Point mutation of a conserved arginine (104) to lysine introduces hypersensitivity to inhibition by glyphosate in the 5-enolpyruvylshikimate-3-phosphate synthase of *Bacillus subtilis*

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Received 19 May 1995; revised version received 4 September 1995

Abstract **The role of a conserved arginine (R104) in the putative phosphoenol pyruvate binding region of 5-enolpyruvyl shikimate-3-phosphate synthase of *Bacillus subtilis* has been investigated. Employing site directed mutagenesis arginine was substituted by lysine or glutamine. Native and mutant proteins were expressed and purified to near homogeneity. Estimation of Michaelis and inhibitor constants of the native and mutant proteins exhibited altered substrate–inhibitor binding mode and constants. Mutation R104K hypersensitized the enzyme reaction to inhibition by glyphosate. The role of R104 in discriminating between glyphosate and phosphoenol pyruvate is discussed.**

Key words: 5-Enolpyruvyl shikimate 3-phosphate synthase; Site-directed mutagenesis; Active site analysis; Glyphosate

1. Introduction

5-Enolpyruvyl shikimate-3-phosphate (EPSP) synthase (EC 2.5.1.9) is an important enzyme of aromatic amino acid pathway. The reaction involves catalytic transfer of carboxyvinyl group of phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P) to yield EPSP and inorganic phosphate (Fig. 1). The enzyme has been investigated in great detail since it was demonstrated to be the target site of a widely used herbicide, *N*-phosphonomethyl glycine (glyphosate; PMG) [1,2]. Glyphosate is a competitive inhibitor of the reaction with respect to phosphoenol pyruvate [3]. In an ordered, sequential reaction mechanism, shikimate-3-phosphate is the first substrate to bind to the polypeptide followed by phosphoenol pyruvate [4]. Amongst PEP utilizing reactions EPSP synthase (EPSPs) is unique, since in all other enzymes PEP is the first substrate to interact with the protein. This unusual reaction mode has generated a lot of interest in identifying the PEP binding region on the EPSPs protein. Random and site-directed mutagenesis investigations of EPSPs isolated from plants and bacteria have tentatively identified region between 90 and 120 amino acid residues to be the site of PEP interaction [5,6]. Clustal alignment of residues in this putative PEP binding region from bacteria, and plants has revealed several conserved residues (Table 1). Mutation P101S of EPSPs from *Salmonella typhimurium* concomitantly altered K_m for PEP and K_i for glyphosate, thereby establishing competitive nature of the inhibition of reaction by glyphosate [5]. Similarly, the role of Gly^{96} within this region has been analysed in EPSPs of plants and bacteria. Substitution G96A increased K_i (app) for glyphosate by 5000-fold and K_m (PEP) by 40-fold, again defining competitive inhibition of the reaction by glyphosate. Besides Pro^{101} and Gly^{96}, there are other conserved amino acids within 90–120 residues span of different EPSPs (Table 1). Since the substrate and the inhibitor are anionic, we have focused on the role of a conserved arginine (R104) in the catalysis. By site-directed mutagenesis we generated mutations R104K and R104Q and assessed the role of arginine in the EPSPs reaction. The results of kinetic analyses of these mutant proteins are reported here.

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2. Materials and methods

All chemicals and biochemicals were purchased from Sigma Chemical Co. (USA). Restriction enzymes were obtained from New England Biolabs (USA). Analytical grade *N*-phosphonomethyl glycine was gifted by Excel Agrochemicals, Bombay, India. Oligodeoxyribonucleotides were synthesised by the phosphoramidite method using an automated DNA synthesiser (model 380B, Applied Biosystems, USA). The vector pBSKII was obtained from Stratagene, USA and the vector bearing *aroE* gene, pUCtrp100 was provided by *Bacillus* Genetic Stock Centre, Ohio, USA. The *aroE* gene was subcloned from pUCtrp100 into pBSKII and maintained in *E. coli* DH5α.

Growth conditions, procedure for plasmid purification and different enzyme manipulations of the DNA were performed according to the published protocols [7].

2.1. Substitution of conserved arginine by site-directed mutagenesis

Mutants of EPSPs were constructed using background minimised cassette mutagenesis protocol using the polymerase chain reaction (PCR) [8]. For convenience, the mutations targeted segment (nucleotides 314–724) of *aroE* was excised and sub-cloned as *Bss*HII fragment. Following nucleotides, corresponding to the desired substitutions, were changed: R104K, CGT→AAA; R104Q, CGT→CAG. Mutations corresponding to the desired substitutions were introduced by PCR using amplitaq DNA polymerase and fragment of *aroE* as the template. The entire mutant cassette was sequenced by Sanger's dideoxy chain termination method [9] using T_7DNA polymerase system and [α-^{32}P]dATP (Pharmacia). The mutant cassette was exchanged with wild type *aroE* gene and the junctions of introduced cassettes were sequenced using primers internal to the gene.

2.2. Expression and purification of EPSPs

The wild type and mutant genes cloned on pBSKII+ were expressed using bacteriophage T_7 RNA polymerase and promoter system [10]. *E. coli* cells bearing the wild type *aroE* (or the mutant) gene were co-transformed with vector pGP1–2. Co-transformed cells were grown in Luria broth at 30°C. At $A_{590} = 0.3$ the expression was induced by raising the temperature to 42°C. After 30 min the temperature was lowered to 40°C and rifampicin added (200 μg/ml). After growing the culture for a further 120 min, the cells were harvested by centrifugation and the pellet was washed with buffer A (50 mM Tris-HCl, pH 7.2, 0.5 mM 2-mercaptoethanol and 1 mM $MgCl_2$). The cell-free suspension was obtained by resuspending the pellet in buffer A containing lysozyme (100 μg/ml) and DNase (10 μg/ml). The suspension was incu-

SSDI 0014-5793(95)01124-2

bated at 37°C for 1 h and centrifuged at 12000 × *g* for 30 min at 4°C. The level of expression of the mutant and wild type gene was approximately 30% of the total protein. Proteins in the supernatant were precipitated with 65% ammonium sulphate. After centrifugation, the pellet was discarded, supernatant was dialysed extensively against buffer A and used as the source of enzyme. Analysis of the dialysate by SDS-PAGE revealed that EPSPs constituted the major band and was more than 90% pure (Fig. 2). The purified enzyme preparation was free from phosphatases.

2.3. Protein estimation

Protein was estimated by the method of Bradford [11] using bovine serum albumin as standard protein.

2.4. Assay of EPSP synthase

Monovalent cations have been demonstrated to modulate EPSPs activity of *B. subtilis* [12]. To avoid aberrant kinetic constant estimations, care was taken to avoid monovalent cations in all components of the reaction mixture.

Tricyclohexylammonium salt of phosphoenol pyruvate was used in the reaction mixture. Shikimate-3-phosphate was isolated from culture supernatant of *Klebsiella pneumoniae* ATCC 25597 by following the protocol outlined by Knowles and Sprinson [13]. Crude shikimate-3-phosphate was purified further by passing through a Sephadex G-10 column (2.5 × 80 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0). Fractions containing shikimate-3-phosphate were pooled and lyophilized. The concentrated sample contained 95% shikimate-3-phosphate and 4.5% shikimate.

EPSP synthase was assayed by estimating the rate of P_i release as described by Lanzetta et al. [14]. The substrates (PEP and S3P) were prepared in buffer A. Glyphosate solution was prepared by titrating it with NaOH to pH 7.0. Reaction mixtures in 200 μl contained buffer A, 1 mM phosphoenol pyruvate, 1 mM shikimate-3-phosphate, 100 mM ammonium chloride and enzyme protein. Incubation was carried out at 37°C for 20 min following which an aliquot of reaction mixture was used for determining the P_i released. Specific activities were determined for protein concentrations that gave linear increase in velocity with increasing protein concentration.

Slab gel electrophoresis in the presence of SDS was performed by the method of Laemmli [15].

3. Results and discussion

Site-directed mutagenesis is a powerful technique that has been effectively used to elucidate the amino acid residues involved in interaction with the substrates and their analogs during enzyme catalysis. The amino acid residues that are highly conserved are the obvious target for such studies. Sequence analysis of EPSPs from several bacteria and plants has revealed stretches of conserved amino acid residues [4]. Thus, a critical role in the EPSPs catalytic reaction has been assigned to highly conserved Gly^{96}, Pro^{101}, His^{385} and Arg^{24} residues [6,16].

The reaction mechanism, order of binding of substrates, shikimate 3-phosphate and phosphoenol pyruvate and the competitive inhibition of the reaction by glyphosate (Vs PEP) is similar in EPSPs of *E. coli* [17], *Bacillus subtilis* [12,16] and *Petunia hybrida* [18]. Therefore, it is logical to presume that the highly conserved residues will represent domains critical for substrate interaction with the enzyme. Keeping in view the anionic nature of the substrates and the inhibitor, we have investigated the role of a conserved arginine residue (R104), located in the putative PEP binding region of the EPSPs molecule. Its role in the catalytic reaction has been assessed by replacing it with lysine or glutamine using site specific mutagenesis.

Table 1
Sequence homology[a] of putative PEP binding segment of EPSPs from different bacteria

Name of bacteria	Sequence	Ref.
Aeromonas salmonicida	^{98}**AMRPLCAALCLGS**^{110}	[20]
Escherichia coli	^{98}**AMRPLAAALCLGS**^{110}	[21]
Bordetella pertusis	^{96}**AFRPLTAALALMG**^{108}	[22]
Bacillus subtilis	^{102}**AGRPFYSAVAGDE**^{114}	[23]
Consensus sequence[b]	**AXRPXXXAXXXXX**	

[a] Amino acids sequences were compared by clustal alignment programme of Pc/Gene version 5.03.
[b] For the consensus sequence 'X' represents non-conserved amino acid.

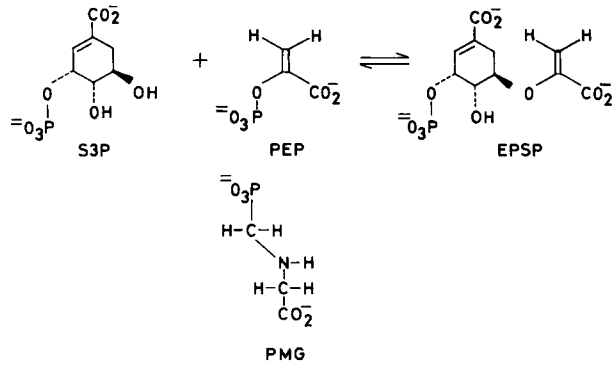

Fig. 1 Enzymatic reaction catalysed by 5-enolpyruvyl shikimate 3-phosphate synthase and the structure of glyphosate.

EPSPs from *Bacillus subtilis* was over expressed in *E. coli* by the two vector system of Tabor and Richardson [10]. Fractionation of expressed proteins with ammonium sulphate yielded more than 90% pure EPSPs (Fig. 2, lane 3). EPSPs of *B. subtilis* has been shown to be modulated by monovalent cations [12]. Of several cations tested ammonium ions were shown to maximally activate the enzyme and also sensitize the enzyme to inhibition by glyphosate [12,16]. Wild type enzyme upon activation by ammonia was 25-fold more sensitive to inhibition by glyposate than the unactivated enzyme [12]. The specific activities of the purified wild type and mutant EPSP synthases are shown in Table 2. For uniformity, all enzyme activity assays were performed in the presence of 100 mM NH_4Cl. The lower activity of the enzyme from R104K is due to total loss of activation by ammonia and other monovalent cations. The mutant enzyme from R104K exhibited increased sensitivity to inhibition by glyphosate than the wild type or R104Q enzyme. Double reciprocal plots of reaction velocity versus phosphoenol pyruvate at several different and fixed concentrations of glyphosate were linear for the wild type or mutant enzymes (data not shown). Substitution of arginine by glutamine (R104Q) reduced the sensitivity of the reaction to inhibition by glyphosate by approximately 3-fold. The K_m (PEP) increased by an identical margin (Table 2). On the other hand, substituting arginine by lysine (R104K) introduced, in the enzyme reaction, hypersensitivity to inhibition by glyphosate. Interestingly, a reduction in K_m for PEP was not observed and the enzymatic reaction of R104K exhibited competitive inhibition by glyphosate (data not shown). Since the binding of glyphosate has been demonstrated to be competitive one would expect that an alteration in Ki for glyphosate will be accompanied by a change in K_m for PEP. In addition to altered K_i (glyphosate) in R104K, ammonia induced activation of the enzyme activity was abolished completely. The kinetic interaction between PEP, glypho-

Table 2
Effects of site-specific mutagenesis of a conserved arginine on the catalytic activity of EPSP synthase of *B. subtilis*[a]

Mutation	Specific activity[b]	*n*-fold activation by NH_4	Kinetic constants[c] (mM)		
			K_m PEP	K_m S3P	K_i PMG
Wild type	10	29	0.10	0.33	0.060
R104K	0.1	1	0.30	0.20	0.005
R104Q	6	7	0.28	0.70	0.175

[a] The results presented are an average of two sets of experiments done in triplicate and the individual values did not vary by more than 10%.
[b] Specific activity is expressed as μmol P_i released/min/mg protein.
[c] For determining K_i (glyphosate), S3P concentrations were fixed at 1.0 mM (wild type), 0.25 mM (R104Q) and 1.0 mM (R104K) and PEP concentrations were varied between 0.05 and 0.50 mM (6 concentration points). Glyphosate concentrations used for determining K_i were 0 μM, 100 μM and 200 μM for wild type enzyme; 0 μM, 125 μM and 500 μM for R104Q enzyme and 0 μM, 10 μM, 20 μM and 40 μM for R104K enzyme. 0.15 μg, 0.40 μg and 22 μg protein was used for K_i determination assays for wild type, R104Q and R104K proteins, respectively.

sate and ammonia can be taken together with our earlier observations. We have recently demonstrated modulation of catalytic and non catalytic PEP interaction sites by ammonia [16]. A secondary, non-catalytic PEP binding site is located around residue His^{385} while the catalytic site is located within residues 90–120. It has been postulated that the interaction of ammonia with the enzyme results in blocking of the non-catalytic PEP binding site resulting in channeling of PEP towards the catalytic site [16]. Ammonia lowers the K_m for PEP probably by blocking the non-catalytic PEP binding site and exposing the reactive catalytic site. Thus, it is likely that in R104K in the absence of ammonia activation of the enzyme, PEP is channeled to non-catalytic site and the catalytic site reaction remains competitive with glyphosate. Interestingly, though ammonia activation of the enzyme from R104K is abolished, it exhibits linear kinetic behaviour (K_i glyphosate). This is in contrast with the sigmoidicity exhibited by the unactivated wild type enzyme [16]. This different kinetic behaviour suggests that the site of ammonia activation is distinct from the site responsible for oligomerization of EPSPs. The Km for S3P varied diversely in two mutant proteins (Table 2). While R104K showed a marginal drop in Km (S3P), substitution R104Q reduced the affinity of shikimate-3-phosphate with the polypeptide. Thus, of the three catalytically interacting molecules (S3P, PEP and glyphosate),

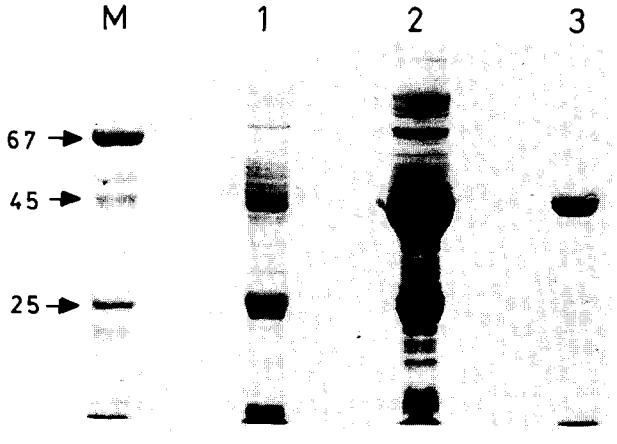

Fig. 2 Purification of 5-enolpyruvyl shikimate 3-phosphate synthase. Coomassie blue stained SDS polyacrylamide gels documenting induction and purification of mutant enzyme R104K. Lane (M) marker; lane 1 = cellular proteins from uninduced culture, lane 2 = cellular proteins from induced culture, and lane 3 = supernatant after 65% ammonium sulphate precipitation.

subsitutions at the conserved R104 result in a significant alteration in the binding of glyphosate to the enzyme.

For substitution R104K, the net charge and the hydrophilicity have not changed and the critical difference being the absence of guanidium side chain. On the other hand, the replacement R104Q, represents the loss of guanidium chain and net charge. The consequences of substitution are dramatic with respect to the K_i of glyphosate. It is likely that in the absence of guanidium chain of arginine the binding of glyphosate to the polypeptide increases by 14-fold, as in R104K. However, abolishing charge (R104Q) reduces the binding of glyphosate by 3-fold. This suggests that the interaction of glyphosate to the enzyme:shikimate-3-phosphate complex is a function of the charge of the interacting residue. This interaction is probably blocked and negatively influenced by guanidium chain of arginine. In contrast, the interaction of PEP with the enzyme–shikimate 3-phosphate complex prefers arginine in the interacting region and is independent of net charge of the residue. Critically the discrimination between glyphosate and phosphoenol pyruvate appears to be a function of guanidium chain of arginine.

These results and the availability of plethora of information for agriculturally important EPSPs polypeptide offers a challenging opportunity for synthesising inhibitors based on glyphosate-enzyme interaction analysis. Results of X-ray (3A) analysis of EPSPs of *E. coli* [19] and intensive mutagenesis of the putative PEP binding domain [6,16] also lead to a speculation on the critical role of conserved arginine in interacting with glyphosate. The structural analysis of the wild type and mutant protein R104K and R104Q with bound glyphosate will yield incisive interaction details for better understanding of this unique reaction involving the transfer of the carboxyvinyl group.

Acknowledgements: We gratefully acknowledge Prof. K.K. Tewari for a critical reading of the manuscript.

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