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Reactivation of metal-requiring apoenzymes by phytochelatin–metal complexes

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The enzymatically inactive, metal-requiring apoforms of diamino oxidase and of carbonic anhydrase were reactivated by copper– and zinc–phytochelatin complexes, respectively. The level and the rate of reactivation effected by metal complexes consisting of poly(γ-glutamylcysteinyl)glycine as well as by the respective free metal ion were compared. An efficient transfer of zinc and copper from phytochelatin-complexes to apoenzymes was observed in vitro.

Metallo-enzyme; Phytochelatin; Metal transfer; Reactivation; Homeostasis

1. INTRODUCTION

We have postulated that metal-binding phytochelatins (PCs) are involved in heavy metal detoxification and homeostasis of essential ions such as zinc and copper in plants [1,2]. PCs are sulfur-rich peptides of the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, $n = 2\text{–}11$ [1,3] which are induced by a variety of heavy metal ions and appear to complex these ions by thiolate coordination. The peptides are synthesized by transpeptidation of glutamylcysteine residues from glutathione catalyzed by the metal-activated and constitutive enzyme phytochelatin-synthase [4]. A role of PCs in metal detoxification was inferred from experiments in which PC biosynthesis was inhibited [2,3,5] and from the analysis of mutants with reduced capacity to synthesize PCs [6] and a consequent hypersensitivity towards heavy metal ions. Furthermore, plant cells selected for increased cadmium tolerance were found to synthesize higher levels of the heavy metal-binding peptides [3].

In addition, PCs appear to be part of a homeostatic system which regulates the availability of zinc and copper ions in the plant cell. Thus, even the low levels of zinc and copper ions required for cultivation of plant cells in nutrient solutions provoke the induction of PCs [7]. The metal–PC complexes are subject to turnover since the PC level strongly decreases during onset of cell growth implying a release of zinc and copper ions [7]. Putative acceptor sites for the ions are inter alia apoforms of metal-requiring enzymes and zinc-finger-forming proteins, while a metal transfer to the vacuole may also occur [8]. A transfer of a metal ion from the metal–PC complex to a metal-requiring enzyme has up to now not been demonstrated and the question is addressed in this paper.

Abbreviation: PC_n, phytochelatin of n glutamylcysteinyl residues.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Diethyldithiocarbamate and dipicolate were from Sigma, all other chemicals were of the highest purity available from Merck, Sigma or Boehringer Mannheim.

2.2. *Apo-metalloenzymes*

Residual metal ions were removed from all buffers and glassware used for isolation and reactivation of apoenzymes as described [9].

2.2.1. Diamine oxidase

The diamine oxidase was isolated from leaves and stems of 9-day-old *Pisum sativum* seedlings (1.1 kg) according to [10]. The metal-free apoform of the copper enzyme was obtained by overnight incubation of the isolated protein (2 mg) in 1 ml of 50 mM potassium-phosphate buffer, pH 7.0, supplemented with 100 mM diethyldithiocarbamate (DIECA). The insoluble metal–DIECA complex was removed by centrifugation (13000 × g, 15 min) and the supernatant chromatographed on a Sephadex G-25 column (Pharmacia, PD-10 column) equilibrated with 50 mM potassium-phosphate buffer, pH 7.0. The protein fraction, as indicated by its UV absorption at 280 nm, was collected and used for subsequent reactivation experiments.

2.2.2. Carbonic anhydrase

The carbonic anhydrase isolated from bovine erythrocytes was obtained from a commercial source (Sigma, Munich). The zinc ion of the metallo-enzyme (1 mg/ml) was removed by successive ultrafiltration in the presence of 100 mM dipicolate in 200 mM potassium-phosphate buffer, pH 7.5, as described [11]. The concentrated enzyme solution was finally chromatographed on a column as described

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above except that 100 mM Tris-HCl buffer, pH 7.5, was used. The apo-enzyme was stored at −20°C.

2.3. *Metal–phytochelatin complexes*

The individual metal-free PC peptides (2–4 mg) were isolated and reconstituted with zinc and copper ions as described previously [12] with the exception that the metal/sulfhydryl ratio was 1 : 4. The PCs bound all the provided metal ions under these conditions, and the complexes were chromatographed on a Sephadex G-25 column (25 × 1.5 cm) in 5 mM Tris-HCl buffer, pH 7.5. Elution of the zinc- and copper-complexes was monitored at 230 nm and 250 nm, respectively.

2.4. *Reactivation of metallo-enzymes*

2.4.1. Diamine oxidase

The reconstitution of the apoform of the oxidase to the enzymatically active holoenzyme was monitored spectrophotometrically using putrescine as a substrate and recording the formation of chinazoline at 435 nm and at 25°C [13]. The assay was performed in the presence of 4 mM 2-aminobenzaldehyde, 2 mM putrescine, 0.01 mg purified enzyme reconstituted in the presence of varying levels of either free or complexed copper ions in 50 mM potassium phosphate buffer, pH 7.5. The reaction was started by addition of 100 μl enzyme solution which had been preincubated with the copper source for 30 min, unless otherwise stated. The total volume of the incubation mixture was 1 ml.

2.4.2. Carbonic anhydrase

The activity of the carbonic anhydrase was determined spectrophotometrically at 400 nm [14] following the cleavage of *p*-nitrophenyl acetate to *p*-nitrophenol and acetate according to [14]. The assay mixture consisted of 5 mM *p*-nitrophenyl acetate, 4 μg apoenzyme, varying levels of either free or complexed zinc ions in a volume of 1 ml 100 mM Tris-HCl, pH 7.5. The specific activity of the untreated holo-enzyme was 37 nkat/mg protein.

3. RESULTS AND DISCUSSION

3.1. *Reactivation of copper-requiring diamino oxidase*

The copper-containing diamine oxidase was purified 300-fold from pea seedlings as described [10] with a 48% yield of activity (990 nkat). The metal chelator DIECA was used to specifically remove the copper ions from the enzyme (specific activity 45.1 nkat/mg) resulting in a residual specific activity of 0.9 nkat/mg protein, i.e. 2% of the activity of the purified enzyme. Addition of various concentrations of $CuSO_4$ to the apoform of the oxidase produced a maximal reactivation of 80% of its initial activity at 10 μM copper ions (Fig. 1). This concentration corresponds to the copper level in the untreated holo-enzyme preparation. Higher copper concentrations resulted in an apparent inhibition of the enzyme activity (Fig. 1). In a previous publication, a copper-requiring apoform of the diamine oxidase from clover leaves was reconstituted by uncomplexed copper ions to the same maximal extent as our enzyme preparation [15].

In order to investigate whether metal–PC complexes could provide metal ions in a transfer reaction to metal-requiring apoenzymes we tested the reactivation of apoenzymes by zinc- and copper–PC complexes. Cu–PC complexes were incubated with apo-diamine oxidase and the enzymatic activity subsequently tested.

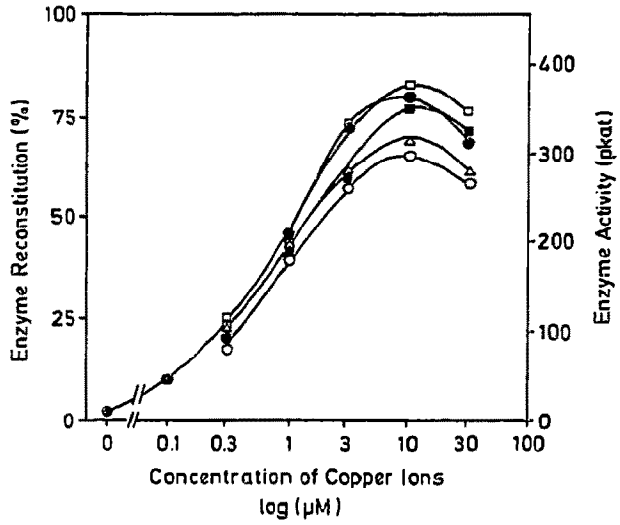

Fig. 1. Reactivation of the metal requiring apoenzyme of diamine oxidase from *Pisum sativum* by free copper ions and copper–phytochelatin complexes. The copper ions were supplied at varying levels as free ions (●—●), complexed to di(γ-glutamylcysteinyl)glycine (PC2, ■—■), tetra(γ-glutamylcysteinyl)glycine (PC4, △—△), penta(γ-glutamylcysteinyl)glycine (PC5, ○—○), or to the copper-phytochelatin complex as isolated from plant tissue (PCn, □—□) which consists predominantly of a mixture of PC2 and PC3. The level of reconstitution reflects maximal observed reactivation during an incubation period of 30 min.

The metal–PC complexes, consisting either of PC2 (di(γ-glutamylcysteinyl)glycine) or the PC mixture as isolated from plant cells containing predominantly PC2 and PC3, were comparably as effective as metal donors as was the free copper ion (Fig. 1). The reactivated enzyme exhibited an activity of 80% of its initial level at 10 μM $CuSO_4$, and 83% and 78% with the Cu–PC mixture from plants and the Cu–PC2 complex, respectively, providing an equal amount of copper. In contrast, the Cu–PC4 and Cu–PC5 complexes were less efficient in the reconstitution experiments with a maximal reactivation of 71% and 65%, respectively (Fig. 1). Enzyme activity in these experiments was determined after preincubation of the enzyme with the copper source for 30 min. Longer preincubation periods did not increase the degree of enzyme reactivation which indicates that a thermodynamic equilibrium between the different copper binding sites had been attained. However, reducing the preincubation period to as low as 1 min revealed substantial differences in the rate of metal transfer from the individual PC complexes to the apoenzyme compared to the free copper ions. These results are summarized in Fig. 2. Free copper ions most readily reactivated the diamine oxidase, while copper–PC complexes reactivated the apoenzyme at a reduced rate. Differences were particularly evident at copper levels of 3 and 10 μM. The rate of reactivation with 3 μM $CuSO_4$ (0.9 pmol/s^2) was approximately twice as high as that with PC complexes (0.5 pmol/s^2,

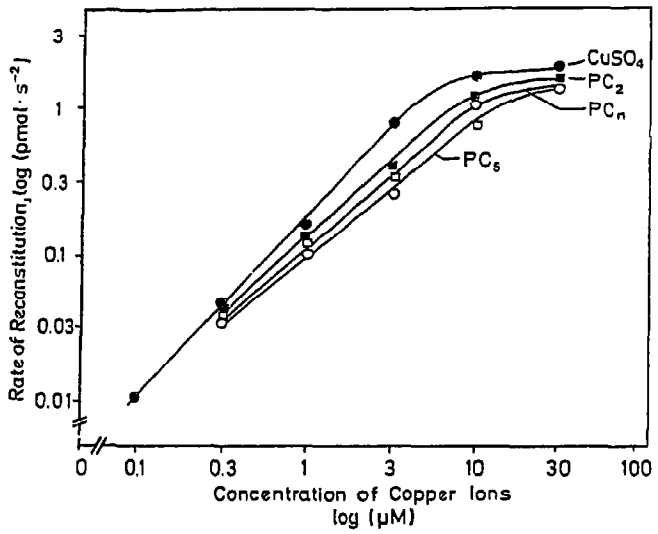

Fig. 2. Rate of reactivation of apo-diamine oxidase by free copper ions and copper–phytochelatin complexes. Rates of reactivation were determined by incubating the apoenzyme for 1, 3, 5, 8, 12, and 15 min with the copper source and were deduced from the linear phase of the reaction. Free copper ions were provided as copper sulfate solution. $CuSO_4$, PC2, PC5 and PCn as explained in the legend to Fig. 1.

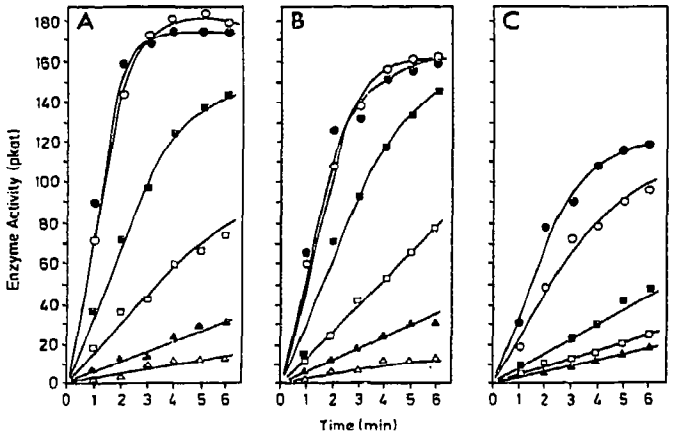

Fig. 3. Time course of reactivation of apocarbonic anhydrase by free zinc ions and zinc-phytochelatin complexes. The zinc ions were administered as (A) free zinc ions, or bound to (B) di(γ-glutamylcysteinyl)glycine (PC2) and (C) hepta(γ-glutamylcysteinyl)glycine (PC7) to the assay mixture containing the apoenzyme. The zinc was provided at 6 nM (Δ—Δ), 30 nM (▲—▲), 100 nM (□—□), 300 nM (■—■), 600 nM (○—○), and 1000 nM (●—●).

0.4 pmol/s^2 and 0.3 pmol/s^2 for PC2, plant Cu–PC and PC5, respectively). The rate of reconstitution as a function of the different PC complexes was generally higher for shorter peptide chains in the PC molecule, with differences in the range of 10–20% (Fig. 2). This could reflect different strengths of ion binding. The plant Cu–PC complex consisted predominantly of PC2 and PC3 [2], and produced intermediate rates compared to the numbers obtained with PC2 and PC5 complexes. In conclusion, the data imply an efficient transfer of copper ions from PC complexes to the plant apo-diamino oxidase in vitro.

3.2. *Reactivation of zinc-requiring carbonic anhydrase*

The largely zinc-free bovine carbonic anhydrase was obtained after removal of the heavy metal ions with dipicolate and subsequent gel filtration. The apoenzyme had a residual specific activity of 1.4 nkat/mg protein, i.e. 4% of that of the untreated holo-enzyme. Titration of the apo-enzyme (at 0.15 mg/ml, corresponding to 5 μM protein-bound zinc) with $ZnSO_4$ resulted in a complete reconstitution at 5–6 μM zinc ions, with specific activities (39 nkat/mg) even 5% higher than the specific activity of the untreated enzyme (data not shown). Addition of micromolar concentrations of zinc ions to the commercial enzyme preparation also increased its specific activity by 5% indicating an incomplete saturation of the original preparation with zinc ions.

In addition, reactivation of the apoenzyme was also achieved with Zn–PC2 (Fig. 3B) and Zn–PC7 (Fig. 3C) but to a lesser extent than with free zinc ions (Fig. 3A) at varying zinc concentrations. The highest level of reconstitution of the enzyme was 94% with the PC2 complex, and 70% with the PC7 complex, compared to 1 μM free zinc ions. The rate of enzyme reactivation also clearly differed between the various zinc donors. For instance, the rate of reactivation was 1.3 pmol/s^2 (100%) with 0.6 μM $ZnSO_4$, and at the same zinc level 0.9 pmol/s^2 (69%) and 0.4 pmol/s^2 (30%) with the corresponding PC2 and PC7 complex, respectively. The significantly lower rates were observed at all zinc concentrations used for reconstitution and indicate a substantially slower release of the zinc ions from PC peptides. In addition, there is also a striking difference between the PC complexes formed by peptides of different chain length with an approximately two-fold higher rate for the PC2 complex. At this point it remains elusive whether this finding reflects a difference in the diffusion rate of the individual PC complexes or a different affinity for zinc binding with a tighter metal sequestration in the complex consisting of longer PC peptides.

The lower degree of the reactivation of both the diamino oxidase and the carbonic anhydrase with metal complexes of higher PC members compared to the corresponding complex of PC2 points to a more efficient binding (higher binding constants) of the metal ions by longer PC molecules. In plants, zinc and copper ions predominantly induce PC2, and PC2 and PC3, respectively [2,8]. $Zn–PC_2$ complexes and also the Cu–PC complex isolated from plants were capable of reactivating apoenzymes in vitro to a degree comparable to that of the corresponding metal salt.

The metal PC complexes are, therefore, not only detoxification forms of heavy metals but are probably

also able to serve as metal ion donors in the plant cell and could thus fulfill a further prerequisite for a homeostatic function of the PC peptides.

The process of activating apoenzymes by the metal–PC complex, rather than by the free metal ion, offers the great metabolic advantage of lesser toxicity towards heavy-metal-sensitive enzymes (Kneer and Zenk, unpublished results). The synthesis of PC helps to prevent adverse reactions of heavy metal ions by their complexation and enables plants to adapt to changes in the supply of metal ions provided by the environment. The roles of PCs appear to be identical in this respect to the functions discussed for metallothioneins in the animal system [16].

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