MECHANISM OF ACTION OF NaCl ON MAIZE ALCOHOL DEHYDROGENASE AND PHOSPHOENOLPYRUVATE CARBOXYLASE

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Alcohol dehydrogenase (ADH) and phosphoenolpyruvate carboxylase (PEPC) are inhibited by NaCl. PEPC is more sensitive to NaCl than ADH. Chloride ions act toward maize ADH as an inhibitor which is competitive with respect to the substrate, *i.e.* ethanol; the inhibition with respect to acetaldehyde is of the mixed, competitive-uncompetitive type. The inhibition constants for Cl^- ions and ADH are of the order of 10^{-1} mol l^{-1}. The binding site for Cl^- ions constitutes most likely the central Zn atom present in the active center of ADH. NaCl also inhibits two PEPC isozymes (PEPC I and PEPC II) isolated from corn leaves. Isozyme PEPC I is inhibited more strongly than isozyme PEPC II. Sodium chloride acts on both PEPC isozymes as a competitive inhibitor with respect to the substrate, *i.e.* phosphoenolpyruvate, the inhibition constants being of the order of 10^{-2} mol l^{-1}. NaCl also acts as an allosteric effector of PEPC I, binding not only to the active center of the enzyme but also to the allosteric site of the protein molecule of PEPC I. The reversible inhibition of PEPC isozymes is converted into irreversible inactivation of the isozymes. The irreversible inhibition is probably a result of a change in the quaternary structure in the protein molecule of PEPC.

Alcohol dehydrogenase (ADH, EC 1.1.1.1) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) are enzymes which play an important role in the metabolism of higher plants.

Alcohol dehydrogenase plays the key role in the anaerobic degradation of saccharides present as stock compounds in the seeds of higher plants. This so-called natural anaerobiosis proceeds during the early stage of germination of seeds of higher plants[1].

Phosphoenolpyruvate carboxylase is the primary enzyme of CO_2 fixation during photosynthesis of the so-called C_4 plants[2]. PEPC is present in maize leaves in the form of two isozymes whose function and properties have intensively been studied during the past few years[3-13]. The properties and the mechanism of catalysis by both enzymes have been characterized in earlier studies (PEPC (refs[3-13]) and ADH (refs[14-22])).

In view of the importance of both enzymes in plant metabolism this study has been undertaken to investigate how these enzymes are affected by chloride ions which occur in the environment in increased concentrations as a result of enhanced

anthropogenetic activity. The increasing penetration of salt into soils and waters leads to a decrease of the production of some crop. The manner in which plants absorb chlorides and the pathways along which they are distributed in plant tissues have been intensively studied[23-26].

It is well known that maize accepts chlorides through the root system and transports them to the shoots afterwards[27,28]. The Na^+ and Cl^- concentrations in the plant cells may lead to the inhibition of metabolically essential enzymes; these inhibitions may be the cause of the decrease of growth of this crop. Even though the inhibition of many enzymes has been demonstrated in *in vivo* experiments[25-27,29-31], information on its molecular basis is so far meagre. Our present study is therefore devoted to the mechanism of inhibition caused by Cl^--ions in metabolically important plant enzymes and to a discussion of the mode of binding of the inhibitor to the protein molecule of both key enzymes.

EXPERIMENTAL

Plant material and chemicals: ADH was isolated from maize (*Zea mays* L., cv. CE-205 S) seeds which had been germinating for 3 days in distilled water. PEPC isozymes were isolated from the leaves of the above maize variety which had been cultivated at 20 to 30°C for three to four weeks in a greenhouse at natural illumination. Tris-(hydroxymetyl) aminomethane was from Merck, A.G., Darmstadt, F.R.G., 2-mercaptoethanol, NAD^+ and NADH from Koch–Light Laboratories, Colnbrook, England, PEP from Sigma Chemical Corp., St. Louis, MO, U.S.A. The remaining chemicals, all of analytical purity grade, were purchased from Lachema, Brno, Czechoslovakia.

The isolation of maize ADH and the determination of its enzymatic activity have been described in our previous work[22,32]. ADH activity was measured in Specord M-40 spectrophotometer (Carl Zeiss, Jena, G.D.R.) in terms of increase (for ethanol oxidation) or decrease (for acetaldehyde reduction) of absorbance at 340 nm. The reaction medium contained 0·1 mol l^{-1} Na-phosphate buffer, pH 8·5, 0·5 mmol l^{-1} NAD^+ or (NADH), 100 mmol l^{-1} ethanol (or 10 mmol l^{-1} acetaldehyde) and 1 μmol l^{-1} ADH. The measurement was carried out at 20°C. The specific activity of isolated maize ADH was 5·2 μmol NAD^+ min^{-1} mg^{-1}.

PEPC isozymes were isolated by a procedure described in our previous study[13]. PEPC activity was assayed spectrophotometrically in the Specord spectrophotometer in two different manners: *1*) by measuring NADH oxidation at 340 nm (decrease of absorbance at 340 nm) after linking the PEPC-catalyzed reaction with exogeneous malate dehydrogenase or *2*) by measuring oxaloacetate formation at 280 nm (increase of absorbance at 280 nm) without linkage with malate dehydrogenase. The reaction medium contained: 50 mmol l^{-1} Tris-acetate buffer, pH 8·1, 10 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} PEP, 0·15 mmol l^{-1} NADH and 1 international malate dehydrogenase unit. When the formation of oxaloacetate was measured as the increase of absorbance at 280 nm an analogous reaction medium was used, yet NADH and malate dehydrogenase were not present. The measurement was carried out at 20°C. The specific activity of isozyme PEPC I was 26·2 μmol PEP min^{-1} mg^{-1} and of isozyme PEPC II 15·83 μmol PEP min^{-1} mg^{-1}.

The values of the Michaelis constants (K_m) were determined according to Lineweaver and Burk[33], for PEPC I also by using the Hill plot[34].

Determination of inhibition constants: The inhibition constant is regarded as the dissociation constant of the enzyme–inhibitor complex. It was determined graphically by the method of Lineweaver and Burk[33] and by the method of Dixon[34].

The interaction constants of ADH were determined graphically according to Yonetani and Theorell[35]. The measurements were performed in 2 ml of reaction medium containing 0·5 mmol . l^{-1} sodium phosphate buffer, pH 8·5, 50 mmol l^{-1} ethanol, 1 μmol l^{-1} ADH and always two different inhibitors; the concentration of one of them remained constant and the concentration of the other one was varied. The measurement was carried out at 0·1 mol l^{-1} concentration of NaCl and at varying concentration of ADP (0—10 mmol l^{-1}) or *o*-phenanthrolline (0 to 2 mmol l^{-1}), then at 0·15 mol l^{-1} concentration of NaCl and identical concentrations of either ADP or *o*-phenanthrolline, and finally at 0·2 mol l^{-1} concentration of NaCl and at identical concentrations of ADP and *o*-phenanthrolline as shown above.

The Hill constants (n) were determined graphically by plotting the values of $\log v/(V_{max} - v)$ *versus* the values of log PEP concentration[34].

The irreversible inactivations of ADH and of PEPC isozymes by chloride ions were carried out in 3 ml test tubes containing 1 ml of the incubation medium. The solutions pipetted into the test tube were 0·7 ml of 50 mmol l^{-1} Tris–acetate buffer, pH 7·0, 0·1 ml of a NaCl solution in the same buffer (NaCl concentration 0·5—2 mol l^{-1}, *i.e.* the final NaCl concentration in the medium was 50—200 mmol l^{-1}) and 0·2 ml of the solutions of ADH or PEPC isozymes in the same buffer (the concentration of the enzymes was 10 μmol l^{-1}). After an appropriate incubation period (19—26 h) 0·1 ml aliquots of the incubation mixture were withdrawn and ADH or PEPC activity was assayed. The inactivation was allowed to proceed at 4°C. All the remaining measurements (enzymatic activity assays) were performed at 20°C. Control incubation of ADH and of PEPC isozymes without NaCl was run simultaneously. Samples of ADH and of PEPC isozymes incubated 96 h with 100 mol l^{-1} NaCl were chromatographed on a Sepharose 4B column (2 × 50 cm) and the changes in quaternary structure of the protein molecule of ADH and of PEPC isozymes were examined. ADH (10 μmol l^{-1}) was incubated in 50 mmol l^{-1} Tris-acetate buffer at pH 7·0 also with 1 mol l^{-1} NaCl 5 h and the incubation medium was frozen (—18°C) afterwards for 1 h. After thawing at 2°C ADH was chromatographed on the Sepharose 4B. column as described above. The Sepharose column was calibrated with urease (483 000), catalase (232 000), gamma-globulin (157 000), and bovine serum albumin (67 000).

RESULTS

Effect of NaCl *on Maize* ADH

Chloride anions act as an inhibitor of maize ADH which is competitive with respect to ethanol and to the coenzyme (NAD^+, NADH) (Table I). Interest deserves the behavior of NaCl with respect to acetaldehyde: mixed (M) inhibition, *i.e.* competitive-uncompetitive (C-U) can be observed. The latter can be explained by postulating that the chloride anions do not form a ternary complex, E–NADH–Cl^-, yet form a complex with the product, *i.e.* E–NAD^+–Cl^-. Since a high chloride concentration was used, there was also competition with NADH.

In addition to the coenzyme-binding sites to which the chloride ions are directly bound there must exist on the enzyme molecule still another binding site permitting the formation of the E–NAD^+–Cl^- complex to occur. The uncompetitive behavior

of chloride ions with respect to acetaldehyde can be explained by postulating the existence of such a binding site. Since the chloride ions behave strictly competitively also with respect to ethanol it is reasonable to assume that the second binding site is the ethanol binding site.

The results of the kinetic measurements carried out in the presence of pairs of inhibitors competitive with respect to the coenzyme, *i.e.* of Cl^- -ions and *o*-phenathrolline, and of Cl^- and ADP have shown that the Cl^--ions bind to the site which is identical to the *o*-phenathrolline binding site. This conclusion follows from the value of the interaction constant α which for the inhibitor pair Cl^--ions–*o*-phenathrolline equals ∞. *o*-Phenathrolline binds to the central Zn-atom of the ADH molecule[36,37]. Because of the effect of the negative charge of the Cl^--ion the chloride could coordinate with the Zn^{2+}-ion.

The chloride shows a different binding site with respect to ADP which, as a partial analog of the coenzyme (NAD^+), is a competitive inhibitor of plant ADH[37]. The interaction constant of the pair ADP–Cl^- equals 8·0 and thus both inhibitors bind to different sites in the ADH molecule and are mutually repulsed in the complex with the enzyme. This repulsion can be explained by the negative charges of both ligands.

Irreversible inhibition of ADH by chloride ions has not been observed so far. Maize ADH is a dimer containing two identical subunits of molecular weight 30 000 (ref.[38]). There is no change in the arrangement of ADH subunits at 200 mmol l^{-1} concentration of NaCl.

Effect of NaCl *on Maize* PEPC *Isozymes*

Two PEPC isozymes isolated from maize leaves are inhibited by NaCl. The inhibition of PEPC isozymes by chloride ions is competitive with respect to PEP, hence the degree of the inhibition strongly depends on the substrate of PEPC isozymes (PEP)

TABLE I

Inhibition of maize ADH by chloride ions. Experimental conditions: 0·1 mol l^{-1} Na-phosphate buffer, pH 8·5, 0·1—0·5 mol l^{-1} NAD^+, 10—100 mmol l^{-1} ethanol, 1 μmol l^{-1} ADH, 0·1 to 0·5 mmol l^{-1} NADH, 0·2—1 mmol l^{-1} acetaldehyde, 0—200 mmol l^{-1} NaCl

Substrate	Inhibition type	K_i, mmol l^{-1}
NAD^+	C	240·0
NADH	C	138·0
Ethanol	C	100·0
Acetaldehyde	M(C-U)	95·0

(Fig. 1). Both isozymes are markedly inhibited by NaCl at low PEP concentrations. Isozyme PEPC I is more sensitive to NaCl than isozyme PEPC II.

Fig. 1 shows the kinetic dependence of NaCl binding to PEPC isozymes. The action of chlorides transforms the hyperbolic dependence of the reaction rate on substrate concentration observed with isozyme PEPC I to a sigmoidal plot (Fig. 1*a*) and the Hill constant n increases (Table II). Neither the kinetic dependence nor the value of the Hill constants are changed by the binding of chlorides to isozyme PEPC II (Fig. 1*b*, Table II). The action of NaCl leads to an increase in the values of Michaelis constants K_m for PEP with both isozymes (Table II).

The inhibition constant K_i with respect to PEP for isozyme PEPC I has a value of 12 mmol l^{-1} (Fig. 2) and a value of 90 mmol l^{-1} for isozyme PEPC II (graph not shown). The reciprocal plot of enzyme activity *versus* NaCl concentration according to Dixon and Webb[34] is nonlinear for isozyme PEPC I (Fig. 2) and this shows that more than one inhibitor molecule can bind to the PEPC I protein molecule[34]. Since sigmoidal kinetics is observed with PEPC I as a result of the action of NaCl we may conclude that NaCl acts as a negative allosteric effector of the PEPC I

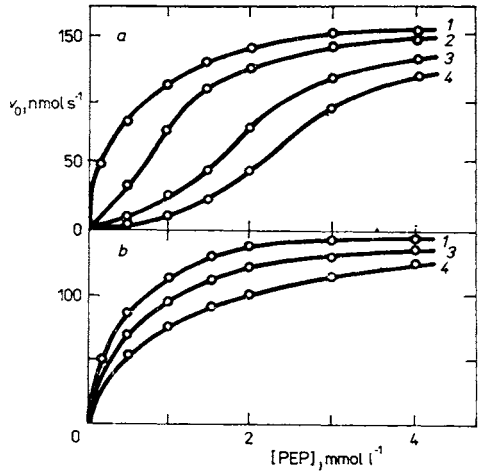

FIG. 1

Effect of NaCl on activity of *a* PEPC I and *b* PEPC II isozymes. *x*: PEP concentration; *y*: initial rate of enzymatic reaction (nmol of oxaloacetate s^{-1}). Experimental conditions: 50 mmol l^{-1} Tris-acetate buffer, pH 8·1, 10 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} $MgCl_2$, 0·1—5 mmol l^{-1} PEP, 1 μmol l^{-1} PEPC I and PEPC II. NaCl concentration, mmol l^{-1}: *1* 0, *2* 10, *3* 50, *4* 100

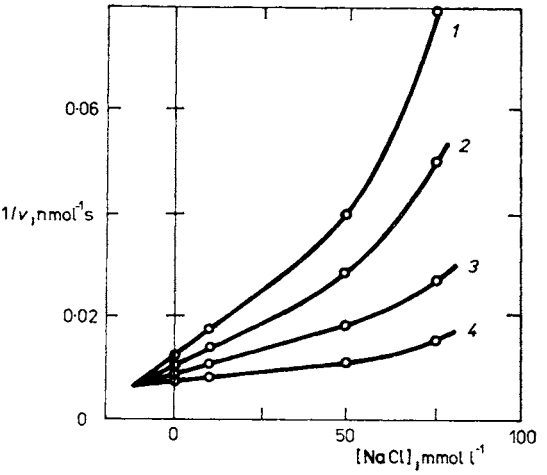

FIG. 2

Plot of reciprocal initial rate of reaction catalyzed by isozyme PEPC I *versus* NaCl concentration. *x*: NaCl concentration; *y*: reciprocal initial rate of enzymatic reaction (nmol^{-1} of oxaloacetate s). Experimental conditions: same as shown in Fig. 1. PEP concentration, mmol l^{-1}: *1* 1·0, *2* 1·5, *3* 2, *4* 2·5

isozyme and binds to the allosteric site of the protein molecule of this isozyme. The other binding site for Cl^--ions on PEPC I can thus be the allosteric binding site. It would thus appear that the reversible inhibition of the PEPC isozymes is merely the initial step of the irreversible inhibition. The incubation of both PEPC isozymes with NaCl results in a time-dependent inactivation of the isozymes (Table III). Isozyme PEPC I is more sensitive to this inhibition than isozyme PEPC II. PEPC I is totally inactivated by NaCl at a 200 mmol l^{-1} concentration in 4 days yet isozyme PEPC II is still partly active after this period (Table III). PEP is an agent which efficiently protects even against this inhibition (Table IV).

The effect of long-term action of NaCl on the isozymes of maize PEPC was examined also with respect to its interference with the tetramer structure of the protein molecule of the isozymes. Both isozymes are homotetramers build up of subunits of molecular weight 100 000 (PEPC I) and 89 000 (PEPC II) (ref.[13]). NaCl brings about dissociation of the tetramer structure of the isozymes into the dimer and the monomer. The dissociation is not complete, however, the tetramer is also present. Moreover, the presence of associates of molecular weight higher than that of the tetramer PEPC molecule was also observed (Fig. 3). Enzymatic activity was observed only together with the tetramer structure of the isozymes (Fig. 3). As follows from Fig. 3 the protein molecule of the PEPC II isozyme is more stable toward structural changes than PEPC I, as evidenced probably also by the lower sensitivity of the former to NaCl.

DISCUSSION

The present paper shows that both enzymes studied (ADH and PEPC) are negatively affected by chloride ions. The values of the inhibition constants show that ADH is

TABLE II

Effect of NaCl on kinetic parameters of maize PEPC. Experimental conditions: 50 mmol l^{-1} Tris-acetate buffer, pH 8·1, 10 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} $MgCl_2$, 0·1–5 mmol l^{-1} PEP, 0–100 mmol l^{-1} NaCl, 1 μmol l^{-1} PEPC I and PEPC II. *n*, Hill coefficient

NaCl concentration mmol l^{-1}	PEPC I		PEPC II	
	K_m mmol l^{-1}	*n*	K_m mmol l^{-1}	*n*
0	0·60	1·2	0·50	0·9
10	0·93	1·9	—	—
50	1·90	1·95	0·55	0·9
100	2·21	3·0	0·60	1·0

less sensitive to NaCl than PEPC. Hence, the metabolism at the stage of maize seed germination, in which ADH plays the key role, is far less affected by NaCl than the metabolic processes involved in photosynthesis in maize. PEPC is the key enzyme

TABLE III

Inactivation of isozymes of maize PEPC by NaCl. Experimental conditions: The incubation of the PEPC isozymes (10 μmol l^{-1}) was carried out in 50 mmol l^{-1} Tris-acetate buffer, pH 7·0, containing 0—200 mmol l^{-1} NaCl. Following 12—96 h incubation the activity of the PEPC isozymes was assayed on 0·1 ml aliquots in a medium containing 50 mmol l^{-1} Tris-acetate buffer, pH 8·1, 10 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} PEP, 1 μmol l^{-1} PEPC isozymes. The incubation of PEPC isozymes with NaCl was carried out at 0°C. The numbers in the Table indicate the activity of PEPC isozymes (I, II) expressed in % of activity at zero time

Incubation time h	NaCl concentration, mmol l^{-1}							
	0		50		100		200	
	I	II	I	II	I	II	I	II
0	100	100	100	100	100	100	100	100
12	101	100	83	99	68	93	51	72
24	100	100	78	98	49	89	40	50
48	98	100	62	85	30	78	20	35
72	98	98	55	73	18	61	7	28
96	97	96	51	70	10	55	0	20

TABLE IV

Effect of substrate (PEP) on inactivation of isozymes of maize PEPC by NaCl (c = 200 mmol l^{-1}). Experimental conditions: see Table III, except that 5 mmol l^{-1} PEP was present in parallel experiments

Incubation time h	PEPC I		PEPC II	
	without PEP	5 mmol l^{-1} PEP	without PEP	5 mmol l^{-1} PEP
0	100	100	100	100
12	51	73	72	80
24	40	58	50	62
48	20	43	35	49
72	7	40	28	45
96	0	38	20	44

for photosynthetic processes which proceed in maize leaves and its activity is limiting for the C_4 photosynthesis[39].

Since the binding of Cl^--ions to ADH is reversible and competitive with the substrates it can be eliminated by higher substrate concentrations. Irreversible ADH inactivation has not been observed and neither has been demonstrated a change in the subunit structure of the enzyme. Partial dissociation of ADH occurs only at 1 mol l^{-1} concentration of NaCl. The enzyme preparation must moreover be frozen before the dissociation in 1 mol l^{-1} NaCl and the process itself must must be performed at 2°C. Reassociation of the subunits to the dimer of molecular weight 60 000 occurs at 20°C.

NaCl inhibits also horse liver ADH (LADH). The inhibition constants K_i with respect to ethanol are identical for both ADH's (100 mmol l^{-1}). The inhibition by Cl^--ions with respect to NAD^+ and NADH are, however, far stronger than with

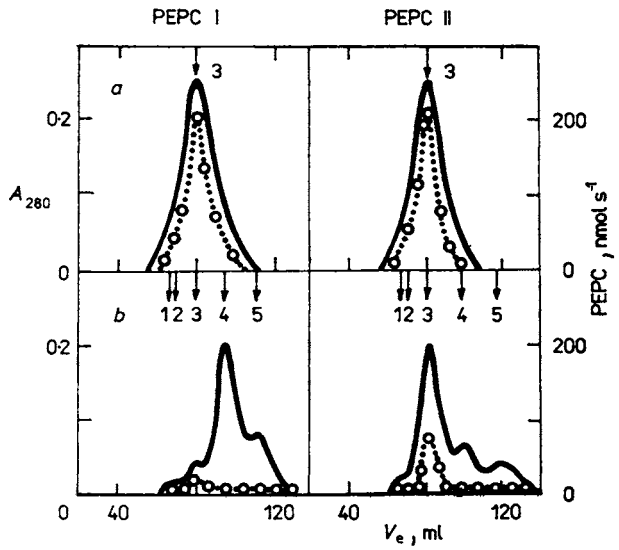

FIG. 3

Gel filtration of isozymes of maize PEPC on Sepharose 4B, *a* without NaCl and *b* with 100 mmol. l^{-1} NaCl. *x*: elution volume (ml); *y*: left-hand side, absorbance at 280 nm, right-hand side: PEPC activity (nmol of oxaloacetate s^{-1}). Experimental conditions: the samples of PEPC isozymes were in cubated 96 h with 100 mmol $^{-1}$NaCl in 50 mmol l^{-1} Tris-acetate buffer, pH 7·0 (10 μmol l^{-1} PEPC I and PEPC II) at 4°C and then chromatographed on a Sepharose 4B column (2 × 50 cm). Control sample (without NaCl) was chromatographed in a parallel run. Full line, proteins, dashed line, PEPC activity. Arrows 1, 2 designate the positions of high molecular weight associates and arrows 3, 4, 5 the positions of the tetra-, di-, and monomer, respectively. The same buffer in which the isozymes had been incubated was used for elution.

maize ADH (the K_i for LADH and competition with NAD^+ has a value of 60 mmol . $.l^{-1}$, for NADH a value of 30 mmol l^{-1}) (ref.[40]). At low chloride concentrations its binding to the phenanthrolline binding site of LADH is observed; binding to still another, so far unknown site, however, can be demostrated at high chloride concentrations[41]. The inhibitory effect on ADH of different origin is obviously of more general character since both plant and animal ADH's are affected by chlorides in a similar manner.

A far more complex effect of chlorides on PEPC isozymes has been observed. The binding of Cl^--ions is also competitive with respect to the substrate of the enzyme (PEP) yet a change in the kinetics of this reaction can be observed with PEPC I. The action of NaCl makes PEPC I to transform the hyperbolic kinetics of PEP binding to sigmoidal kinetics in the sense of positive cooperativity. PEPC I is an enzyme which shows sigmoidal kinetics under certain conditions. Such conditions constitute, above all, the effects of pH and several factors which lead to sigmoidal kinetics (effectors such as glycine, glucose-6-phosphate, glycerol) (refs[6,11,42]). The Cl^--ions can play a role similar to that of the above factors. The action of NaCl on PEPC I results in the appearance of sigmoidicity of the kinetics and the Hill constant also increases. It is apparent that the Cl^--ions bind to at least two sites in the PEPC I molecule. One of these sites is the PEP binding site. The other one is the so-called allosteric site since the binding of NaCl to PEPC I brings about the changes described above. Isozyme PEPC II does not show allosteric characteristics[6,11]. Hence, neither the Cl^--ions bring about its allosteric behavior.

Far more important than the reversible inhibition of PEPC is its irreversible inhibition. The latter obviously results from a change of tetramer arrangement of the quaternary structure of the PEPC molecule. Even though the substrate can also decrease this irreversible inhibition, this phenomenon is significant because the chloride content in maize tissues increases with the still increasing presence of salt in the soils[28] and because of its long-term action on the plant.

Additional studies on the regulation of NaCl-caused inhibition by the substrate of the enzyme (PEP) or other compounds are likely to provide valuable information on the possibilities of decreasing the negative effect of Cl^--ions on this enzyme of key importance.

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