Identification of reactive lysines in phospho*enol*pyruvate carboxykinases from *Escherichia coli* and *Saccharomyces cerevisiae*

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Abstract ***Escherichia coli*** **and** ***Saccharomyces cerevisiae*** **phospho*enol*pyruvate carboxykinases (PEPCKs), were inactivated by pyridoxal 5′-phosphate followed by reduction with sodium borohydride. Concomitantly with the inactivation, one pyridoxyl group was incorporated in each enzyme monomer. The modification and loss of activity was prevented in the presence of ADP plus Mn^{2+}. After digestion of the modified protein with trypsin plus protease V-8, the labeled peptides were isolated by reverse-phase high-performance liquid chromatography and sequenced by gas-phase automatic Edman degradation. Lys^{286} of bacterial PEPCK and Lys^{289} of the yeast enzyme were identified as the reactive amino acid residues. The modified lysine residues are conserved in all ATP-dependent phospho*enol*pyruvate carboxykinases described so far.**

Key words: Phospho*enol*pyruvate carboxykinase; Lysyl residues; Chemical modification

1. Introduction

Phospho*enol*pyruvate carboxykinase (PEPCK) (ATP: oxaloacetate carboxylyase (transphosphorylating), E.C. 4.1.1.49) catalyses the decarboxylation of oxaloacetate in the presence of ATP and a divalent metal ion to give CO_2, ADP and PEP. The primary role of this enzyme is the catalysis of PEP formation as the first commited step in gluconeogenesis [1].

ATP-dependent PEPCKs are found in yeast [2], bacteria [3], plants [4], and trypanosomastids [5]. The *Saccharomyces cerevisiae* enzyme is composed of four identical subunits of 61.3 kDa each [6,7], meanwhile the *Escherichia coli* PEPCK is a monomer of 51.3 kDa [8]. These two PEPCKs share 42% of protein sequence identity [8]. Chemical modification experiments have indicated that the yeast enzyme is rapidly inactivated by sulfhydryl-directed reagents [9,10], and we have identified Cys^{364} and Cys^{457} as the reactive residues [11,12], meanwhile, *E. coli* PEPCK does not contain reactive sulfhydryl groups of functional significance [13]. Despite the above differences, the common thread running through chemical modification studies is the involvement of essential arginines [13,14], and lysines [13,15] in *S. cerevisiae* and *E. coli* PEPCKs.

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Abbreviations: PEPCK, phospho*enol*pyruvate carboxykinase; PLP, pyridoxal 5′-phosphate; HPLC, high-performance liquid chromatography, TFA, trifluoracetic acid; PTH, phenylthiohydantoin; OAA, oxalacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; PEP, phospho*enol*pyruvate.

In order to shed light on the active site structure and reaction mechanism of ATP-dependent PEPCKs, we have identified the reactive lysines of yeast and bacterial PEPCKs by a combination of enzyme labeling with pyridoxal 5-phosphate and peptide analysis.

2. Materials and methods

ADP, ATP, PEP, PLP, $NaBH_4$, $NaCNBH_3$, NADH and malate dehydrogenase were from Sigma. OAA, endoproteinase glu-C from *S. aureus* and sequencing grade trypsin were from Boehringer-Mannheim. All other reagents were of the purest commercially available grade. *E. coli* and *S. cerevisiae* PEPCKs were obtained as described before [8,11]. Enzyme concentrations were determined from the extintion coeficient at 280 nm, 75,000 $M^{-1} \cdot cm^{-1}$ for the subunit yeast enzyme [6], and 57,000 $M^{-1} \cdot cm^{-1}$ for the *E. coli* enzyme [3,8].

2.1. Enzyme assays

The rate of OAA formation from PEP and ADP was determined spectrophotometrically at 30°C as described by Malebrán and Cardemil [14] for the yeast carboxykinase, and by Bazaes et al. [13] for the bacterial PEPCK.

2.2. Modification by PLP

Inactivation by PLP was performed at 0 °C in 50 mM K-HEPES buffer pH 7.5 containing 5 mM $NaCNBH_3$. At time intervals, aliquots were removed and transferred to tubes containing 0.1 ml of 40 mM $NaBH_4$ in 25 mM K-HEPES buffer. From these solutions, aliquots were taken for enzyme activity assay. For stoichiometry of PLP binding the $NaCNBH_3$ was omitted and the reaction was directly stopped with 40 mM $NaBH_4$, following the procedure already described for the *E. coli* PEPCK [13].

2.3. Isolation of labeled peptides

The labeled carboxykinases were carboxymethylated [16], dialyzed against 50 mM NH_4HCO_3 pH 8.0 and subjected to proteolysis with 1% trypsin plus 1% of protease V-8 for 24 h at 30°C. The peptides were separated on a Merck-Hitachi HPLC system equipped with a LiChrocart C-18 reverse-phase column (0.4 × 25 cm) equilibrated with 0.05% TFA at a flow rate of 1 ml/min. After 20 min, the column was eluted for 120 min with an acetonitrile gradient (0–60%) in 0.05% TFA. The fluorescence (excitation at 410 nm and emission at 325 nm) of the effluent was continuosly monitored. The collected peaks were concentrated under a stream of nitrogen at 30°C, and rechromatographed on a Supelco C-4 column (0.46 × 25 cm), eluted in the same conditions. N-terminal sequencing by Edman degradation was performed at the Protein Sequencing Service at the Universidad de Chile.

3. Results

S. cerevisiae PEPCK was rapidly inactivated at pH 7.5 by low concentrations of PLP: the inactivation was time-dependent and a constant value of activity was reached which varied with the initial concentration of PLP. Fig. 1 shows the inactivation course in the absence and in the presence of several single

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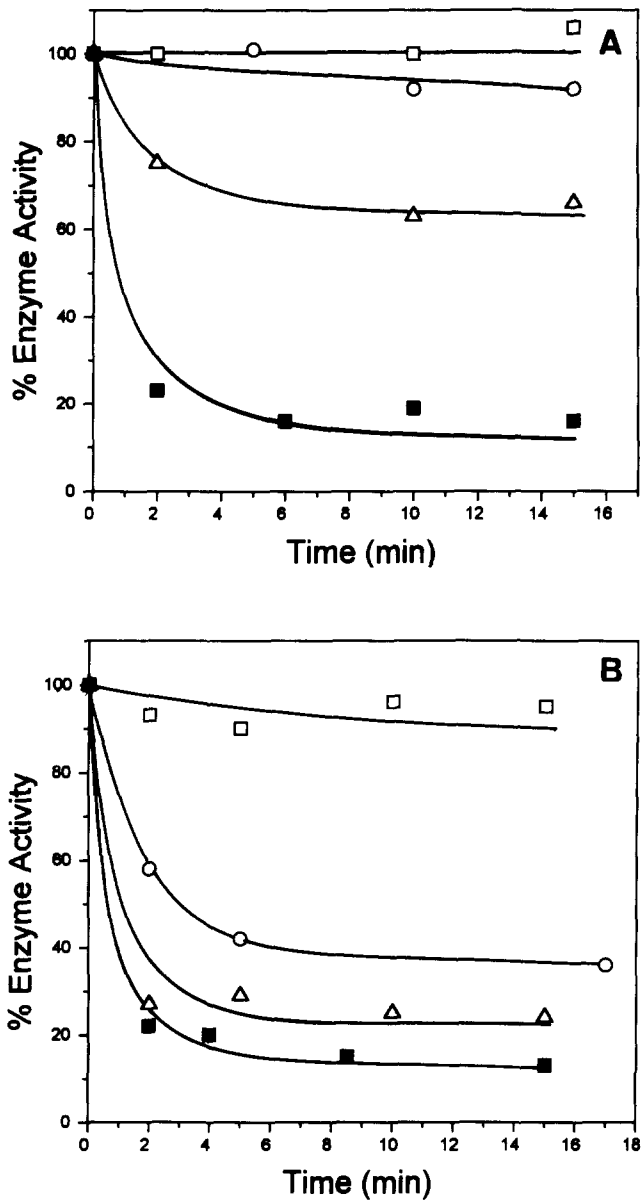

Fig. 1. Inactivation of *Saccharomyces cerevisiae* PEPCK by PLP. The enzyme (16 μM subunits) was incubated with 50 μM PLP: (A) in the absence (■) or in the presence of 5 mM ADP plus 5 mM $MnCl_2$ (□) 5 mM PEP (△) , 5 mM PEP plus 5 mM $MnCl_2$ (○); (B) in the absence (■) or in the presence of 5 mM OAA (○), 5 mM OAA plus 5 mM $MnCl_2$ (□), 5 mM $MnCl_2$ (△).

substrates and combination with Mn^{2+}. ADP in the presence of Mn^{2+} afforded the best protection followed by PEP plus Mn^{2+}.

The incorporation of pyridoxyl phosphate was determined on samples of enzymes incubated in the absence and in the presence of protecting substrates (2.5 mM ADP and 2.5 mM Mn^{2+}).

After reduction with sodium borohydride and removal of excess of pyridoxol phosphate, the absorbance at 325 nm was measured. The number of pyridoxyl groups bound was estimated as aproximately one per subunit of the tetrameric enzyme. In the presence of ADP plus Mn^{2+} the incorporation of the reagent was aproximately 0.1 mol per mol of subunit.

Samples of both *E. coli* and *S. cerevisiae* PEPCK were labeled with PLP in conditions of incorporation of about one mol of pyridoxyl per enzyme subunit (*S. cerevisiae*) or per mol of the monomeric enzyme (*E. coli* [14]). When the PEPCK from *E. coli* was treated with trypsin, a single labeled pyridoxyl-peptide appeared in HPLC. When isolated and sequenced, we found that it had the following sequence: ^{263}L-I-G-D-D-E-H-G-W-D-D-D-G-V-F-N-F-E-G-G-S-Y-A-X-T^{287}, according to the published amino acid sequence of the enzyme [8]; In this peptide Lys^{286} was missing, and after that position only traces of the Thr^{287} were detected. Since this PLP tryptic peptide was too long, we decided to treat both *E. coli* and *S. cerevisiae* PEPCKs with a mixture of trypsin and protease V-8, in order of determine with more precision the location of the labeled residue. As can be seen in the above sequence, the tryptic peptide obtained from the *E. coli* enzyme, has several possible points of attack by protease V-8, and if PLP modified the homologous Lys residue in the *S. cerevisiae* PEPCK, the region around this Lys also contained several points of cleavage by the protease [7].

Therefore, both samples were digested with trypsin plus protease V-8 and the resulting peptides were resolved in a C-18 reverse phase column, as described in section 2. Fig. 2 shows the fluorescent profile obtained for the *E. coli* enzyme in which only one labeled peak was detected. No fluorescent peaks were obtained when the enzyme was labeled in the presence of ADP plus Mn^{2+} . Fig. 3 shows the results for the *S. cerevisiae* PEPCK, where the lower profile was obtained from an enzyme labeled in the presence of ADP plus Mn^{2+}. It can be seen that in the presence of the protecting substrates, two peaks were absent: a minor peak eluting at 34.5 min and a major peak eluting at 87.8 min. The fractions corresponding to the mayor peak from the *S. cerevisiae* PEPCK and the single peak from the *E. coli* enzyme were concentrated and repurified on a C4 reverse phase columnn. The minor peak from the *S. cerevisiae*

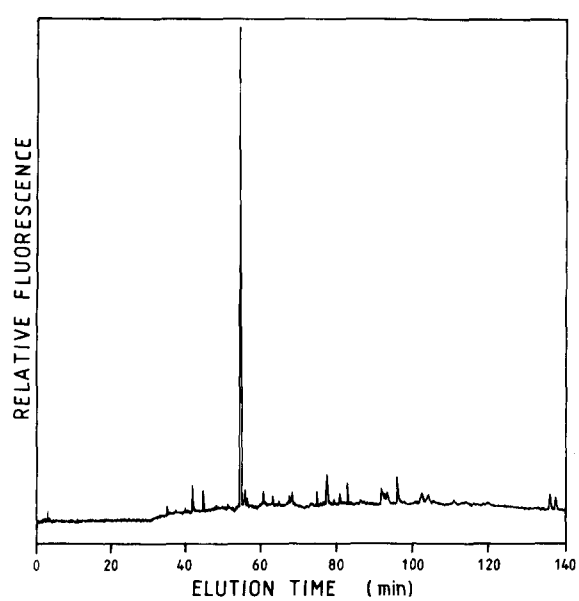

Fig. 2. Separation of PLP-labeled peptide from *E. coli* PEPCK. Digested PLP-enzyme was prepared and subject to reverse-phase HPLC on a C_{18} column as described in section 2. The fluorescent peak eluting at 55 minutes was collected for further analysis.

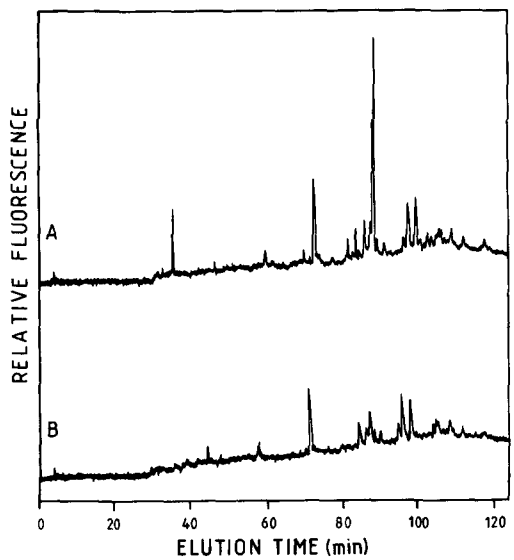

Fig. 3. Reverse-phase separation of PLP-labeled peptides from *S. cerevisiae* PEPCK. The PLP-modified enzyme was digested and applied to a C_{18} column as described in section 2. (A) elution profile obtained from the modification of the enzyme in the absence of protectors; (B) in the presence of 2.5 mM ADP plus 2.5 mM $MnCl_2$.

PEPCK was not retained in the C4 column and it was washed out. No further sudies were done on this peak.

The labeled peptides were sequenced and the result of the analysis is shown in Table 1. The peptide from the *E. coli* PEPCK was nine residues long, starting in Gly^{281} and ending in Lys^{289} [8]; Lys^{286} was again missing in cycle number six and should correspond to the modified residue. This sequence confirms the one found in the previously obtained tryptic peptide, hence protease V-8 cleaved between Glu^{280} and Gly^{281}, and the peptide ended in Lys^{289}, a tryptic cleavage.

The *S. cerevisiae* peptide was 13 residues long, from Gly^{284} to Glu^{296}, according to the primary structure of the enzyme [7], both ends were protease V-8 cleavages. No residue was detected in cycle number six corresponding to Lys^{289} and it should be the modified lysine. Cycle number 11, corresponding to a Ser residue was not identified, probably for insufficient PTH-amino acid product.

4. Discussion

We have labeled *E. coli* and *S. cerevisiae* PEPCKs with pyridoxal 5'-phosphate. This compound has been widely used as a group-specific reagent directed to lysine residues [17], and in many cases has shown to act as a real affinity reagent for phosphate binding sites in enzymes [18]. Both enzymes were inactivated by PLP with the incorporation of one mol of the reagent per mol of active unit of enzyme, being the enzymes protected against the inactivation and modification, by ADP (or PEP) plus Mn^{2+}. The target lysines in both PEPCKs are very reactives since the reaction with PLP was very fast and the decrease in activity reached a plateau in a few minutes, in agreement with the previously reported second order inactivation rate constant of $11.7 \times 10^3\ M^{-1} \cdot min^{-1}$. for the reaction of *E. coli* PEPCK with PLP [13].

A high reactivity for a particular lysine residue may be explained by a hydrophobic environment surrounding the residue, lowering the pK of the amino group [19]. However, there is no information about the physicochemical characteristic of the substrate binding site of the *E. coli* PEPCK. With respect to the *S. cerevisiae* enzyme, it has been reported that the nucleotide-binding site is a low polarity region [10], and a pK of 8.1 has been estimated for the reaction of the 2',3'-dialdehyde derivative of ATP with a lysyl residue, probably located in its neighborhood [15]. A similar microenvironment polarity has been deduced for the cytosolic rat liver PEPCK [20].

Reactive lysines have also been found in the GTP-dependent chicken mitocondrial PEPCK, where a highly reactive lysine residue of this enzyme reacts with PLP with second order inactivation kinetics [22]. The sequencing of the modified tryptic peptide showed that the label was at Lys^{141} [23], a residue conserved between GTP-dependent PEPCKs [24].

Our results also show a discrepancy with the reported sequence of the *E. coli* PEPCK, which was deduced from the DNA sequence [8]. This discrepancy concerns five residues with the sequence ^{279}F-E-G-G-S^{283}. The reported sequence was ^{279}L-K-A-A-G^{283}. The DNA of the pckA gene also has been resequenced in this region and found to be compatible with the sequence ^{279}F-E-G-G-S^{283} (X. Xu and H. Goldie, in preparation).

The modified lysine residues occupy equivalent positions in these PEPCKs, as shown in Table 2, and they are located in a highly conserved part of the central region of these enzymes, at a distance of about 30 residues from the first consensus sequence described for the binding of the phosphoryl group of nucleotides ('P loop', [21]). The comparative examination of the primary structures of the five ATP-dependent PEPCKs show that only four lysine residues are completely conserved. Among them Lys^{286} of *E. coli* PEPCK and the equivalent Lys^{289} of the *S. cerevisiae* enzyme that are the PLP-reacting residues determined in this work. Another conserved lysine is located in the 'P loop' (Lys^{253} of *E. coli* and Lys^{256} of *S. cerevisiae*) and the other are two neighboring lysines (Lys^{211} and Lys^{212}, respectively).

Preliminary crystalographic X-ray studies performed on the *E. coli* PEPCK have shown that Lys^{286} is surface exposed and close to other basic residues including Lys^{253}, the one participat-

Table 1
Edman degradation of modified peptides from *E. coli* and *Saccharomyces cerevisiae* PEPCKs

Cycle number	*E. coli*	*S. cerevisiae*
1	G^{281}	G^{284}
2	G	G
3	S	CMC
4	Y	Y
5	A	A
6	X	X
7	T	CMC
8	I	I
9	K^{289}	N
10		L
11		(S)
12		A
13		E^{296}

In positions indicated as X no PTH-amino acid were detected, which correspond to lysines according to the known primary structure of each enzyme [8,7]. No PTH-residue was detected in cycle No. 11 for the *S. cerevisiae* peptide, corresponding to serine [7]. CMC stands for cysteine detected as the carboxymethyl derivative.

Table 2
Partial sequences of the *E. coli* and *S. cerevisiae* PEPCKs containing the reactive lysines. Comparison with the corresponding sequences of other ATP-dependent PEPCKs

E.c.	FFGLSGNGKTAFP-RPKRRLIGDDEHGWDDDGVFNFEGGSYAKTIKLSKE	293
S.c.	FFGLSGNGKTTLSADPHRLLIGDDEHCWSDHGVFNIEGGCYAKCINLSAE	296
NGR	FFGLSGNGKTTLSADPNRTLIGDDEHGWSDKGVFNFEGGCYAKAIRLSEA	291
T.b.	FFGLSGNGKTTLSADPRRNLIGDDEHVWTDRGVFNIEGGCYAKAIGLNPE	268
T.c.	FFGLSGNGKTTLSADPHRNLIGDDEHVWTDRGVFNIEGGCYAKAIGLNPK	268

Identity of amino acid residues are indicated by *continuous lines* and conservative substitutions by *dotted lines*. The 'P loop' is indicated by a *continuous line* below the sequences. The identified peptides are shown in *bold type*. A star is placed over the modified lysines. Sequences of *Escherichia coli* (E.c.), *Saccharomyces cerevisiae* (S.c.), *Rhizobium sp. NGR 234* (NGR) and *Trypanosoma brucei* (T.b.) were taken from [25]; sequence of *Trypanosoma cruzi* (T.c.) was taken from [26].

ing in the 'P loop'in the active site of the enzyme (A. Matte, H. Goldie and L. Delbaere, unpublished data). This is in agreement with the good protection against the modification of the enzyme by PLP, afforded by ADP plus Mn^{2+} or PEP plus Mn^{2+}, and suggest that the phosphoryl moiety of both substrates interact with the ε amino group of the lysine residue. Experiments are in progress toward the further analysis of these active lysines through site-directed mutagenesis.

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