EFFECT OF HEAVY METAL IONS ON BARLEY RIBULOSE-1,5--BISPHOSPHATE CARBOXYLASE

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Heavy metal ions (Cu^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}) in concentrations of 10^{-4} mol l^{-1} inhibit barley ribulose-1,5-bisphosphate carboxylase (RuBPC) (EC 4.1.1.39). The enzyme is inhibited most strongly by Cd^{2+} and Cu^{2+} and only very little by Zn^{2+}-ions. Cu^{2+} and Zn^{2+}-ions in concentrations of the order of 10^{-6} mol l^{-1} stimulate the RuBPC activity. The reaction of metal ions with the protein molecule of the enzyme is time dependent. The activation of the enzyme by Cu^{2+}- and Zn^{2+}-ions is the highest after 10-min incubation. Cd^{2+}-ions in concentrations of 10^{-6} mol l^{-1} lead to a time dependent irreversible inhibition of the enzyme.

Ribulose-1,5-bisphosphate carboxylase (RuBPC) (EC 4.1.1.39) is an enzyme catalyzing the so-called CO_2 dark fixation to ribulose-1,5-bisphosphate (RuBP) during photosynthesis in green plants and other photosynthesizing organisms.

RuBPC is localized in the stroma of chloroplasts[1]. It is a high molecular weight protein with subunit structure[2,3]. RuBPC consists of two subunit types, namely of eight large subunits and eight small subunits[2]. The molecular weight of the former is 51 000—58 000 and of the latter 12 000—18 000 according to the type of plant from which RuBPC was isolated[4]. The enzyme composed of the larger subunits is active even in the absence of the smaller subunits[4]. The larger subunit obviously is responsible for the catalytic function and the smaller one for the regulatory function[5,6]. The functions of both RuBPC subunits, however, have not been elucidated completely (there exists evidence of the necessity of the presence of the smaller subunits for catalysis by RuBPC in certain plant species) and have been the object of intensive studies[7]. Lysine[4] and cysteine[8] are present in the active center of the enzyme and are essential for its activity. The lysine residue is present in the binding site for RuBP and participates in the binding of this substrate to the protein molecule of the enzyme. The active center of the enzyme is being intensively studied[8].

The enzyme acts not only as a catalyst of RuBPC carboxylation but it also catalyzes RuBP oxygenation to glyceraldehyde-3-phosphate and to phosphoglycolate. The same active center of the enzyme is involved in both reactions[10-14]. The catalytic function of the enzyme requires activation in the presence of CO_2 and Mg^{2+}-ions. The activation of the enzyme proceeds at a slower rate than the CO_2 fixation to RuBP itself. CO_2 participating in the activation is not identical with CO_2 carboxylating RuBP.

The molecule of RuBPC contains firmly bound copper. It is not known as yet whether the latter affects the structural characteristics of the enzyme and whether it is necessary for substrate binding[15,16]. The role of Cu^{2+} in the catalysis of the oxygenation reaction has been discussed[17].

In view of the importance of this enzyme for photosynthetic processes in higher plants this study is designed to investigate the effect of those heavy metals on the enzyme which accumulate in increasing amounts in the environment. Two biogenous metals, Cu^{2+} and Zn^{2+}, which also represent essential ions for plants and are present in low concentrations in plant structures[18,19], have been chosen, and on the other hand, also Pb^{2+} and Cd^{2+} which represent inessential elements. The way in which the enzyme can be influenced by these compounds *in vivo* and *in vitro* is studied and their mechanism of action is discussed.

EXPERIMENTAL

Plant material and chemicals: The leaves of barley (*Hordeum vulgare* L., cv. Spartan) grown 3 weeks at 20–30°C in a greenhouse were used for the isolation of RuBPC. Tris-(hydroxymethyl)-aminomethane was from Merck AG, Darmstadt, F.R.G., 2-mercaptoethanol and dithiothreitol from Koch-Light Laboratories Ltd., Colnbrook, England. Sephadex G-25 and Sepharose 4B from Pharmacia Fine Chemicals, Sweden, DEAE-cellulose (DE-32) from Whatman--Maidstone, England, RuBP from Serva GmbH, Heidelberg, F.R.G., $Na_2{}^{14}CO_3$ from the Institute for Research, Production, and Utilization of Radioisotopes, Prague, Czechoslovakia and all the remaining chemicals (analytical reagent purity) from Lachema Brno, Czechoslovakia.

Isolation of barley RuBPC: Barley leaves (100 g) were homogenized in a blender and extracted with cold 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 2 mmol l^{-1} mercaptoethanol, 0·5 mmol l^{-1} EDTA, and 20 mmol l^{-1} $NaHCO_3$ (200 ml). The homogenate was filtered through two layers of gauze and the filtrate centrifuged 15 min at 15 000 g (4°C). The supernatant was fractionated by precipitation with ammonium sulfate. The fraction showing the highest specific activity of RuBPC (30—60% saturation with ammonium sulfate) was dissolved in the minimal volume of 50 mmol l^{-1} Tris-HCl buffer, pH 7·8 containing 2 mmol l^{-1} mercaptoethanol and desalted on a column of Sephadex G-25 (2 × 18 cm). The elution was effected by the above buffer. The fractions containing active RuBPC were pooled and chromatographed on a column of DEAE-cellulose (1 × 17 cm). The proteins were eluted by a linear $NaHCO_3$ gradient (0—0·5 mol l^{-1}) in 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 2 mmol l^{-1} mercaptoethanol (150 ml). The fractions with the highest protein content were pooled, the proteins precipitated with ammonium sulfate (70% saturation), centrifuged off, and dissolved in the minimal volume of 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 2 mmol l^{-1} mercaptoethanol. The RuBPC activity of these fractions was then assayed. The fraction with the highest RuBPC activity was subsequently chromatographed on a Sepharose 4B column (2 × 50 cm), and rechromatographed on the same column. The elution was effected in both cases by 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 2 mmol l^{-1} mercaptoethanol. The enzyme isolated was stored before the measurement in the form of an ammonium sulfate suspension (70% saturation) at 0°C in 50 mmol l^{-1} Tris-HCl buffer, pH 7·8 containing 0·4 mmol . l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} $NaHCO_3$, and 1 mmol l^{-1} mercaptotehanol. The same buffer was used for dilution whenever necessary.

The molecular weight of RuBPC was determined by gel filtration on a column of Sephadex 4B (2 × 50 cm). Urease (M_r = 483 000), maize phosphoenolpyruvate carboxylase (M_r = 400 000),

catalase (232 000) and γ-globulin (157 000) were used as standards. RuBPC was chromatographed on the above Sepharose 4B column which was also used for the standards. The elution was effected by 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 2 mmol l^{-1} mercaptoethanol. The molecular weight of the enzyme was read off from a graph in which elution volumes of standards were plotted versus the logarithm of their known molecular weights.

Assay of RuBPC activity: The enzyme (2 mg/ml) was dissolved before the assay in 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 0·4 mol l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} $NaHCO_3$, and 1 mmol l^{-1} mercaptoethanol. A 0·1 ml aliquot was pipetted into 0·5 ml of a reaction mixture containing 100 mmol l^{-1} Tris-HCl, pH 8·0, 0·4 mmol l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} dithiothreitol, 2 mmol l^{-1} RuBP, and 5 mmol l^{-1} $Na_2{}^{14}CO_3$. The reaction was terminated by the addition of 0·5 ml of 6 mol l^{-1} HCl after 5 min. The RuBPC activity was measured as ^{14}C fixed to the stable reaction product. The radioactivity of ^{14}C incorporated into this product was measured in a toluene scintillation solution in Packard TRI CARB 300 Scintillation Counter.

Effect of heavy metals on RuBPC in vivo: Barley seedlings were cultivated in solutions of heavy metal ions (concentration 1 μmol l^{-1} – 1 mmol l^{-1}) in deionized water. The metal ions were used in all experiments as sulfates and nitrate ($ZnSO_4$, 3 $CdSO_4$.8 H_2O, $CuSO_4$.5 H_2O, $Pb(NO_3)_2$). The shoots of the plants were homogenized after 7-day cultivation and RuBPC was extracted with 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 2 mmol l^{-1} mercaptoethnol, 0·5 mmol l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, and 20 mmol . l^{-1} $NaHCO_3$. The extracts were filtered through double gauze and centrifuged 15 min at 4°C and 15 000 × *g*. The RuBPC activity was measured in the supernatant after 15-min incubation of the extracts at 20°C. Aliquots of the extracts (0·1 ml) were pipetted into a reaction medium (total volume 0·5 ml)containing all the components necessary for Ru BPC activity assays (see above). The activity was measured as described above.

Effect of heavy metals on RuBPC isolated: The inactivation studies with metal ions were carried out in test tubes containing a total volume of 1 ml of the incubation medium. The following solutions were pipetted into the test tube: 0·7 ml of 50 mmol l^{-1} Tris-HCl buffer, pH 7·2, 0·1 ml of the metal ion solution (concentration 5 . 10^{-3}, 5 . 10^{-4}, and 5 . 10^{-5} mol l^{-1}) in the same buffer, and 0·2 ml of the enzyme solution (enzyme concentration 3–4 μmol l^{-1}). After 20 min of incubation at 20°C an aliquot (0·1 ml) of the mixture was pipetted into the reaction medium for RuBPC activity assay as described above. The buffer solution (0·1 ml) was pipetted instead of the metal solution (0·1 ml) into controls.

Effect of length of incubation on RuBPC inactivation by heavy metal ions: The inactivation was carried out as described above except that only one heavy metal ion concentration was used (5 . 10^{-6} mol l^{-1} in the test tube, 0·1 ml of 5 . 10^{-5} mol l^{-1} solution was pipetted). An aliquot (0·1 ml) of the incubation mixture was pipetted after 5, 10, and 20 min of incubation into the reaction medium for enzyme assays. The activity was measured as described above.

Effect of ligands on RuBPC inhibiton by Cd^{2+}-ions: The following solutions were pipetted into the incubation medium: 0·6 ml of 50 mmol l^{-1} Tris-HCl buffer, pH 7·2, 0·1 ml of 5 . . 10^{-5} mol l^{-1} Cd^{2+} in the same buffer, 0·1 ml of either 20 mmol l^{-1} Na_2CO_3 or of 20 mmol . . l^{-1} RuBP or of 20 mmol l^{-1} $MgCl_2$ in the buffer, and 0·2 ml of the RuBPC solution (enzyme concentration 3–4 μmol l^{-1}). The mixture was incubated at 20°C and an aliquot (0·1 ml) withdrawn after 5, 10, and 20 min of incubation was pipetted into the medium for RuBPC activity assays. The activity was determined as described above. The buffer (0·1 ml) instead of 0·1 ml of Cd^{2+} solution was pipetted into controls.

RESULTS

Isolation of Ribulose-1,5-bisphosphate Carboxylase

RuBPC was isolated by a procedure involving ammonium sulfate fractionation, desalting on a Sephadex G-25 column, chromatography on DEAE-cellulose, and chromatography and rechromatography on Sepharose 4B. The course of DEAE-cellulose chromatography is illustrated in Fig. 1, of the gel filtration on Sepharose 4B in Fig. 2. The enzyme was homogeneous as regards its molecular weight. When the protein peak showing enzymatic activity was rechromatographed on the Sepharose 4B column one symmetrical protein peak only with enzymatic activity was eluted.

The molecular weight of the enzyme determined by gel filtration was 550 000 ± 5 000, *i.e.* a value which corresponds to molecular weight values obtained with RuBPC's of higher plants[4].

Effect of Heavy Metal Ions on Ribulose-1,5-bisphosphate Carboxylase

Extracts were prepared of the shoots of barley seedlings which had been cultivated in solutions of the ions examined for 7 days and the RuBPC activity of the extracts was determined. The activity was examined as a function of the increasing metal ion concentration in the culture medium (Table I).

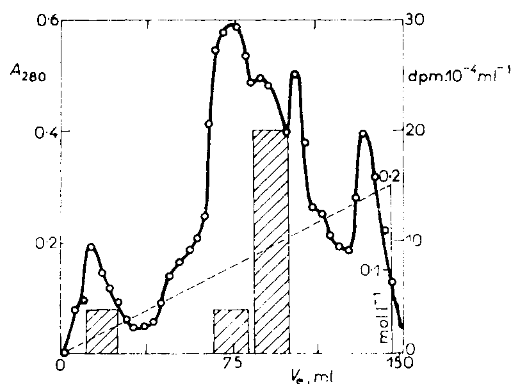

FIG. 1

Chromatography of RuBPC on DEAE-cellulose. ml elution volume; full line absorbance at 280 nm; dashed line NaCl concentration (mol l^{-1}); hatched columns RuBPC radioactivity in cpm . 10^{-4} ml^{-1}. The experimental conditions are described in the text

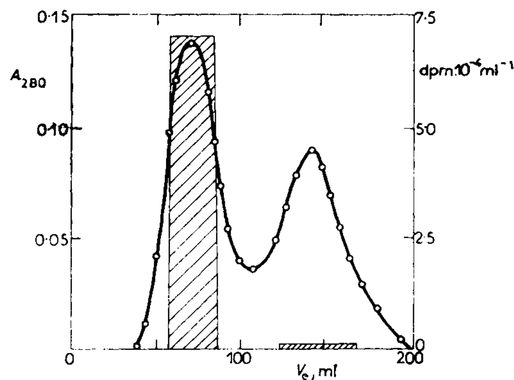

FIG. 2

Gel filtration of barley RuBPC on Sepharose 4B. ml elution volume; A_{280} absorbance at 280 nm; hatched columns RuBPC radioactivity in cpm . 10^{-4} ml^{-1}. The experimental conditions are described in the text

Cd^{2+}-ions are the most toxic ones for RuBPC activity in the *in vivo* experiments, next follow Cu^{2+}- and Pb^{2+}-ions whereas Zn^{2+}-ions are without any effect.

Having obtained the above results of the *in vivo* experiments we decided to investigate the effect of heavy metal ions also on the isolated enzyme.

The heavy metal ions investigated affect also the isolated enzyme. All metals in concentrations of the order of 10^{-4} mol l^{-1} markedly inactivate RuBPC (Table II). The strongest inactivators in in vitro experiments are Cd^{2+}- and Cu^{2+}-ions, next follow Pb^{2+}-ions, whereas Zn^{2+}-ions inactivate the enzyme at the highest concentrations only and on the contrary activate the enzyme at lower concentrations (Table II).

The reaction of the heavy metal ions with the protein molecule of the enzyme depends on the length of the preincubation period. The time profile of the reaction was examined with the lowest ion concentrations, 5 μmol l^{-1}, *i.e.* concentrations at which both biogenous (Cu^{2+} and Zn^{2+}) and exogeneous (Pb^{2+}, Cd^{2+}) metals are present in plant tissues. Inessential metals appear in plant tissues after the plants have been exposed to media containing higher concentrations of these metal ions[18]. Low concentrations of ions of the two biogeneous metals mentioned above activate the enzyme. The activation was most marked after 10 min of incubation. Cd^{2+}-ions, by contrast, cause a time-dependent, irreversible inhibition of the enzyme. Pb^{2+}-ions do not affect the RuBPC activity when used in these concentrations (Table III).

Cd^{2+} shows the strongest inhibitory effects and we decided therefore to examine how this inhibition is affected by certain ligands binding to the enzyme molecule.

Effect of Ribulose-1,5-bisphosphate, Na_2CO_3, and $MgCl_2$ on Inhibition of Ribulose-1,5-bisphosphate Carboxylase by Cd^{2+}-ions

RuBPC and Na_2CO_3, which liberate CO_2 in the reaction medium, affect the inactivation in different ways. Whereas RuBP is practically without any effect on the inactivation of the enzyme, Na_2CO_3 protects the enzyme almost completely (Table IV). Similarly to CO_2 also Mg^{2+}-ions playing the role of an activator in the catalysis by the enzyme prevent the enzyme from inactivation.

DISCUSSION

Heavy metals enter the tissues of higher plants, both the roots and the shoots, when the plants grow in media containing higher concentrations of these elements. An oversupply of metals to the plants leads first to limitation of root development resulting in a decrease of the adsorption of nutrients (P, K, Fe) to a level limiting plant growth[20]. Chlorosis is observed during the subsequent phase. It can be caused by higher concentrations of heavy metals in the plant leaves but its appearance has also been explained by an interference with the proper function of Fe in plants[18]. The totally impaired plant growth results in a strong decrease of the yield of the crop[21].

The present state of information on the effect of heavy metal ions on plant metabolism is meagre. Heavy metals affect certain enzymes present in the germinating seed[22,23]; heavy metals

inhibit chlorophyll biosynthesis[24], electron transfer during photosynthesis and also affect the transpiration[25]. Some heavy metal ions markedly inhibit also the enzyme participating in CO_2 fixation in the so-called C_4 plants, *i.e.* phosphoenolpyruvate carboxylase[26–28].

In view of the important role of RuBPC in photosynthesis in C_3 plants the manner in which this enzyme is affected by selected heavy metals was examined and the mechanism of their action is discussed.

TABLE I

Effect of heavy metal ions on barley RuBPC *in vivo*. Experimental conditions: RuBPC was extracted with 50 mmol l^{-1} Tris-HCl buffer, pH 7·8, containing 2 mmol l^{-1} mercaptoethanol, 0·5 mmol l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, and 20 mmol l^{-1} $NaHCO_3$. The activity of the enzyme was assayed in a reaction medium containing 100 mmol l^{-1} Tris-HCl, pH 8·0, 0·4 mmol . . l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} dithiothreitol, 2 mmol l^{-1} RuBP, and 5 mmol . . l^{-1} $Na_2{}^{14}CO_3$ (35 counts s^{-1} $nmol^{-1}$); *t* 20°C. The values given in the table show RuBPC activity expressed in % of the activity of the enzyme extracted from plants cultivated in 1 μmol l^{-1} metal solutions

Ion concentration in culture medium μmol l^{-1}	Metal			
	Cu^{2+}	Zn^{2+}	Pb^{2+}	Cd^{2+}
1	100·0	100·0	100·0	100·0
10	66·3	95·9	100·0	51·4
100	58·1	80·9	82·3	21·4
1 000	44·1	99·9	63·1	—[a]

[a] The plants did not grow.

TABLE II

Effect of heavy metal ions on RuBPC isolated. Experimental conditions: 50 mmol l^{-1} Tris-HCl, pH 7·2, 4 μmol l^{-1} RuBPC. An aliquot (0·1 ml) was pipetted after 20-min incubation in the metal solution into a reaction medium containing 100 mmol l^{-1} Tris-HCl, pH 8·0, 0·4 mmol l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} dithiothreitol, 2 mmol l^{-1} RuBP, and 5 mmol l^{-1} $Na_2{}^{14}CO_3$; *t* 20°C. The values in the table show % of activity of the enzyme preincubated in the absence of metal ions (incubation period 20 min)

Ion concentration in incubation medium μmol l^{-1}	Metal			
	Cu^{2+}	Zn^{2+}	Pb^{2+}	Cd^{2+}
5	109·0	178·0	99·5	39·0
50	31·0	208·0	83·8	26·0
500	0	59·0	53·6	16·0

Cu^{2+}-, Zn^{2+}-, Pb^{2+}-, and Cd^{2+}-ions affect the RuBPC activity in different manners. All metals inactivate the enzyme in concentrations of the order of 10^{-4} mol l^{-1}. The strongest inhibitors are Cd^{2+} and Cu^{2+}, the weakest inhibition is observed with Zn^{2+}. The inhibitory effect of heavy metal ions on certain enzymes can be explained by the formation of disulfide bonds between free SH-groups of the enzyme or by the formation of mercaptides with thiol groups[26] or by the formation of complicated unknown complexes[29]. The SH-groups of cysteine are present in the molecule of RuBPC where they are localized in the active center of the enzyme protein (Cys 173, Cys 458) (ref.[8]). These groups are essential for enzymatic activity[11,30]. The inhibitory effect of heavy metal ions examined in this study can be explained by the reaction of these elements with the SH-groups of cysteine which are essential for enzymatic activity.

We have also observed activation of the enzyme by low concentrations of Zn^{2+}- and Cu^{2+}-ions. The activation of RuBPC by low concentrations of Cu^{2+} can be

TABLE III

Effect of heavy metal ions on RuBPC isolated as function of the preincubation time. Experimental conditions: see Table II

Incubation time, min	Metal Cu^{2+}	Zn^{2+}	Pb^{2+} (5 μmol l^{-1})	Cd^{2+}
0	100·0	100·0	100·0	100·0
10	162·0	211·0	104·0	65·0
20	109·0	178·0	99·5	39·0

TABLE IV

Effect of ligands on RuBPC inhibition by Cd^{2+}-ions. Experimental conditions: see Table II. The values in the table show % of the activity of the enzyme preincubated in the absence of Cd^{2+}

Incubation time, min	Cd^{2+} (5 μmol l^{-1}) without ligand	+RuBP	$+Na_2CO_3$ (2 mmol l^{-1})	$+MgCl_2$
0	100·0	100·0	100·0	100·0
10	65·0	54·0	121·0	112·0
20	39·0	31·0	93·0	117·0

also related to the fact that copper is present in the enzyme molecule[15] and is therefore an endogeneous metal of the enzyme. Activation of RuBPC by Cu^{2+}-ions has also been observed in earlier studies[17,31,32] and an activating role of Cu^{2+} during the catalysis of the oxygenation reaction has been considered[17]. Zn^{2+} in low concentrations obviously plays the same role as endogeneous Cu^{2+}. This is also evidenced by the fact that RuBPC can be activated by ions of other metals, such as, *e.g.*, Co^{2+} (ref.[33]). Higher concentrations of these metal ions, however, attack the SH-groups of the enzyme and thus inactivate it.

The RuBPC inactivation caused by Cd^{2+}-ions is affected by enzyme substrates and activators. Whereas RuBP itself does not prevent the enzyme from inactivation, Na_2CO_3 protects the enzyme against inactivation. Similarly also act Mg^{2+}-ions, an activator. The protective effect of these compounds can have a physiological meaning since the compounds mentioned are naturally present as metabolites in the leaves of green plants and can thus interfere with the negative effects caused by certain heavy metals.

The mechanism of the protective effect of Na_2CO_3 and Mg^{2+} can be explained as follows: *1*) In case the enzyme is preincubated with Cd^{2+}-ions and with Na_2CO_3 a reaction giving rise to $CdCO_3$ takes place; this leads to a decrease of the instantaneous cadmium concentration in the solution. *2*) There is another possibility, namely that the formation of the enzyme–CO_2 complex from the enzyme and from the CO_2 liberated may render the SH-groups of the active center less accessible to Cd^{2+}. The protective effect of Mg^{2+}-ions can again be explained by their binding to the active center of the enzyme. We may also consider with both compounds a protection caused by conformational changes in the structure of the protein molecule of the enzyme which may occur during the binding of CO_2 or Mg^{2+}-ions[8]. These conformational changes in the enzyme structure may then make the SH-groups inaccessible for interaction with Cd^{2+}-ions.

The above hypothesized mechanisms of the protective effect of the two compounds will have to be verified in other experiments.

It follows from the inhibitory action of heavy metal ions on RuBPC activity that this enzyme may represent one of the target sites for the toxic effects of these metals during photosynthesis in C_3 plants.

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