Limited proteolysis by trypsin influences activity of maize phosphoenolpyruvate carboxylase

Gururaj B Maralihalli and Anil S Bhagwat*
Molecular Biology and Agriculture Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085

Received 7 March 2001; revised and accepted 13 July 2001

Maize phosphoenolpyruvate carboxylase (PEPC) was rapidly and completely inactivated by very low concentrations of trypsin at 37°C. PEP+Mg²⁺ and several other effectors of PEPC carboxylase offered substantial protection against trypsin inactivation. Inactivation resulted from a fairly specific cleavage of 20 kDa peptide from the enzyme subunit. Limited proteolysis under catalytic condition (in presence of PEP, Mg²⁺ and HCO₃⁻) although yielded a truncated subunit of 90 kDa, did not affect the catalytic function appreciably but desensitized the enzyme to the effectors like glucose-6-phosphate glycine and malate. However, under non-catalytic condition, only malate sensitivity was appreciably affected. Significant protection of the enzyme activity against trypsin during catalytic phase could be either due to a conformational change induced on substrate binding. Several lines of evidence indicate that the inactivation caused by a cleavage at a highly conserved C-terminal end of the subunit.

Phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) plays a key role in C-4 photosynthesis catalyzing carboxylation of phosphoenolpyruvate to provide four-carbon skeleton for source of carbon dioxide for the Calvin cycle¹. This enzyme is the most abundant protein in mature mesophyll cells of maize comprising 10-15% of the soluble protein².³. The gene encoding the enzyme was earlier thought to be specifically expressed in the mesophyll cells in response to illumination. However, substantial accumulation of transcripts encoding the C-4 isoenzyme in other parts such as inner leaf sheath, husks and tassels has been reported⁴. The mRNA level markedly increases during leaf development and greening of etiolated plants⁵. In addition to its role in C-4 photosynthesis, PEPC has various other physiological roles common to both C-3 and C-4 plants⁶. The enzyme is allosteric in nature and multiple effectors regulate its activity. In C-4 plants, PEPC carboxylase is allosterically activated by glucose-6-phosphate⁷ and is inhibited by carboxylic acid such as oxaloacetic acid, aspartate and malate, which are primary products of carboxylation⁸.⁹.¹¹. A molecular weight of 400,000 has been reported for the purified phosphoenolpyruvate carboxylase from maize and other plants and is composed of four identical subunits².¹². Topography of the active site of maize PEPC has been studied using various compounds that have structural similarity with PEPC¹³-¹⁷. The chemistry of the active site of PEPC has been elucidated by chemical modification studies using phenylglyoxal¹⁸, eosine isothiocyanate¹⁹, pyridoxal phosphate²⁰, tetranitromethane, O-phthalaldehyde and Woodward’s reagent K²¹-²³. Using site-directed mutagenesis, the role of His⁵⁷⁹ in catalysis and regulatory function of E.coli PEPC has been demonstrated²⁴.

Conformational change in an enzyme elicited by effectors can be detected by examining the change in the susceptibility of the enzyme to limited proteolytic attack in the native state. Desensitization of the enzyme to the effectors caused by proteolytic attack has been investigated for several allosteric enzymes. Fructose-1, 6-bisphosphatase was desensitized to AMP²⁵. Action of papain, subtilisin and trypsin on aspartate transcarbamylase results in complete loss of allosteric activity²⁵. Trypsin, subtilisin and other proteases have been previously used to specifically modify the native protein structure²⁶-³⁰. Limited trypsic proteolysis of spinach Rubisco resulted in ordered release of two adjacent N-terminal peptides from the large subunit. Protection of the catalytic site against trypsin during catalysis was due to a conformational change at the N-terminal domain of the large subunit¹³. Amino terminal truncation of Rubisco small
subunit has been shown to influence catalysis and subunit interaction. Several reports show that PEP carboxylase was partially proteolysed during purification when protease inhibitors like leupeptin and chymostatin; as well as protective agents like malate, glycerol and glucose-6-phosphate were not used. The N-terminal peptide encompassing the phosphorylation site Ser in maize was removed by such proteolytic cleavage resulting in reduced Vmax and loss of regulatory properties including malate sensitivity. The negative charge introduced on phosphorylation seems to be responsible for enhancing the activity and the regulatory effects like malate sensitivity.

Our study on carboxyl group modification by Woodward’s reagent K that also introduces some negative charge on the enzyme molecule also decreased the affinity of the enzyme to the negative effector malate.

Here, we present data to show that PEPC from maize was inactivated by trypsin under non-catalytic condition, resulting in a truncated enzyme having molecular mass of about 80 kDa; however, in the presence of the substrates very little inactivation was observed although the subunit molecular mass was reduced to 90 kDa. Truncated enzyme having small residual activity shows significantly reduced affinity for bicarbonate and malate inhibition was considerably reduced.

Materials and Methods

Chemicals

PEP (trisodium salt), DTNB, trypsin (TPCK treated), tris, dithiotreitol, HEPES, ANS and protein kinase-catalytic subunit A were obtained from Sigma Chemical Co. [32P] ATP was a product of Amersham Life Sciences.

Purification of the enzyme

PEP carboxylase was purified to homogeneity from maize leaves essentially by the method of Bhagwat and Sane with slight modification in the extraction buffer and fractionation with ammonium sulphate. The freshly purified enzyme showed a specific activity of 20 μ moles/min/mg and migrated as a single protein band on SDS-PAGE, indicating that the enzyme was more than 95% pure. The malate sensitivity was not affected during purification and the purified enzyme could be phosphorylated under in vitro conditions indicating that the native enzyme was intact and the full-length protein was not proteolysed during purification.

Protein determination and enzyme assay

Protein concentration was estimated by either measuring A at 280 nm assuming A 280 nm of 1= 0.88 mg/ml or by the method of Lowry et al. after removal of reducing agents by Sephadex G-25 column. The enzyme activity was measured by a linked assay using malate dehydrogenase. The reaction mixture (1 ml) contained HEPES, 100 mM (pH 8.0), MgCl2, 2 mM; MDH, 4 units; PEP, 2 mM; NaHCO3, 10 mM; NADH, 100 mM and the enzyme. The enzyme activity was monitored from change in absorbance at 340 nm at room temperature.

Limited proteolysis

Limited proteolysis of PEPC catalyzed by trypsin was performed at 37°C using a fixed ratio of trypsin (TPCK treated from Sigma) to the enzyme as specified in the Figure legends. The carboxylase concentration was usually 1 mg/ml in 25 mM Tris-HCl (pH 7.0) containing 1 mM EDTA and 5 mM β-mercaptoethanol. The proteolysis was terminated by addition of soybean trypsin inhibitor 6 fold molar excess over trypsin. The trypsin inhibitor had no effect on the enzyme activity. For SDS-PAGE, the samples were quenched by boiling for 1 min in an equal volume of Laemmli SDS-PAGE buffer. When the sensitivity of the modified enzyme to the effectors was to be determined, the modified enzyme (residual activity 40-50%) was treated with the indicated concentrations of the effectors for 10 min prior to the assay.

Gel electrophoresis

Electrophoresis in 10% polyacrylamide gels was performed using a slab apparatus and discontinuous buffer system of Laemmli. Gels and reservoir buffer were supplemented with 0.1% (w/w) SDS for electrophoresis of denatured protein. Gels were stained with Coomassie brilliant blue.

Sulphydryl group analysis

The method of Ellman was used to determine free and total sulphydryls. The enzyme was completely denatured with 5% SDS to determine total sulphydryl groups.

Phosphorylation of maize PEP carboxylase

The method described by Terada et al. was used for in vitro phosphorylation studies on native and proteolysed maize PEP carboxylase. The trypsinised sample having 35% residual activity was obtained by treating the native enzyme (1 mg/ml) with 5 µg of
trypsin for 1 min at 37°C. The reaction was terminated by trypsin inhibitor and the samples were passed through Sephadex G-25 column before phosphorylation.

PEPC (0.5 mg/ml) with and without trypsin treatment was incubated at 37°C for 1 hr with the catalytic subunit A-kinase (5.6×10^−3U/ml) in a reaction mixture containing 2 ml MES-NaOH (pH 6.0), 5 mM magnesium acetate, 0.2 mM [32P] ATP, (70.2 CPM/p mole). The reaction was terminated with an equal volume of 20% TCA. A portion of 32P labelled PEPC samples were subjected to SDS-PAGE and distribution of radioactivity was detected by autoradiography on X-ray film, Jindhal Photo films, Japan at -70°C for 30 days.

Fluorescence measurements
The protein fluorescence emission was recorded with Hitachi spectrofluorimeter. The excitation and emission spectra indicated maxima at 283 nm (excitation) and 338 nm (emission). At these wavelengths all measurements were carried out with enzyme samples of 110 μg/ml concentrations. Fluorescence emission of the enzyme-ANS (extrinsic) complex was measured in 25 mM HEPES-NaOH buffer (pH 7.0) containing 0.2 mM ANS and 0.2 mg/ml enzyme in a total volume of 3 ml. The excitation beam was passed through a narrow bandpass filter of 365 nm and fluorescence emission monitored at 470 nm.

Results and Discussion
Kinetics of inactivation
Phosphoenolpyruvate carboxylase from maize leaves was rapidly and completely inactivated in Tris-HCl buffer (pH 7.0) by treatment with very low concentrations of trypsin. The inactivation followed pseudo-first-order reaction kinetics (Fig. 1). The susceptibility of the carboxylase to proteolysis could be due to general effect of proteolytic enzymes like trypsin to cleave the native protein at random sites of the subunits at the exposed basic residues like lysine and arginine. Lysine residues of PEPC may have a role in catalysis.\(^{10,11}\) Similarly, arginine residues have also been implicated in binding of the phosphate group of the substrate PEP\(^ {13}\). Since the data of Wedding and Black\(^ {14}\) on Crassula show that the proteases which attack at the Arg residues are not very effective inhibitors of the enzyme, it seems likely that lysine residues may be the target of the proteolytic attack in the present case. Three lysines and seventeen arginines are conserved in the amino acid sequence of PEPC from different sources and two arginines are unique to maize PEPC\(^ {16,24}\).

Substrate PEP + Mg\(^ {2+}\) offered substantial protection against trypsin attack (Table 1). Several other effectors like phosphoglycolate, glycine and glucose-6-phosphate offered considerable protection against trypsin inactivation.

Kinetic properties of trypsinized PEPC
Proteolysis of PEP carboxylase for 1 min with trypsin, exhibited 60% reduction in \(K_{cat}\), a 2.5 fold increase in \(K_m\) (PEP) and 2-fold increase in \(K_m\) (Mg\(^ {2+}\)). The residual 40% of the active enzyme species displayed modified kinetic constants (results not included).

Fluorescence properties of the modified enzyme
Since trypsinization of PEPC from maize caused inactivation as well as desensitization to G-6-P and

![Fig. 1—Time course of inactivation of the enzyme at carboxylase to trypsin ratio of 400:1 (●) and 200:1 (○).](image-url)
malate, it was thought that trypsin treatment either removes some essential residues required for activity and / or causes a conformational change resulting in loss of activity and desensitization. Fluorescence is a powerful tool for monitoring conformational change in a protein molecule. The quenching of fluorescence emission by binding of ligands is generally used as a measure of their binding affinities (Kd). Such data on binding of various substrates to the proteolysed enzyme with and without substrate protection are presented in Table 2. The data indicate that binding of Mg\(^{2+}\), PEP and HCO\(_3^-\) sequentially caused quenching of the intrinsic fluorescence of the native enzyme indicating normal binding constants. When similar experiment was done with the trypsin treated enzyme having 20% residual activity it was seen that HCO\(_3^-\) binding was considerably affected Table 2 (line 4 column 3 vs 4) as evident from drastically reduced fluorescence quenching by HCO\(_3^-\). However, PEP+Mg\(^{2+}\) protected enzyme having more than 85% of initial activity, on trypsinization showed HCO\(_3^-\) binding comparable to that of control. Similar results were also obtained using ANS as an extrinsic fluorescence probe (data not included). Thus, trypsin inactivation could be due to altered binding affinity of the enzyme for bicarbonate. The data in Table 2 further show that trypsinization caused about 50% decrease in the total intrinsic fluorescence as compared to the control in the absence of any added ligands, which closely correlates to the loss of tryptophan.

**Table 2—Effect of trypsin treatment on intrinsic fluorescence of maize PEP carboxylase**

The enzyme in 25 mM HEPES-NaOH buffer (pH 7.0) was passed through Sephadex G-25 column to remove bound substrate. The dialysed enzyme was protected (where indicated) with 50 mM PEP, 10 mM MgCl\(_2\) and 10 mM HCO\(_3^-\) before trypsin treatment. The TPCK treated trypsin at a protein to trypsin ratio of 200:1 was used for proteolytic treatment for 7.5 min at 37°C. The reaction was quenched with trypsin inhibitor. The intrinsic fluorescence was measured as described under "Material and Methods". The values in the parenthesis represent % of control. The value without any added ligand is taken as 100%.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Control E</th>
<th>E+ trypsin</th>
<th>PEP+Mg(^{2+})-protected E+trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2400 (100.0)</td>
<td>210 (100.0)</td>
<td>2380 (100)</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>2280 (95.0)</td>
<td>830 (68.5)</td>
<td>2210 (92.8)</td>
</tr>
<tr>
<td>PEP+PEP</td>
<td>1820 (75.8)</td>
<td>790 (65.2)</td>
<td>1750 (73.5)</td>
</tr>
<tr>
<td>3+HCO(_3^-)</td>
<td>350 (14.6)</td>
<td>780 (64.4)</td>
<td>320 (13.4)</td>
</tr>
</tbody>
</table>

![Fig. 2 — Mobility of intact and modified maize PEP carboxylase on SDS-PAGE with and without protection by PEP+MgCl\(_2\).](image-url)

Electrophoretic analyses of trypsinized and phosphorylated PEPC

Inactivation of PEPC by trypsin was caused by fairly specific cleavage of the enzyme molecule. This was evident from the SDS-PAGE data shown in Fig. 2. Trypsin treatment for about 7.5 min at an enzyme to trypsin ratio of 200:1 resulted in an approximate 20 kDa decrease in the subunit molecular mass of the enzyme. Intermediates between intact (100 kDa) and the modified (80 kDa) were not seen even at very early period of digestion. Thus, the conversion of the intact (100 kDa) to the inactive modified 80 kDa subunit was specific and was caused by single or very few "hits".

The purified maize PEP carboxylase, with and without trypsin treatment, was subjected to in vitro phosphorylation in a reconstituted system mediated by cyclic AMP dependent protein kinase from bovine heart. The autoradiography data of the separated subunits on 10% SDS-PAGE clearly indicate that both native (100 kDa) and proteolysed (80 kDa) enzyme could be phosphorylated in vitro indicating that the N-terminal Ser\(^{15}\) was not cleaved during purification (in...
the native enzyme) and also on limited proteolysis by trypsin in trypsinised enzyme in our studies. Proteins in their native typically globular conformation are generally resistant to proteolytic attack; however, in many proteins the C-terminal or the N-terminal end may be accessible to proteolytic attack and if this were involved in either catalysis or maintaining the active conformation, the cleavage would cause enzyme inactivation. The site of action of trypsin on PEPC during catalysis i.e. in presence of PEP, Mg and bicarbonate, was different as compared to the unprotected or non-catalytic enzyme. The data in Fig. 2 further show that the enzyme subunit molecular mass was reduced to 90 kDa from 100 kDa, but surprisingly, the modified enzyme retained more than 80% of the original activity. This possibly suggests that the 10 kDa portion cleaved by trypsin during catalytic phase may not be required for activity. The molecular weight determination of the subunit after trypsin treatment in presence and absence of substrates is shown in Fig. 2. In the case of many enzymes, the active site is located at the N or C terminal domain and in many cases these domains interact with each other. In the case of Rubisco, the two domains share the active site and the C-terminal domain has been shown to form a lid to close the active site after the substrate has been bound to the site of action. Treatment of Rubisco with carboxypeptidase also indicated that C-terminal domain of large subunit of spinach, which is not located near the active region, play a role in determining catalytic activity of the enzyme.

Free and total sulfhydryl group estimation

Any changes in number of free and total sulfhydryl groups in proteins may indicate a conformational change in protein or a loss of –SH group. The free and total sulfhydryl groups were same in the control and trypsinised PEPC samples having 10-15% residual activity indicating no gross conformational change or a reduction in the total sulfhydryl groups (data not included). These data also suggest that trypsin did not cleave the N-terminal end which contains 2-SH groups in the 20 kD stretch and cleavage at this site would have reduced the titratable –SH groups.

Allosteric properties of the modified enzyme and evidence for C-terminal cleavage

The enzyme treated with trypsin under both catalytic and non-catalytic conditions showed different responses to various activators. Under non-catalytic condition, trypsin did not desensitize the enzyme to positive effectors like glycine and glucose-6-phosphate. However, negative effector malate sensitivity was reduced (Table 3). Westhoff et al. have studied the molecular evolution of C-4 PEPC in genus Flaveria. They have tried to identify C-4 specific determinant for expected glucose-6-phosphate activation and malate inhibition. They have proposed that glucose-6-phosphate activation appears to be predominantly located in the amino terminal residue of the enzyme, whereas, the major determinant of malate sensitivity are not carried ny N-terminal residues but are located at the C-terminal end. Our results presented here strongly corroborate these findings as the proteolysed enzyme was not desensitized against glucose-6-phosphate because the N-terminus was not affected by trypsin; however, malate sensitivity was considerably reduced in case of the treated enzyme since the C-terminal end was specifically cleaved by trypsin under our experimental conditions. Under catalytic conditions trypsin caused appreciable increase in activity at pH 7.0. Since the catalytic site was not affected by trypsin, it was thought worthwhile

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Control</th>
<th>Glucose-6-Phosphate (10 mM)</th>
<th>Glycine (10 mM)</th>
<th>Malate (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>178</td>
<td>450</td>
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<td>1</td>
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</tr>
<tr>
<td>3</td>
<td>27</td>
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<td>240</td>
<td>200</td>
</tr>
</tbody>
</table>
to check the effect of trypsin on the allosteric site under these conditions. The results show that the enzyme was desensitized by trypsin to the effectors like malate and glucose-6-phosphate and glycine under catalytic conditions. Thus, it seems likely that the allosteric property of the enzyme may require some functional group(s) located at the terminal end.

From the sequence of maize PEP carboxylase, it was seen that the 20 kDa stretch at the C-terminal end contains 4 tryptophans as opposed to only one in the 20 kDa N-terminal region. Thus, the decrease in fluorescence was clearly due to loss of 4 tryptophans at the C-terminal end, out of total 12 tryptophan per subunit. This observation along with our data on sulfhydril group estimation and in vitro phosphorylation strongly suggest that trypsin specifically attack at the C-terminal portion of the enzyme under the experimental conditions employed here. As mentioned earlier, C-terminal region of PEPC is highly conserved. A recent study on removal of conserved QNTG tetramotif was indispensable for maximum catalytic activity of C-4 PEPC. This motif was not essential for either assembly of the 110 kDa polypeptide into stable tetramer or the phosphorylation if its regulatory terminal domain. Thus, the C-terminus of PEPC warrants continued structure/function analysis.

It has been observed that an enzyme from different sources behaves differently as to its susceptibility towards trypsin. In the case of Rubisco, the enzyme from spinach is very sensitive to trypsin attack whereas the R. rubrum enzyme is resistant to trypsin attack. Similarly, FBP aldolase from wheat is rapidly inactivated by trypsin, but the aldolase from animal sources like chicken muscle, rabbit muscle and Aseris muscle are very resistant to trypsin attack. Thus, it would be interesting to check whether the PEPC from other sources like C-3 plants is susceptible to trypsin, since in C-3 plants PEPC has a very different role.

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