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A tryptophan residue is identified in the substrate binding of penicillin G acylase from *Kluyvera citrophila*

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Abstract

Penicillin acylases are important enzymes in pharmaceutical industry for the production of semi-synthetic β-lactam antibiotics *via* the key intermediate 6-aminopenicillanic acid. The penicillin G acylase purified from *Kluyvera citrophila* (*Kc*PGA) on modification with tryptophan-specific reagents such as *N*-bromo succinamide (NBS) and 2-hydroxy 5-nitrobenzylbromide (HNBB) showed partial loss of activity and substrate protection. Various solute quenchers and substrate were used to probe the microenvironment of the putative reactive tryptophan through fluorescence quenching. Homology modeling of *Kc*PGA structure has been carried out. Docking substrate on this modeled *Kc*PGA structure identifies the tryptophan residue that is directly influenced by substrate binding. To confirm the biological significance of this particular tryptophan, we did a sequence comparison of PGAs from various organisms. The sequence alignment clustered the matches into two sets, those closer to (>40% identical) *Kc*PGA and had the tryptophan of interest present in them formed the first set, while those less identical (<30%) to *Kc*PGA and the particular tryptophan absent in them formed the second set. It is clear from the reported kinetic parameters of representative members of these two sets that the affinity for penicillin G (penG) of the former class is several times better. Thus, based on our studies we suggest that the tryptophan residue in the identified position is important for binding substrate penG by the acylases.

Keywords: *K. citrophila*; Penicillin G acylase; Tryptophan modification; Fluorescence measurement; Substrate-docking; Sequence alignment

1. Introduction

The microbial enzymes such as penicillin G acylases (penicillin amidohydrolases, PGAs, EC 3.5.1.11) have a high impact on the pharmaceutical industry by their application in the production of antibiotics. They are employed in the deacylation of benzyl penicillin to 6-aminopenicillianic acid (6-APA), the precursor molecule for production of semi-synthetic penicillins [1–3]. Their high efficiency has resulted in the replacement of conventional chemical processes in favor of enzymatic ones by the industry [4].

The penicillin G acylases (PGAs) have been purified and characterized from various sources [5–11]. The PGA from *Kluyvera citrophila* (*Kc*PGA) has attracted attention due to its better and more suitable features for industrial applications as compared to PGA from *Escherichia coli* (*Ec*PGA). It is comparatively easier to immobilize *Kc*PGA and shows more stability towards extreme conditions of temperature, pH, and presence of organic solvents [12–15]. The stabilization of the enzyme by immobilization is also reported [16]. *Kc*PGA is translated as a single-chain precursor consisting of 844 amino acid residues in the cytoplasm. Subsequent processing by removal of a 26 residue signal peptide and a 54 residue spacer peptide produces in periplasm the mature enzyme in the form of a heterodimer consisting of an α- and a β-chain of 209 and 555 amino acid residues, respectively [17]. It has been reported that altering amino acid residue Gly β21 affects protein maturation [18]. The serine residue with a newly generated free α-amino group at the N-terminus of β-subunit acts both as nucleophile and as base in catalysis. Thus, *Kc*PGA can be placed in the Ntn hydrolase family, since having Ser at the N-terminus of β-chain and the modeled three-dimensional structure showing characteristic Ntn-hydrolase 'α–β–β–α' tertiary fold [19,20]. There have been reports of this enzyme being cloned and overexpressed in *E. coli* [21,22].

The active site of the enzyme has drawn special attention due to its importance in understanding the catalytic mechanism and substrate specificity for application in protein engineering

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[23–25]. Thus, it is important to identify the residues that influence the specificity, activity and contribute to the stability of the enzyme. The residues Arg α145, Phe α146 and Tyr β31 have been previously identified to participate in substrate binding [26,27]. Here, we show by using active site chemical modification studies, kinetic analysis, fluorescence spectroscopy, homology modeling, molecular dynamics and bioinformatics techniques the importance of a particular tryptophan residue in substrate binding of PGA enzymes.

2. Materials and methods

Benzylpenicillin (penicillin G or penG) and 6-APA were kindly provided by Hindustan Antibiotics Ltd., India. Tryptophan specific reagent *N*-bromo succinamide (NBS) and 2-hydroxy 5-nitrobenzylbromide (HNBB) were purchased from ICN Biochemicals (Ohio, USA). Other analytical grade reagents were procured from Sigma (USA).

2.1. Enzyme assay

*Kc*PGA was prepared and purified using reported procedures [27]. The PGA activity was assayed by incubating 32 μg of *Kc*PGA (0.37 μmol) at 40 °C for 10 min. The volume of reaction mixture was made up to 1 ml in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM penG. 6-APA formed was estimated using *p*-dimethyl aminobenzaldehyde [28]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of 6-APA per min under standard assay conditions. Kinetic constants K_m and k_{cat} were determined by incubating the enzyme at various concentrations of penG (0.1–20 mM) under standard assay conditions, then fitting a linear regression curve to data points using Lineweaver–Burk plot.

2.2. Protein estimation

Protein concentration was determined according to the method of Lowry et al. [29] using BSA as standard.

2.2.1. Modification of tryptophan with N-bromo succinimide

To measure the effect of Trp modification on enzyme activity, 320 μg (3.72 nM) *Kc*PGA in 10 mM sodium acetate buffer, pH 5.5 was incubated with different concentrations of NBS (10–100 μM). Ten microlitre aliquots were withdrawn every 2 min intervals for 15 min and the residual activity measured under standard assay conditions.

The number of tryptophan residues that reacted with NBS was calculated by measuring the decrease in absorbance at 280 nm as described by Spande and Witkop [30]. Under same conditions of enzyme as described above NBS (5–200 μM) was added in aliquots of 10 μl in 10 installments at every 2 min intervals and absorbance recorded. After each addition, an aliquot of 10 μl was removed and the reaction arrested by the addition of 90 μl of 50 mM L-tryptophan. The residual activity was determined under standard assay conditions. NBS mediated inactivation was monitored by measuring the decrease in absorbance at 280 nm. The same procedure was followed in the presence of enzyme inhibitor phenylacetate. The number of tryptophan residues modified was determined using an estimated value of 5500 M^{-1} cm^{-1} for extinction coefficient. Enzyme samples incubated in the absence of NBS served as control.

2.2.2. Modification of tryptophan with 2-hydroxy 5-nitrobenzylbromide

*Kc*PGA in same conditions as used for reactions with NBS was incubated with 10–40 mM HNBB. The solution of HNBB was freshly prepared in dry acetone. Aliquots of 10 μl were withdrawn every 2 min after an initial lapse of 10 min till the end of 20 min and the residual activity measured under standard assay conditions. The number of tryptophan residues modified was determined based on estimated OD at 410 nm and assuming a molar absorption coefficient of 18,000 M^{-1} cm^{-1}.

2.3. Substrate protection studies

The protection provided by substrate and a competitive inhibitor during modification reactions of NBS and HNBB was determined by incubating the enzyme with varying concentrations of benzylpenicillin (substrate) or phenylacetate (competitive inhibitor) to final concentration of 50 mM, prior to treatment with modifying reagents under the reaction conditions. Thereafter, the reacted mixture as well as control was passed through sephadex PD 10 desalting column and the residual activity determined under standard assay conditions. Deactivation in the column was estimated using control.

2.4. Circular dichroism analysis

Both the NBS and HNBB modified *Kc*PGA samples were passed through sephadex G25 column to remove excess reagent and then the CD spectra were recorded. Untreated enzyme passed through the same column is used to record native spectra.

2.5. Fluorometric studies

Fluorescence measurements were performed on a Perkin-Elmer spectroflourimeter LS 5B, using an excitation and emission slit width of 5 nm, 3.81 μM of *Kc*PGA in 10 mM sodium acetate buffer, pH 5.5 was excited at 280 nm and the emission spectra was recorded in the range of wavelength 300–400 nm.

Fluorescence quenching due to modification with NBS and HNBB was measured by adding 2 μl aliquots of the modifier from 500 μm and 20 mM stocks, respectively, till the relative fluorescence intensity lowered substantially. Suitable controls were included to correct for changes in enzyme dilution.

Fluorescence quenching due to substrate and competitive inhibitor was measured by the progressive addition of 10 μl aliquots of either penG or phenylacetate from a 500 mM stock. Correction was applied to compensate for changes in enzyme concentration due to addition of substrate and inhibitor solutions.

Substrate protection against chemical modification was determined by incubating the enzyme sample with 0.5 mM penG prior to the addition of 200 μM NBS, the fluorescence quenching was then measured.

To assess the extent of tryptophan participation in substrate binding, fluorescence measurement was carried out with the addition of penG in aliquots of 0.5 mM (starting with 0.5 mM and final concentration of 2 mM) to NBS modified *Kc*PGA.

The intensity of PGA saturated with penG (F_∞) was obtained from experimental data by plotting $1/(F_0 - F)$ versus $1/[S]$ and extrapolating the curve to *Y*-axis. Here, F_0 is defined as the fluorescence intensity of the enzyme alone and F is the fluorescence intensity of the enzyme at particular penG concentration $[S]$. $\log[(F_0 - F)/(F - F_\infty)]$ was plotted against $\log[S]$ to estimate the association constant (K_a) of the complex which was same as the value of $[S]$ when $\log[(F_0 - F)/(F - F_\infty)] = 0$. The change in free energy of association was determined using the equation:

$$\Delta G = -RT \ln K_a \tag{1}$$

The thermodynamic parameters *viz*. enthalpy change (ΔH) and entropy change (ΔS) were calculated from vant Hoff's analysis of temperature dependence of K_a by using the equation:

$$\ln K_a = \frac{-\Delta H}{RT} + \frac{\Delta S}{R}. \tag{2}$$

Enthalpy change was calculated from the slope of the curve of $\ln K_a$ versus $1/T$ which equals $(-\Delta H/R)$. The entropy change was then obtained from the equation:

$$\Delta G = \Delta H - T\Delta S. \tag{3}$$

To study the nature of the microenvironment of tryptophan residue 8 M acrylamide, 5 M potassium iodide (KI) and 5 M cesium chloride (CsCl), each separately, has been added to the enzyme sample and tested. 10 mM sodium thiosulfate was included in case of KI to prevent formation of I_3^-