Photo-oxidation of 5-enolpyruvoylshikimate-3-phosphate synthase from *Escherichia coli*: evidence for a reactive imidazole group (His³⁸⁵) at the herbicide glyphosate-binding site

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Photo-oxidation of *Escherichia coli* 5-enolpyruvoylshikimate-3-phosphate synthase, a target for the non-selective herbicide glyphosate (*N*-phosphonomethylglycine), in the presence of pyridoxal 5'-phosphate resulted in irreversible inactivation of the enzyme. The inactivation followed pseudo-first-order and saturation kinetics with a K_{inact} of 50 μM . The inactivation is specifically prevented by preincubation of the enzyme with the combination of shikimate 3-phosphate and glyphosate. Increasing glyphosate concentration during preincubation resulted in a

decreasing rate of inactivation. On 95% inactivation, approximately one histidine per molecule of enzyme was oxidized. Tryptic mapping of the enzyme modified in the absence and presence of shikimate 3-phosphate and glyphosate as well as analyses of the histidine content in the isolated peptides indicated that His³⁸⁵, in the peptide Asn³⁸³-Asp-His-Arg³⁸⁶, was the site of oxidation. These results suggest that His³⁸⁵ is the most accessible reactive imidazole group under these conditions and is located close to the glyphosate-binding site.

INTRODUCTION

5-Enolpyruvoylshikimate-3-phosphate synthase (EPSP synthase, EC 2.5.1.19) catalyses the reversible condensation of phosphoenolpyruvate (PEP) and shikimate 3-phosphate (S3P) to yield 5-enolpyruvoylshikimate 3-phosphate (EPSP) (Scheme 1), which is an intermediate in the biosynthetic pathway leading to aromatic amino acids [1]. The enzyme reaction is one of two known examples in which the enolpyruvoyl group is transferred unchanged to an acceptor molecule, the other being UDP-GlcNAc enolpyruvoyltransferase [2]. In addition to the interest in the unique reaction, the enzyme is of considerable agronomic interest because it is known to be the primary target of the broad-spectrum non-selective herbicide glyphosate (*N*-phosphonomethylglycine) [3,4] which inhibits the enzyme by competing with the substrate PEP for binding to the active site [4–6]. The enzyme is a monomeric protein with a molecular mass of about 46 kDa

[6]. Its kinetic properties and reaction mechanism have been extensively investigated [4–10]. The complete amino acid sequences of the enzymes from various sources including bacteria, fungi and plants are known [7,11–16], and the mutant enzyme from *Salmonella typhimurium* with the replacement of Pro¹⁰¹ by Ser is reported to be glyphosate-resistant [11]. In an effort to obtain information about reactive amino acid residues in the active site of this enzyme, we have performed chemical modifications using site-specific reagents and site-directed mutagenesis. These studies are important in the understanding of the catalytic mechanism of EPSP synthase and its inhibition by glyphosate. We have reported the presence of Cys⁴⁰⁸, Lys⁴¹¹, Lys³⁴⁰, Glu⁴¹⁸, Arg²⁷ and Arg¹³⁴ in the active centre [17–21]. The importance of the cationic group of Lys²² in substrate binding has been explored by chemical modifications of the enzyme with pyridoxal 5'-phosphate (PLP) [22], *o*-phthalaldehyde [19] or pyruvate in the presence of sodium cyanoborohydride [23] and by site-directed mutagenesis of Lys²² to Arg, Glu and Ala [24]. Furthermore, we also suggested the existence of a histidine residue in the active site by modification of the enzyme with diethyl pyrocarbonate [25] but its position in the amino acid sequence was unclear because of the instability of *N*-carbethoxy-histidine during peptide purification [26]. In the present study, in view of the fact that PLP has also been reported to act as a photosensitizing agent for histidine residues of many enzymes [27–30], we have examined the effect of PLP on the enzyme activity in visible light. The results provide evidence for the existence of an essential histidine (His³⁸⁵) which might be located at the glyphosate-binding site.

EXPERIMENTAL

Materials

EPSP synthase was purified to homogeneity from *Escherichia coli* as described [22]. S3P, EPSP and analytical-grade glyphosate

Scheme 1 Reaction catalysed by EPSP synthase and the structure of glyphosate

were from the Agricultural Group of Monsanto Company. PEP, PLP and L-cysteine ethyl ester were obtained from Sigma Chemicals. Tos-Phe-CH₂Cl (TPCK)-treated trypsin and chymotrypsin were from Worthington Biochemicals. Other reagents used were of the highest grade commercially available.

Photo-oxidation of EPSP synthase in the presence of PLP

EPSP synthase (1 mg/ml) in 50 mM Hepes buffer, pH 7.8, containing appropriate concentrations of PLP was irradiated at 25 °C in a 1 cm × 13 cm Pyrex tube placed 10 cm from a 300 W Kodak Ektagraphic III AMT Projector. At various timed intervals, a sample of the reaction mixture was withdrawn and diluted 100-fold in 50 mM Hepes buffer, pH 7.0, containing 10 mM L-cysteine ethyl ester. After 5 min of incubation, the diluted enzyme was assayed for activity as described below. In the protection experiments, glyphosate and other ligands were preincubated with the enzyme for 5 min before the addition of PLP. Apparent first-order rate constants (k_{obs}) were calculated by least-squares analysis from the slopes of plots of $\ln(E/E_0)$ against time where E_0 and E are activities observed at zero time and times t respectively.

Enzyme assay

Enzyme activity was determined by measuring the rate of release of P_i as described by Lanzetta et al. [31]. A typical assay mixture contained 50 mM Hepes, pH 7.0, 2 mM S3P, 1 mM PEP and enzyme in a total volume of 0.1 ml. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of P_i per min at 25 °C. Specific activity is expressed as units/mg of protein.

Other methods

Proteins (or peptides) were digested with trypsin (or chymotrypsin) in 1% NH₄HCO₃ for 8 h at 37 °C at an enzyme/substrate ratio of 1:50 (w/w). Purity of EPSP synthase was confirmed by SDS/PAGE [32]. Procedures for preparation of the thiocyanylated enzyme, protein carboxymethylation, peptide isolation, amino acid analysis and peptide sequencing were as described in previous papers [17–24]. Tryptophan was determined after hydrolysis with mercaptoethanesulphonic acid in order to prevent its destruction during hydrolysis as described by Matsuda et al. [33]. The presence of histidine in isolated peptides was confirmed using Pauly reagent [34] and Whatman No. 1 paper. The spectral determination of tryptophan was carried out as described by Edelhoch [35]. The content of thiol groups was also determined by titration of the enzyme with 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 50 mM Hepes buffer, pH 7.0, containing 5 M guanidinium chloride. The numbering of amino acid residues of the enzyme in this study proceeds continuously from Met¹ at the N-terminus to Ala⁴²⁷ at the C-terminus of the reported sequence from *E. coli* [7].

RESULTS

Photo-oxidation of EPSP synthase in the presence of PLP

As shown in Figure 1, irradiation of EPSP synthase in the presence of PLP resulted in a significant decrease in enzyme activity. In contrast with the enzyme modified with PLP in the dark [22], the activity of the inactivated enzyme could not be restored by treatment with amino thiol-containing compounds

Figure 1 Photo-oxidation of EPSP synthase in the presence of PLP

The enzyme (1 mg/ml) in 50 mM Hepes buffer, pH 7.8, was exposed to visible light at 25 °C in the presence of 50 (●), 25 (○), 10 (▲), 5 (△) and 0 μM (■) PLP. At various timed intervals, a sample of the reaction mixture was withdrawn and diluted 100-fold in 50 mM Hepes buffer, pH 7.8, containing 10 mM L-cysteine ethyl ester. After a 5 min incubation, the diluted enzyme was assayed for activity as described in the Experimental section. EPSP synthase incubated with 50 μM PLP in the dark lost no activity under these conditions. Inset: double-reciprocal plot of the apparent first-order rate constant for inactivation (k_{obs}) with respect to PLP concentration.

such as L-cysteine ethyl ester, cysteamine, DL-penicillamine and L-cysteine. Loss of enzyme activity, however, was not complete on extended irradiation. After 30 min of prolonged incubation, 5% of the initial enzyme activity remained. The inactivation displayed pseudo-first-order kinetics with various concentrations of PLP (Figure 1). The apparent first-order rate constant for inactivation (k_{obs}) was found to approach a maximal value as the inactivator (PLP) concentration increased. This result is expected if enzyme (E) and PLP form a reversible complex as an obligatory intermediate preceding the irreversible formation of the inactive enzyme [E_{inact} , defined in eqn. (1)]

$$K_{\text{inact}} = [E][\text{PLP}]/[E \cdot \text{PLP}] \quad (2)$$

According to this mechanism, the dependence of k_{obs} on inactivator concentration is represented by eqn. (3) [35–38]

$$k_{\text{obs}} = k_2 / \{1 + (K_{\text{inact}}/[\text{PLP}])\} \quad (3)$$

Eqn. (3) may be rearranged to give a linear rate expression corresponding to eqn. (4) [36–39]

$$1/k_{\text{obs}} = (K_{\text{inact}}/k_2)(1/[\text{PLP}]) + (1/k_2) \quad (4)$$

where k_{+2} is the first-order rate constant for the formation of the inactive complex and K_{inact} is the apparent dissociation constant. Eqn. (4) predicts that $1/k_{\text{obs}}$ should be a linear function of the reciprocal of the concentration of PLP. When the calculated $1/k_{\text{obs}}$ is plotted according to eqn. (4), a straight line is obtained (Figure 1, inset). From the slope of the line shown in this Figure, a K_{inact} of 50 μM for PLP was calculated.

Table 1 Effect of added ligands on photo-oxidation of EPSP synthase in the presence of PLP

EPSP synthase (1 mg/ml) in 50 mM Hepes buffer, pH 7.8, was preincubated with various ligands as indicated for 5 min before the addition of 50 μ M PLP. After irradiation for 10 min, a portion of the reaction mixture was removed, incubated with 10 mM L-cysteine ethyl ester for 5 min and then assayed as described in the Experimental section.

Added ligands	Residual activity (% of native)
Native enzyme	100
+ 50 μ M PLP	5
+ 0.1 mM PEP and 50 μ M PLP	7
+ 0.1 mM S3P and 50 μ M PLP	15
+ 50 μ M glyphosate and 50 μ M PLP	6
+ 0.1 mM S3P, 50 μ M glyphosate and 50 μ M PLP	100
+ 0.1 mM EPSP and 50 μ M PLP	90

Figure 2 Effect of glyphosate in the photo-oxidation of EPSP synthase in the presence of PLP

Experimental conditions were the same as in Figure 1 except that the concentration of PLP was 50 μ M throughout. The competitive inhibitor, glyphosate, was added simultaneously with 0.1 mM S3P 5 min before the addition of PLP. The apparent first-order rate constants (k_{obs}) were obtained as in Figure 1.

Effect of added ligands on photo-oxidation

Photo-oxidation of EPSP synthase in the presence of PLP was prevented by preincubation of the enzyme with S3P, EPSP and the combination of S3P and the competitive inhibitor glyphosate but not by PEP. Glyphosate alone had no effect on the enzyme activity. This is not unexpected since glyphosate binding to the enzyme in the absence of S3P has never been observed [4–6]. Of these ligands, the combination of S3P plus glyphosate provided the greatest protection (Table 1). This suggests that the modified amino acid residue(s) might be located at the glyphosate-binding site. If the binding of PLP to the enzyme is mutually exclusive with that of the competitive inhibitor, glyphosate in the reaction scheme of eqn. (1), the apparent first-order rate constant for inactivation (k_{obs}) should vary according to the relationship

$$k_{\text{obs}} = k_{+2}/(1 + K_{\text{inact}}\{1 + ([\text{Glyphosate}]/K_d)/[\text{PLP}]\}) \quad (5)$$

Figure 3 Absorption spectra of native and photo-oxidized EPSP synthases

The enzyme (1 mg/ml) was irradiated in the presence of 0 (---) and 50 μ M PLP (—) as described in Figure 1. The proteins were then dialysed against 50 mM Hepes buffer, pH 7.8, and their absorption spectra were recorded with a Beckman spectrophotometer model DU-40.

where K_d is the dissociation constant of the enzyme–glyphosate complex. The linear form of this expression [eqn. (6)] [37,38]

$$1/k_{\text{obs}} = \{K_{\text{inact}}/(k_{+2}[\text{PLP}])\} \{1 + ([\text{Glyphosate}]/K_d)\} + (1/k_{+2}) \quad (6)$$

indicates that $1/k_{\text{obs}}$ should correlate in a linear manner with increasing concentration of glyphosate at a constant level of PLP. A plot of the calculated $1/k_{\text{obs}}$ as a function of glyphosate concentration (Figure 2) produces a straight line. From Figure 2, the dissociation constant of the enzyme–glyphosate complex was calculated to be 1.2 μ M which agrees reasonably well with the reported K_d value of about 1 μ M for glyphosate [4–6]. These kinetic results thus provide evidence that the modification of amino acid residue(s) located close to the glyphosate-binding site might be responsible for inactivation of EPSP synthase produced by irradiation in the presence of PLP.

Characterization of the photo-oxidized enzyme and recovery of amino acids after photo-oxidation

Native and photo-oxidized EPSP synthase showed identical mobilities on SDS/PAGE and Superose 12 gel filtration and similar c.d. spectra at u.v. wavelengths (R. J. Schilling and Q. K. Huynh, unpublished work), indicating that the photo-oxidized enzyme has not undergone any gross changes in physical structure. Photo-oxidation, however, is accompanied by a small increase in absorption at 325 nm (Figure 3). Although photo-oxidation of free histidine does not give a new absorption maximum at 325 nm, photo-oxidation of certain histidine residues in protein has been reported to result in an increase in absorption at 325 nm [40]. However, since the possibility remains that photoinactivation was due to oxidation of tryptophan to *N*-formylkynurenine or an additional reaction between a Schiff-base intermediate of PLP and a nucleophilic side chain next to this

Table 2 Amino acid composition of native and photo-oxidized EPSP synthases

EPSP synthase (1 mg/ml) was irradiated in 50 mM Hepes buffer, pH 7.8, in the absence and presence of 50 μ M PLP. After irradiation for 10 min, the reaction mixtures were incubated with 10 mM L-cysteine ethyl ester for 5 min and then passed over a Sephadex G-25 column (1 cm $\times$ 10 cm) equilibrated in 50 mM Hepes buffer, pH 7.0. The void volume fractions were analysed for amino acid content.

Amino acid	Content (mol/mol of enzyme)		
	Native enzyme	Photo-oxidized enzyme	From sequence*
Cys†	6.1	5.9	6
Met	13.7	13.6	14
Tyr	12.5	12.1	12
Trp	2.2‡ (1.8§)	2.1‡ (1.8§)	2
His	7.8	6.7	8

* From amino acid sequence of the *E. coli* enzyme [7].

† Determined by titration with DTNB as described in the Experimental section.

‡ Determined after hydrolysis with mercaptoethanesulphonic acid to prevent its destruction during hydrolysis [33].

§ Determined spectrophotometrically [35].

Figure 4 Elution profile of peptides derived from EPSP synthases on C_{18} reversed-phase column

The enzyme (1 mg/ml) was irradiated in the presence of 50 μ M PLP for 30 min in the presence (a) and absence (b) of 0.1 mM S3P plus 50 μ M glyphosate. The modified proteins were then carboxymethylated and digested with trypsin. Peptides were separated on a Vydac C_{18} column (4.6 mm $\times$ 250 mm) operated with a Waters h.p.l.c. system model 510. A flow rate of 1 ml/min was used with a gradient system of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 80% acetonitrile (—). Peptides were detected by monitoring changes in the absorbance at 214 nm (—). Peptide fraction 1 of (a) was further digested with chymotrypsin and reappplied to the same column (c). Darkened bars indicate peptide fractions found to react with histidine-specific Pauly reagent [34].

intermediate, possibly a thiol group, we have investigated the effect of photoinactivation on the catalytically active thiocyanylated enzyme. In addition, the content of other amino acid

residues that may have been oxidized during photo-oxidation was also determined. The results indicate that the thiocyanylated enzyme was irreversibly inactivated by irradiation in the presence of PLP at the same rate as the native enzyme (results not shown). Furthermore, the content of tryptophan, methionine, tyrosine and cysteine residues remained unchanged after photo-inactivation. The possibility of tryptophan oxidation was also examined by determination of the fluorescence spectra (from 300 to 500 nm at an excitation wavelength of 295 nm) of the native and photo-oxidized enzymes. The results show no difference in fluorescence spectra of the two enzymes (R. J. Schilling and Q. K. Huynh, unpublished work). Photo-oxidation of EPSP synthase, however, resulted in the oxidation of approximately one histidine residue per molecule of enzyme (Table 2). These results may rule out the modification of tyrosine, cysteine, tryptophan or methionine residues and suggest that the oxidation of a histidine residue might be responsible for photoinactivation of the enzyme in the presence of PLP.

Identification of the photo-oxidized residue

To identify the location of the photo-oxidized histidine residue, EPSP synthase (1 mg/ml) was irradiated in the presence of 50 μ M PLP and the absence and presence of 0.1 mM S3P and 50 μ M glyphosate. The modified proteins were then denatured with 5 M guanidinium chloride, carboxymethylated with iodoacetic acid and digested with trypsin as described in the Experimental section. Although the structure of the modified histidine residue was still not clear and radioactive compound was not involved in the modification process, we have attempted to identify the modified histidine residue by conventional methods such as monitoring the modified histidine-containing peptide using its possible new absorbance at 325 nm. These attempts were not successful. We have therefore identified the modified histidine residue by monitoring the histidine-containing peptide in the enzyme modified in the presence of S3P and glyphosate which was not found for the enzyme modified in the absence of S3P and glyphosate. The tryptic digests were then applied to a Vydac C_{18} reversed-phase column, and histidine-containing peptides were identified by paper chromatography using the histidine-specific Pauly reagent [34]. In contrast with a total of eight tryptic fractions found to react with Pauly reagent for the enzyme modified in the presence of S3P and glyphosate (Figure 4a), only seven tryptic fractions were found for the enzyme modified in the absence of S3P and glyphosate (Figure 4b). Peptide fraction 1 of the enzyme modified in the presence of S3P and glyphosate (Figure 4a) thus must contain a histidine residue that was oxidized in the enzyme modified in the absence of S3P and glyphosate (Figure 4b). Peptide fraction 1 was further digested with chymotrypsin and reappplied to a C_{18} reversed-phase column (Figure 4c). Of the peptides collected, only peptide 2 showed positive reaction with the Pauly reagent for histidine. Amino acid analysis and sequencing of this peptide showed that it contained Asx (2.2 mol), His (1.1 mol) and Arg (1.0 mol) and has a sequence of Asx-Asp-His-Arg which corresponds to that of the segment Asn³⁸³-Asp-His-Arg³⁸⁶ of EPSP synthase [7], indicating that His³⁸⁵ was the oxidized residue.

DISCUSSION

As previously described [17–24,41], covalent-modification studies almost always present difficulties in interpretation. Are observed losses in activity due to modification of residues essential for substrate binding or catalysis, or do they reflect subtle conformational changes in the enzyme molecule produced by a

	380	390
	↓	
<i>E. coli</i>	NFAE	IATYNDHRMAMCFSLVA
<i>S. typhimurium</i>	QHAD	IGTYNDHRMAMCFSLVA
<i>P. hybrida</i>	NVTD	IDTYDDHRMAMAFSLAA
<i>L. esculentum</i>	NVTD	IDTYDDHRMAMAFSLAA
<i>A. nidulans</i>	PVGG	VFCYDDHRVAFSFSVLS
<i>A. thaliana</i>	KTAE	IDTYDDHRMAMAFSLAA
<i>S. cerevisiae</i>	GPVG	VCTYDDHRVANSFSLLA
<i>B. pertussis</i>	RDAN	IGTWDDHRNANCFLLAA

Figure 5 Amino acid sequence around His³⁸⁵ of EPSP synthases from *E. coli* [7], *S. typhimurium* [11], *Petunia hybrida* [12], *Lycopersicon esculentum* (tomato) [12], *Aspergillus nidulans* [13], *Arabidopsis thaliana* [14], *Saccharomyces cerevisiae* [15] and *Bordetella pertussis* [16]

The arrow indicates the essential histidine residue identified in this work.

modifying group of different size and charge? The latter possibility is well illustrated by modification of EPSP synthase with DTNB which resulted in inactivation of the enzyme [18]. However, when the TNB group from the DTNB-modified enzyme was replaced with the smaller uncharged CN group, the enzyme regained its activity indicating that the modified thiol group is not essential for activity. In contrast with covalent modification, although the mechanism of photo-oxidation has not been fully established, it is a useful mild technique for modifying histidine residues in proteins [42]. The results presented here show that PLP irreversibly inactivated EPSP synthase in visible light by oxidation of a histidine residue (Table 2). The specificity of the reaction is supported by the following observations: (1) native and photo-oxidized enzymes showed identical mobilities when subjected to SDS/PAGE and gel filtration as well as identical fluorescence and c.d. spectra (R. J. Schilling and Q. K. Huynh, unpublished work); (2) the catalytically active thiocyanylated enzyme was also irreversibly inactivated by irradiation in the presence of PLP, ruling out the possibility of oxidation of thiol groups; (3) the content of other amino acids that may be oxidized under these conditions, such as methionine, tyrosine and tryptophan, remained unchanged (Table 2). Inactivation of the enzyme by irradiation in the presence of PLP followed pseudo-first order and saturation kinetics with a K_{inact} of 50 μM (Figure 1, inset). On 95% inactivation, approximately one histidine residue per molecule of the enzyme was oxidized (Table 2). Since the kinetic pattern of photo-oxidation of the enzyme by PLP is competitive with glyphosate in the presence of S3P (Figure 2), the modified histidine residue might be located close to the glyphosate-binding site. In order to identify the modified histidine residue, EPSP synthase was irradiated with PLP in the absence and presence of S3P and glyphosate. The modified proteins were then carboxymethylated and digested with trypsin. Isolation of the tryptic peptides by C_{18} reversed-phase h.p.l.c. (Figure 4), as well as subsequent digestion and analyses of the isolated peptides, indicated that His³⁸⁵ was the modified site. This residue is conserved in the primary structure of all EPSP synthases studied to date (Figure 5) [7,11–16].

In the proposed mechanism of action of EPSP synthase, a nucleophile would probably be involved in the protonation of C-3 of PEP to form an enolenzyme [9] or in the addition of the hydroxy group of S3P to the double bond of PEP to give an intermediate in which phosphate is subsequently released to yield EPSP [8,9]. Charged amino acid residues might also be required for S3P and PEP binding. On the basis of sensitivity of the enzyme to bromopyruvate [17], 1-ethyl-3-(3-dimethylamino-

propyl)carbodi-imide [20], *o*-phthalaldehyde [19], phenylglyoxal [21], PLP [22]; Lys⁴¹¹, Glu⁴¹⁸, Lys³⁴⁰, Arg²⁷, Arg¹³⁴ and Lys²², respectively have been suggested as candidates for the proposed nucleophile or substrate binding. Of these suggested active-site residues, the role of Lys²² was confirmed as essential for substrate binding by site-directed mutagenesis studies [24]. Although the roles of Lys³⁴⁰, Lys⁴¹¹ and Glu⁴¹⁸ are still unclear, it has been suggested that the positively charged residues, Arg²⁷ and Arg¹³⁴, might be important for substrate binding [21]. His³⁸⁵ identified in this work is of particular interest since it is conserved in all EPSP synthase sequences studied to date [7,11–16], and, furthermore, crystallographic studies of the *E. coli* EPSP synthase indicate that the protein is folded into two domains with an open structure. It is possible that His³⁸⁵ from one domain and residues Lys²² and Arg²⁷ from the other are in nearby proximity when the two domains close together to form the active site [43] (W. C. Stallings, personal communication).

In conclusion, the results in this study indicate that the oxidation of His³⁸⁵ is responsible for inactivation of the EPSP synthase by irradiation in the presence of PLP. The reaction is prevented by preincubation of the enzyme with S3P and glyphosate. These results suggest that His³⁸⁵ might be located in the active site but do not clearly distinguish between a role in substrate binding or catalysis. If we assume that His³⁸⁵ is essential for the catalytic action of the enzyme, then its oxidation should result in complete inactivation of the enzyme. Our results show that this is not the case and therefore indicate that His³⁸⁵ might be important in substrate binding. The role of His³⁸⁵ in the overall mechanism of action of EPSP synthase, however, needs to be confirmed by site-directed mutagenesis of His³⁸⁵ to other amino acid residues such as arginine, glutamine or alanine and characterization of the purified mutant enzymes.

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