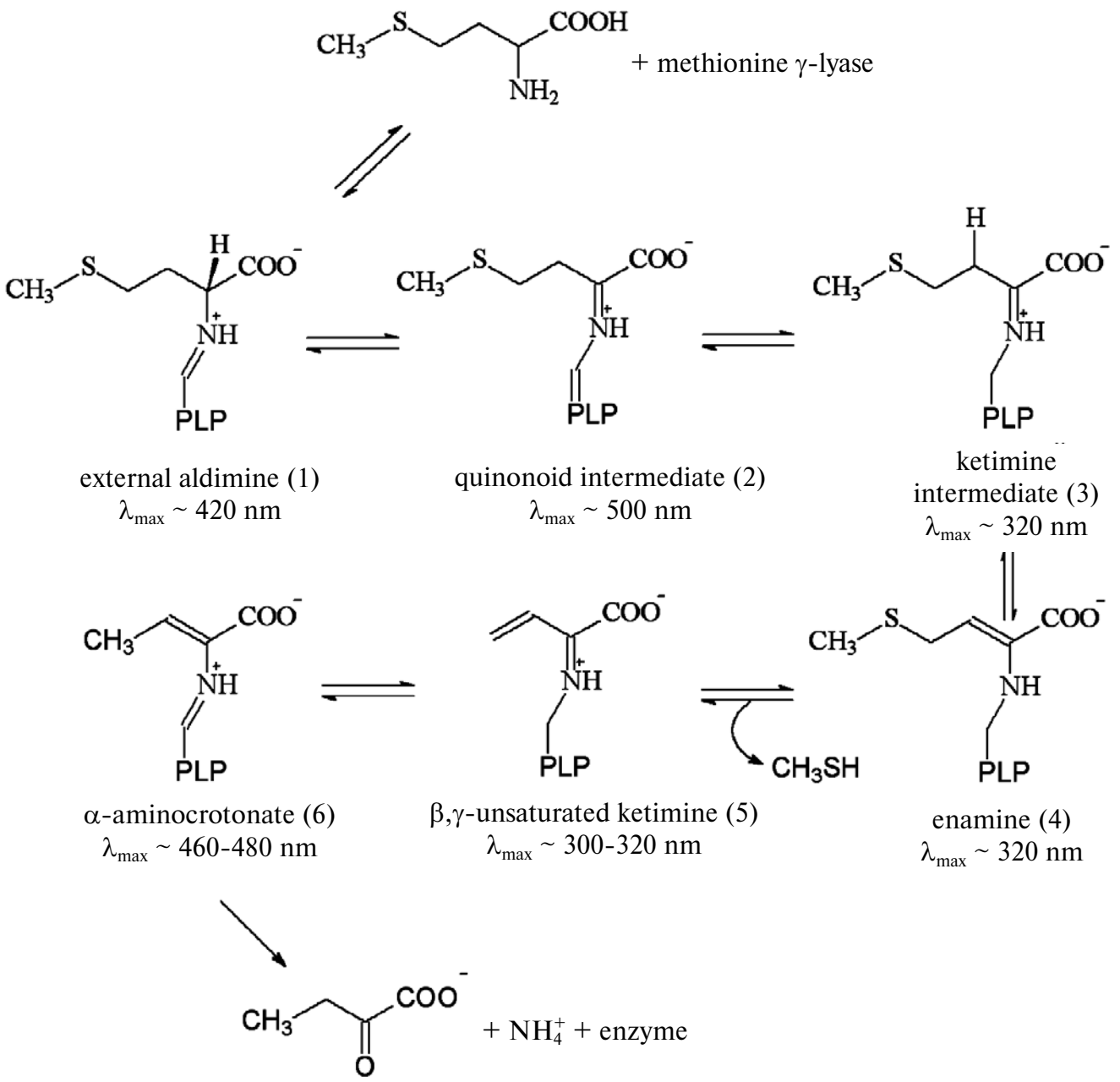

Mechanism of γ-elimination of L-methionine

of pyridoxal 5′-phosphate. Due to presence of the coenzyme, pyridoxal 5′-phosphate-dependent enzymes possess unique spectral features, which allow individual intermediates of the enzymatic reaction to be identified. The Scheme presents major absorption bands of intermediates of the γ-elimination reaction [10]. However, up to now kinetic and spectral data for the pyridoxal 5′-phosphate-dependent enzymatic γ-elimination have been very scarce. In this work, spectral and kinetic parameters of the interaction of MGL with some amino acids are presented.

MATERIALS AND METHODS

Reagents and materials. The following reagents were used: L-methionine, L-norvaline, L-norleucine, L-α-aminobutyric acid, glycine, L-alanine, DL-homoserine, L-methionine sulfone, L-methionine sulfoxide, dinitrophenylhydrazine, phenylmethylsulfonyl fluoride, lactate dehydrogenase from rabbit muscle, dithiothreitol (DTT), and NADH from Sigma (USA); pyridoxal 5′-phosphate from Merck (Germany); EDTA and protamine sulfate from Serva (USA); lactose from Panreac (Spain); glucose,

glycerol, magnesium sulfate, ammonium sulfate, potassium phosphate monobasic, sodium phosphate bibasic, acetic acid, acetic anhydride, triethanolamine, and $HClO_4$ from Reakhim (Russia); yeast extract and tryptone from Difco (USA); DEAE-cellulose from Whatman (England); Superdex 200 from Amersham Biosciences (Sweden); *O*-acetyl-DL-homoserine was prepared by acetylation of DL-homoserine as described earlier [11].

Preparation of bacterial mass and purification of the enzyme. *Escherichia coli* BL21(DE3) cells containing the gene of methionine γ-lyase from *C. freundii* in the plasmid pET-mgl [12] were grown on an "auto-inducing" medium [13] at 37°C with stirring at 180 rpm for 24 h. The cells were collected by centrifugation and stored at –80°C.

The enzyme was isolated as described earlier in [14]. The protein concentration during the purification was determined by the Lowry method [15]. The MGL activity during the purification was determined using S-ethyl-L-cysteine as a substrate by measuring the rate of pyruvate production in the coupled reaction with lactate dehydrogenase by decrease in the NADH absorption at 340 nm ($\Delta\varepsilon = 6220\ M^{-1}\cdot cm^{-1}$) at 30°C. The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 0.1 mM pyridoxal 5′-phosphate, 5 mM DTT, 0.2 mM NADH, 10 U lactate dehydrogenase, and 2.5 mM S-ethyl-L-cysteine. The enzyme amount catalyzing production of 1 μmol/min pyruvate was taken as the enzyme activity unit. Concentration of preparations of purified enzyme was determined by absorption at 278 nm. The absorption coefficient $A^{0.1\%}_{1cm}$ calculated based on determinations of the concentrations of the preparations by the Lowry method [15] and the corresponding absorption values of these preparations at 278 nm was 0.8. The purity of the resulting preparations was tested using SDS-PAGE by the Laemmli method [16]. Enzyme preparations with 95% purity had specific activity of 12 U/mg.

Determination of pyridoxal 5′-phosphate content. The pyridoxal 5′-phosphate contents in the enzyme preparations were determined in 0.1 M NaOH taking $6600\ M^{-1}\cdot cm^{-1}$ [17] as the molar absorption coefficient of pyridoxal 5′-phosphate at 390 nm.

Preparation of 2-[^{2}H]-amino acids. Amino acids were prepared enzymatically from corresponding non-deuterated analogs using tryptophane-indole lyase by the method described in [18].

Kinetic studies. On determination of kinetic parameters of γ-elimination, the reaction mixtures contained 100 mM potassium phosphate buffer (pH 8.0), 0.1 mM pyridoxal 5′-phosphate, 1 mM EDTA, 5 mM DTT, and varied amounts of the substrates. The rate of the enzymatic reaction was determined using dinitrophenylhydrazine by the rate of α-ketobutyrate production [19]. The reaction was initiated by addition of the enzyme (1-5 μg). The mixture was incubated for 15 min at 30°C, and the reaction was stopped by addition of trichloroacetic acid to the final concentration of 12.5% (w/v).

Kinetic parameters were obtained by processing the data according to the Michaelis–Menten equation using the EnzFitter program. In the calculations the molecular weight of the enzyme subunit was taken as 43 kDa.

Inhibition of γ-elimination of L-methionine by different amino acids was studied under the above-described conditions. Values of the inhibition constants were determined using the EnzFitter program. The data were processed in Dixon's coordinates [20].

Spectral studies. Absorption spectra of the holoenzyme and its complexes with substrates and inhibitors were recorded at 25°C with a Cary-50 spectrophotometer (Varian, USA). Circular dichroism spectra were recorded with a Mark III dichrograph (Jobin-Yvon, France) and a portable SKD-2 dichrometer (Institute of Spectroscopy, Russian Academy of Sciences, Troitsk, Moscow Region). The enzyme concentration was 1-2 mg/ml. The spectra were recorded in 100 mM potassium phosphate buffer (pH 8.0).

^{1}H-NMR studies. Kinetics of MGL-catalyzed substitutions of C-α- and C-β-protons of inhibitors by deuterium were studied by ^{1}H-NMR spectroscopy. The reaction mixture contained 0.05 M potassium phosphate buffer (pH 7.6) and 2 mg/ml of the corresponding inhibitor in 0.5 ml of D_2O. The reaction was initiated by addition of 0.01 ml of the enzyme solution (0.5 mg/ml) at 37°C. ^{1}H-NMR spectra were recorded using a Bruker AMXIII-400 spectrometer with operating frequency of 400 MHz. Series of spectra were recorded at certain time intervals using a special automation program. Signals of α- and β-protons were integrated using the modified "enzkin" automation program, which is a part of the XWIN-NMR programs. Kinetic curves of accumulation of deuterated products were calculated using the method described in paper [21].

RESULTS AND DISCUSSION

Interaction of MGL with substrates and inhibitors. In this work we have determined kinetic parameters of the interaction of the enzyme with three substrates of γ-elimination that contain easily leaving groups: L-methionine sulfone, L-methionine sulfoxide, and *O*-acetyl-DL-homoserine. Table 1 presents these parameters together with data obtained earlier for the natural substrate and some of its analogs. Comparison of K_m values has shown, first, that the sulfur atom contributes significantly to the effective binding of the substrates and that its oxidation to sulfonic or sulfoxide group markedly worsens the binding of the corresponding analogs of methionine; second, that k_{cat} values for substrates not requiring catalysis at the stage of elimination of the substituent in the γ-position and also for L-vinylglycine lacking this stage are lower than the k_{cat} values for L-methionine and DL-homocysteine. Therefore, it was suggested that the elimination of a γ-substituent should not be a rate-limiting stage of the enzymatic reaction.

Table 1. Kinetic parameters of γ-elimination by MGL of *C. freundii*

Substrate	MGL of *C. freundii*	
	k_{cat}, sec^{-1}	K_m, mM
L-Methionine	6.2 ± 0.42*	0.7 ± 0.11*
DL-Homocysteine	8.51 ± 0.41*	0.97 ± 0.15*
DL-Homoserine	0.52 ± 0.017*	56.5 ± 6.5*
O-Acetyl-DL-homoserine	2.1 ± 0.053	5.82 ± 0.36
L-Methionine sulfone	2.09 ± 0.14	4.02 ± 0.59
L-Methionine sulfoxide	2.52 ± 0.002	6.21 ± 0.84
L-Vinylglycine	1.9 ± 0.043*	6.7 ± 0.66*

* Data from work [14].

The production of external aldimine (Scheme, structure 1) is the common stage for various reactions (β,γ-elimination, β,γ-substitution, transamination, racemization, α-decarboxylation, aldol cleavage) catalyzed by pyridoxal 5′-phosphate-dependent enzymes; therefore, amino acids are usually competitive inhibitors of these enzymes, and studies of their interactions with the enzymes can reveal regularities responsible for the binding efficiency in pyridoxal 5′-phosphate-dependent catalysis and investigate chemical and kinetic characteristics of stages of the enzymatic reactions. We have studied inhibition of γ-elimination of methionine by some amino acids: glycine, L-alanine, L-α-aminobutyric acid, L-norvaline, and L-norleucine. All these amino acids were shown to be competitive inhibitors of MGL.

The obtained constants of competitive inhibition are presented in Table 2. X-Ray crystallographic analysis of MGL [5, 7] has confirmed that the active site of the enzyme contains many hydrophobic residues. Correspondingly, for amino acids with a linear side chain (L-α-aminobutyric acid, L-norvaline, L-norleucine) the binding is improved along with an increase in the number of methylene groups in the amino acids. Such feature has been observed for the enzyme from *P. putida* [22].

Exchange kinetics of α- and β-protons of competitive inhibitors of MGL. Along with its action on substrates or competitive inhibitors in D_2O, MGL can catalyze the isotope exchange of their α- and β-protons with deuterons. This finding is in agreement with the above-described action mechanism of MGL (Scheme) suggesting a reversible dissociation of α- and β-protons of amino acids bound in the active site (during the enzymatic reaction) at certain catalytic stages. According to data for MGL of *P. putida* [22], in the case of glycine the isotope exchange concerns that of two enantiotopic α-protons, which have a pro-(R)-configuration. In the same work it was qualitatively demonstrated that in some amino acids with a linear side chain α-proton and both β-protons are subjects of the exchange, whereas the exchange rates of β-protons in S-methyl-L-cysteine were different. It was also shown [23] that, using mutant *P. putida* MGL, in addition to α-proton only one of β-protons is exchanged. Therefore, it was concluded that for the wild type *P. putida* MGL the stereoselectivity of the isotope exchange of two diastereotopic β-protons was much lower than the exchange stereoselectivity of α-protons of glycine. Nevertheless, the question about the true stereoselectivity (or its full absence) during the exchange of β-protons remained unclear because integral curves of the isotope exchange were not analyzed in detail. In the present work we have analyzed integral curves of the isotope exchange of both α- and β-protons catalyzed by the wild type *C. freundii* MGL for some amino acids with a linear side chain. The exchange of α-protons was analyzed on examples of both usual amino acids and their mono-deuterated analogs prepared enzymatically. This allowed us to compare the findings and to assess the kinetic effect of the

Table 2. Kinetic parameters of isotope exchange of α- and β-protons of L-amino acids

Amino acid	K_i ($K_m = K_p$), mM	k_{ex}, sec^{-1}		Number of exchanged α- and β-protons
		α-H	β-H	
Glycine	49	20.2	–	1; –
L-Alanine	3.4	2.71	2.63	1; 3
L-α-Aminobutyric acid	8.3	4.52	3.04	1; 2
L-Norvaline	4.7	20.6	11.1	1; 2
L-Norleucine	0.6	41.8	4.74	1; 2
2-[^{2}H]-L-alanine	3.4	–	1.84	–; 2
2-[^{2}H]-L-α-aminobutyric acid	8.3	–	3.04	– ; 2
2-[^{2}H]-L-norvaline	4.7	–	11.7	–; 2
2-[^{2}H]-L-norleucine	0.6	–	3.26	–; 2

introduction of deuterium atom into the α-position on the exchange of β-protons and also to exclude the influence of varying deuterium contents in the α-position on the rate of β-proton exchange during the reaction. We have established that in all cases under consideration during the exchange of both α- and β-protons the parameter $\ln([S]_0/([S]_0 - [P])$ (where $[S]_0$ is the initial concentration of the amino acid and [P] is the concentration of the isotope exchange product) changes proportionally with the reaction time up to 80-85% of the exchange. Characteristic dependences are shown in Fig. 1. Thus, we conclude that for the isotope exchange reactions under study, which occur under conditions of inhibition by the product, the ratio is realized:

$$K_m = K_p, \quad (1)$$

where K_m is the Michaelis constant and K_p is the inhibition constant by the product, which characterizes the enzyme binding with the product of the isotope exchange.

Table 2 presents values of kinetic parameters for isotope exchange reactions calculated from slopes of the corresponding time dependences [21] according to the equation:

$$\ln([S]_0/([S]_0 - [P])) = k_{cat}[E]_0 t/K_m(1 + ([S]_0/K_p)). \quad (2)$$

The findings do not show a noticeable stereoselectivity during the exchange of β-protons for the amino acids under study. This finding seems to be rather unexpected considering chiral features of the active site and a limited free rotation around the C_α–C_β bond in structure 4 (Scheme), which is crucial during the exchange of β-protons.

Corresponding to the supposed action mechanism of MGL, the necessary and sufficient condition for α-proton isotope exchange is the reversible production of a quinonoid intermediate (Scheme, structure 2), which suggests deprotonation of the external aldimine (Scheme, structure 1), the isotope exchange in the acceptor group, and the subsequent attachment of the deuteron to the C-α atom. During such an exchange mechanism β-protons should not be affected. On the other hand, to provide for the mobility of β-protons, the generation of structure 3 suggesting deprotonation of the C-α atom and protonation of the C4′ atom must occur. This process is also reversible and can occur either stepwise (with production of a quinonoid intermediate) or in concerted manner in one stage. Because the acidic–basic group(s) involved in the above-mentioned interconversion of structures 1 and 3 can realize the isotope exchange with the solvent, the exchange of β-protons must be inevitably accompanied by the exchange of α-proton. Table 2 shows that the exchange rates of α- and β-protons are close in the case of L-α-aminobutyric acid and virtually equal in the case of L-alanine. It seems that the isotope exchange of α-proton in the case of these amino acids is essentially associated with interconversion of structures 1 and 3. During the transition to L-norvaline and L-norleucine, the elongation of the side chain is accompanied by acceleration of the α-proton exchange as compared to β-protons, and this seems to indicate an increase in the contribution of the simplest mechanism of the α-proton exchange (exclusively via the quinonoid intermediate) not affecting β-protons.

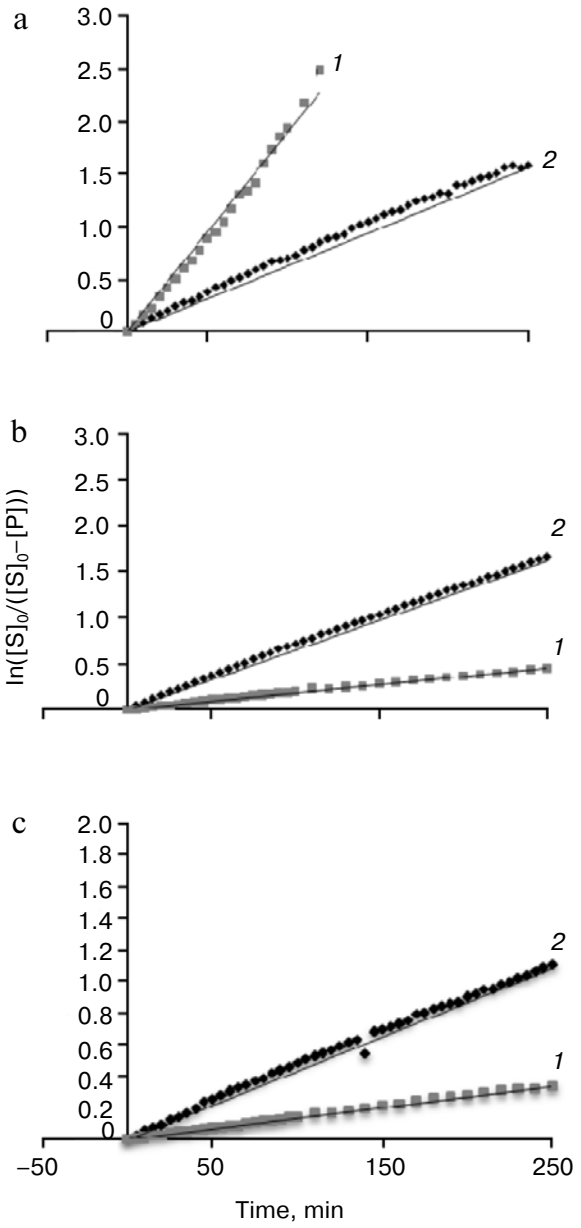

Fig. 1. Time dependence of changes in the parameter $\ln([S]_0/([S]_0 - [P]))$ for L-alanine and L-norleucine. Curves: *1*) calculated for C-α- and C-β-protons of L-alanine and for β-protons of 2-[^{2}H]-L-alanine (a-c, respectively); *2*) calculated for α- and β-protons of L-norleucine and for β-protons of 2-[^{2}H]-L-norleucine (a-c, respectively). The amino acid and protein concentrations in the sample were 2 and 0.01 mg/ml, respectively.

Values of kinetic isotope effects caused by the substitution of α-proton by deuteron on the exchange rates of β-protons are not high (1.43-1.45), and the effects are absent in the case of L-α-aminobutyric acid and L-norvaline. Thus, the contribution of stages suggesting the α-proton detachment to the total energy barrier of the β-proton exchange is not determining, although it is noticeable in the case of L-alanine and L-norleucine.

Spectral properties of the holoenzyme. The absorption spectrum of the MGL holoenzyme at pH 8.0 in the region of 300-500 nm has appearance typical for that of pyridoxal 5′-phosphate aldimines with amino acids, with a specific major band at 420 nm.

To determine the ionization state of the internal aldimine of MGL necessary for understanding mechanisms of the enzymatic reaction, the absorption and circular dichroism spectra recorded at pH 8.0 were resolved into bands corresponding to individual electron transitions of pyridoxal 5′-phosphate aldimines with amino acids. To describe the absorption and circular dichroism bands, logarithmically normal functions and parameters were used of bands of tautomers and conformers of two ionic forms of model aldimines, cationic and neutral, with the transition p*K* between them no more than 7 [24]. The method has been described in detail earlier [25].

The correctness of the model used for description of the resolved spectrum was mainly assessed by correlation of the number of tautomers and conformers to the amount of pyridoxal 5′-phosphate bound in the enzyme. The coincidence or closeness of parameters of the experimental absorption bands with parameters of the corresponding absorption bands for model aldimines was used as another test. Data obtained by resolution using the two models were analyzed, and in the cationic model the amount of pyridoxal 5′-phosphate bound in the enzyme was in correlation with the total amount of tautomers and conformers ($\Sigma C_i = 1.00 \pm 0.02$). For the neutral model there was a pronounced discrepancy ($\Sigma C_i = 1.35 \pm 0.04$).

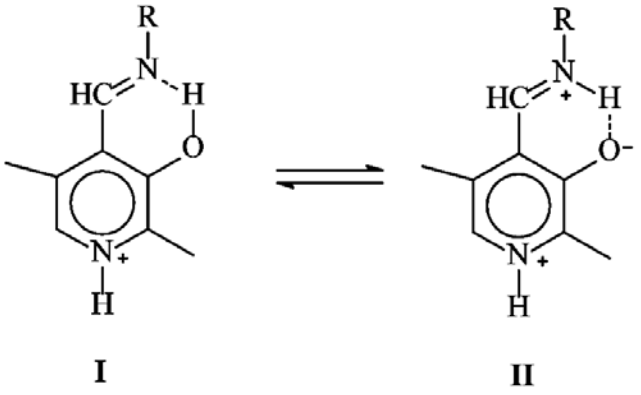

Fig. 2. Equilibrium of tautomers of the internal aldimine cationic form of *C. freundii* MGL.

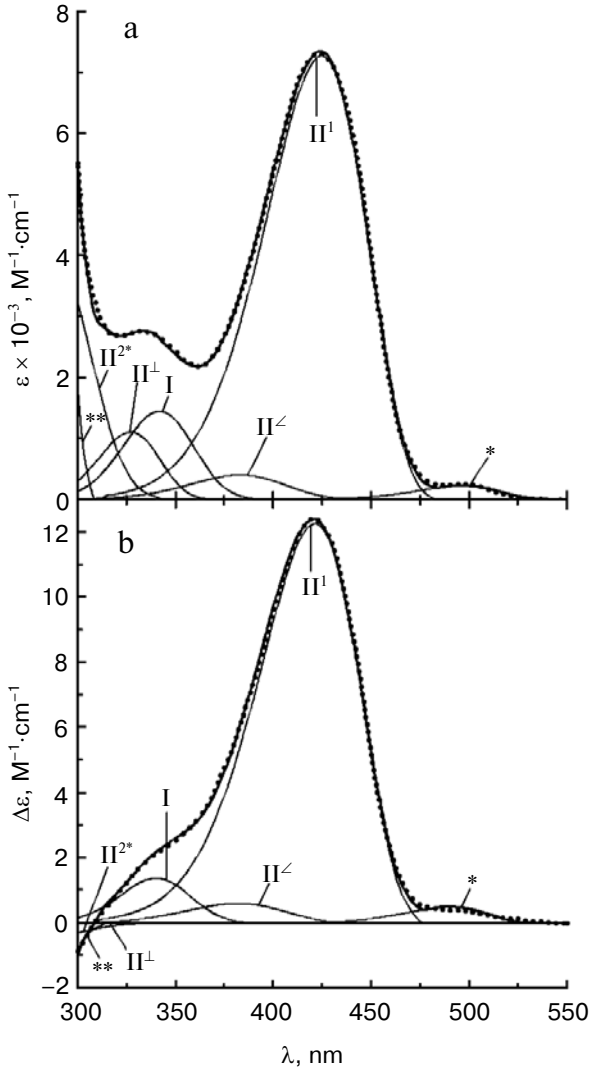

Fig. 3. Resolution of spectra of absorption (a) and of circular dichroism (b) of the internal aldimine of MGL (pH 8.0) onto bands of individual electron transitions of its tautomers and conformers; points present experimental data, bold lines are calculated data, and thin lines present bands corresponding to individual electron transitions. The structures are labeled as in Table 3.

Consequently, the internal aldimine of MGL can be adequately described by the cationic model (Fig. 2), which contains two tautomers, ketimine (structure II) and enolimine (structure I).

Figure 3 presents the resolution of the absorption and circular dichroism spectra of the holoenzyme at pH 8.0 according to this model. Experimental parameters of the bands are presented in Table 3. Parameters of the dominant band with $\lambda_{max} = 424.5$ nm with molar absorption coefficient 10,760 $M^{-1}\cdot cm^{-1}$ coincided with the corresponding parameters for the absorption band of structure II of the model compound and with parameters of the absorption band of ketimine of the holoenzyme tyrosine-phenol lyase of *C. freundii* [25].

Table 3. Parameters of bands of absorption and circular dichroism (CD) spectra of internal aldimines of MGL. Designations: E, electron transition energy; ν, wave number; λ, wavelength; ε, molar absorption coefficient; $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$, molar circular dichroism coefficient; W, half-width; ρ, asymmetry; f, oscillator force; n, contents of tautomers and conformers; Δε/ε, anisotropy factor

Spectrum	Structures	E, eV	$\nu \times 10^{-3}$, cm^{-1}	λ, nm	$\varepsilon \times 10^{-3}$ (Δε), $M^{-1}\cdot cm^{-1}$	$W \times 10^{-3}$, cm^{-1}	ρ	f(med.)	n, %	$(\Delta\varepsilon/\varepsilon) \times 10^3$
Absorption	II^1	2.92	23.55 ± 0.02	424.5 ± 0.3	10.76 ± 0.027	3.53	1.53	0.23 ± 0.01	67.6 ± 1.2	
	$II^{\angle}$	3.24	26.12 ± 0.01	383.0 ± 0.2	8.11 ± 1.93	4.00	1.37	0.02 ± 0.01	5.2 ± 1.8	
	I	3.63	29.25 ± 0.01	341.9 ± 0.1	9.26 ± 0.58	3.65	1.23	0.05	16.0 ± 1.1	
	$II^{\perp}$	3.79	30.58 ± 0.01	327.0 ± 0.1	8.94 ± 1.67	3.47	1.29	0.03 ± 0.01	12.1 ± 3.2	
	II^{2*}	4.28	34.52 ± 0.01	289.6 ± 0.1	5.59 ± 0.75	5.06	1.20	0.16 ± 0.03		
	*	4.56	36.76	272.0	21.51 ± 5.76	5.06	1.39 ± 0.05	0.93 ± 0.29		
CD	II^1	2.94	23.76 ± 0.02	421.0 ± 0.4	18.18 ± 2.00	3.52 ± 0.1	1.55 ± 0.02			1.690
	$II^{\angle}$	3.26	26.31 ± 0.03	380.0 ± 0.4	12.82 ± 6.27	4.00	1.37			1.581
	I	3.65	29.46 ± 0.04	339.4 ± 0.5	7.99 ± 2.57	3.65	1.23			0.863
	$II^{\perp}$	3.82	30.78 ± 0.02	324.9 ± 0.2	–0.16 ± 2.62	3.47	1.29			–0.018
	II^{2*}	4.31	34.73 ± 0.03	287.9 ± 0.2	–0.81 ± 0.70	5.06	1.25 ± 0.09			
	*	4.57	36.90 ± 0.10	271.0 ± 0.7	–4.38 ± 6.9	5.06	1.32 ± 0.10			

Note: Above-line indices (1, 2) correspond to the first and second electron transitions of structure II. Above-line indices (⊥, ∠) correspond to two conformers of structure II (the conformer with the aldimine group in the plane perpendicular to the pyridine cycle plane and the conformer with the aldimine bond released from the coenzyme ring plane but with retained coupling and hydrogen bond between the aldimine nitrogen atom and the coenzyme oxygroup).

* Experimental information about these bands is insufficient.

Resolution of the spectra has shown that, to sufficiently describe the holoenzyme spectrum, it is necessary in addition to ketimine (Fig. 2, structure II), which is in the tautomeric equilibrium with enolimine (Fig. 2, the structure I), to introduce into the model two structures with absorptions in the regions of 310-335 and 366-400 nm. In the case of model aldimines of pyridoxal 5′-phosphate with amino acids, such structures can be aldimine conformers [26]. A conformer with an aldimine group completely released from coupling with π-electrons of the coenzyme pyridine ring, i.e. the conformer with the aldimine group located in the plane perpendicular to the pyridine cycle is designated as "$II^{\perp}$", and the conformer with the aldimine bond released from the coenzyme ring plane but with retained coupling and hydrogen bond between the nitrogen atom of aldimine and 3′-oxygroup of the coenzyme is designated as "$II^{\angle}$". In addition to these bands, the spectra contain a low intensity band with maximum in the region of 500 nm. This band is present in the spectra of tryptophane-indole lyase [27, 28], tyrosine-phenol lyase (unpublished data), and cystathionine γ-lyase [29]. This band may belong to tightly bound quinoid intermediates produced by unknown compounds that are present in cell extracts.

Our findings indicate that the internal aldimine of MGL at pH 8.0 exists as one ionic form with predominance of tautomer II (both heteroatoms are protonated, whereas the 3′-oxygroup is deprotonated and bound by a hydrogen bond with the nitrogen atom of the aldimine group). This suggests that the pK_a value of the pyridine nitrogen atom in the holoenzyme must be not lower than 9.0, whereas for model aldimines this value is no more than 7.0. In the three-dimensional structure of the holoenzyme the side carboxyl group of residue Asp185 is located at hydrogen bond distance from the pyridine nitrogen atom of the coenzyme [7]. The high value of pK_a of the pyridine nitrogen atom in the holoenzyme as compared to the pK_a values for model aldimines of pyridoxal 5′-phosphate seems likely to be caused by the presence of the hydrogen bond and by electrostatic interaction with the side carboxyl group of residue Asp185.

Spectral characteristics of complexes of the enzyme with amino acids. In the absorption spectra of MGL complexes with glycine, L-alanine, and L-norvaline, the intensity of the absorption band is decreased in the region with λ_{max} at 420 nm and increased in the region of 320-340 nm (Fig. 4). The absorption band with λ_{max} in the region of 420 nm should be assigned to absorption of the external aldimine because in the complexes the exchange of C-α- and C-β-protons occurs. The increase in the absorption intensity in the region of 320-340 nm may be due to presence in the equilibrium mixture of intermediates with the tetrahedral C4′-atom of pyridoxal 5′-phosphate. For glycine and L-alanine such an intermediate

must be ketimine intermediate 3, and for L-norvaline both intermediate 3 and enamine 4 (Scheme). In addition to intermediates, an enolic tautomer similar to tautomer I (Fig. 2) of the external aldimine should be also responsible for absorption. A pronounced difference in intensities of the absorption bands of external aldimines in the region of 420 nm in the three complexes can be caused by different equilibria between the external aldimine and the produced intermediates and, moreover, there is also a probability of difference in the ionic composition of the external aldimines. The absorption region of 320-340 nm is not considered for the absorption spectra of complexes of substrates, L-methionine, and L-vinylglycine (Fig. 5) because the absorption in this region is mainly contributed by α-ketobutyric acid produced during the reaction. The most pronounced difference of the absorption and circular dichroism spectra from the spectra of enzyme–inhibitor complexes is the presence of two chromophores with absorption in the region of 440-480 nm with positive circular dichroism of their absorption bands. For pyridoxal 5′-phosphate-dependent enzymes, absorption in the region of 460-480 nm is conventionally assigned to absorption of aminocrotonates (Scheme, structure 6) or of aminoacrylates, which are intermediates in the reactions of β- and γ-elimination [10].

Spectra of the enzyme–substrate complexes in the long-wavelength region are similar to spectra of the *P. ovalis* MGL complex with methionine [30] and of the *P. putida* MGL complexes with L-methionine and *O*-acetyl-L-homoserine [23]. Two overlapping absorption bands

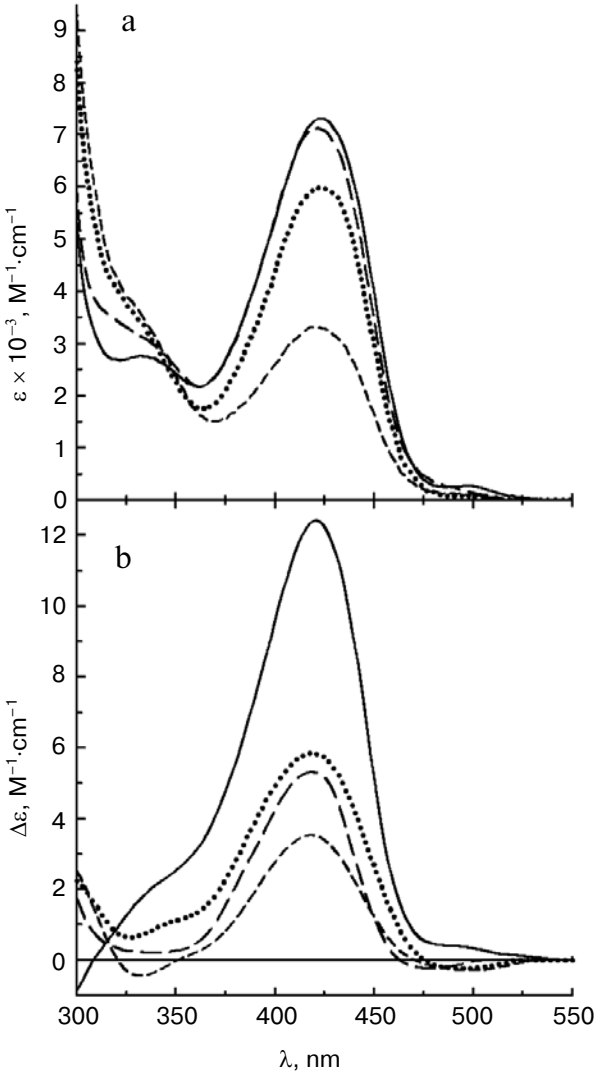

Fig. 4. Spectra of absorption (a) and of circular dichroism (b) of the holoenzyme (solid line) and of its complexes with glycine (long-dashed line), L-alanine (short-dashed line), and L-norvaline (dotted line). Concentrations of inhibitors in the samples were 20 K_i. The spectra were recorded immediately upon the addition of MGL into the sample containing amino acids.

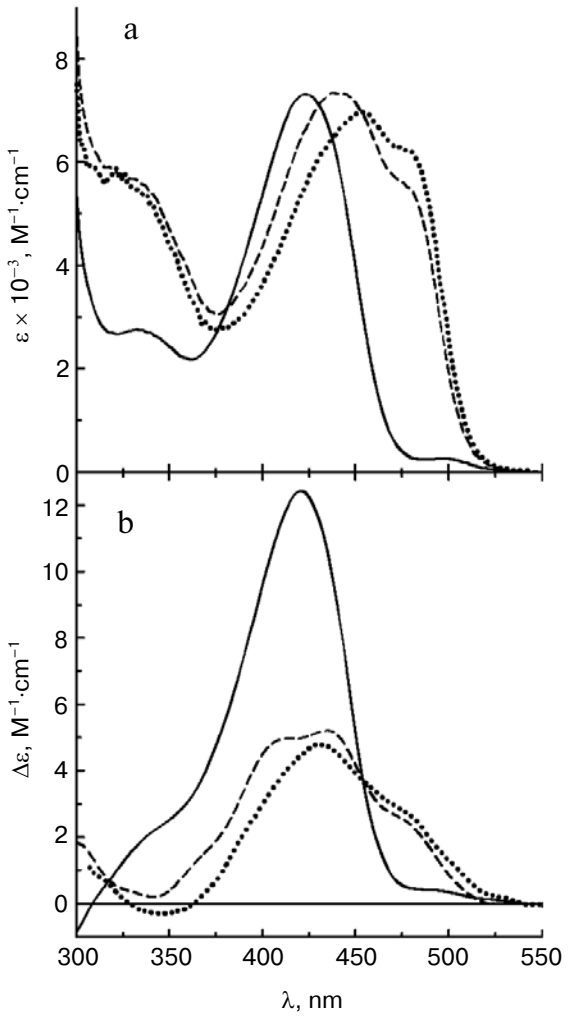

Fig. 5. Spectra of absorption (a) and of circular dichroism (b) of the holoenzyme (solid line) and of its complexes with L-methionine (dashed line), and L-vinylglycine (dotted line). Concentrations of substrates in the samples were 20 K_m. The spectra were recorded immediately upon the addition of MGL into the sample containing substrates.

with maxima at ~460 and ~485 nm were observed in the β-elimination reaction catalyzed by cystathionine γ-synthase [31] and in the γ-elimination reactions catalyzed by cystathionine γ-synthase [8] and cystathionine γ-lyase [32]. Based on rapid kinetics data, the authors of work [8] suggested that these bands should be assigned to the same intermediate (to its different tautomeric or protonated forms), but in works [32] and [33] different rates of changes in the intensity of bands at 460 and 485 nm in the substrate–enzyme complexes were recorded. Spectral data for adequate model compounds are very scarce. The band with the absorption maximum at 460 nm has been shown in the absorption spectrum of the serine dehydratase apoenzyme complex with a synthetic analog of aminoacrylate [34].

Although it is impossible to accurately assign the absorption bands in the MGL complexes with L-methionine and L-vinylglycine in the region of 440-480 nm, the absorption in this region undoubtedly belongs to an intermediate (or intermediates) of the γ-elimination produced during the stages of the enzymatic reaction after the stage of enamine.

It seems that the rate-limiting stage of the γ-elimination is after the stage of ketamine production. First, for substrates with easily separable groups and for L-vinylglycine the rates of γ-elimination are the same as for the natural substrate. Second, the detachment rates of α- and β-protons of inhibitors the most similar in structure to the natural substrate are greater or comparable to the rate of the enzymatic reaction. Third, in the stationary state complexes with L-methionine and L-vinylglycine contain aminocrotonate. A similar hypothesis was proposed earlier for MGL of *P. putida* [23].

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