THE EXOCELLULAR DD-CARBOXYPEPTIDASE OF *STREPTOMYCES ALBUS* G: A METALLO (Zn^{2+}) ENZYME

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1. Introduction

Early experiments [1] carried out with the exocellular, 18 000 M_r DD-carboxypeptidase excreted by *Streptomyces albus* G (in short the G enzyme) had shown that the activity of this enzyme was substantially inhibited by buffers, such as phosphate, which were able to complex divalent cations, and was suppressed by 2 mM ethylenediaminetetraacetate (EDTA). Since the presence of Mg^{2+} (or Ca^{2+}) seemed to be required for optimal enzyme activity and stability, the G enzyme was routinely purified and assayed in the presence of 2–5 mM $MgCl_2$ [2]. Contrary to various other DD-carboxypeptidases known to be serine–enzymes [3–6], the G DD-carboxypeptidase is, most likely, a metallo (Zn^{2+}) enzyme.

2. Materials and methods

All the reagents used were 'pro analysis' Merck compounds. The native G enzyme (95% pure) was in 10 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ [2]. The protein concentrations were estimated on the basis of an $A^{1\%}_{1\ cm}$ value of 10, at 280 nm. The DD-carboxypeptidase activity was measured by incubating the enzyme with 1.7 mM Ac_2-L-Lys–D-Ala–D-Ala at 37°C and by estimating enzymatically the amount of C-terminal D-alanine liberated [2]. The reaction mixtures were in 10 mM Tris–HCl buffer (pH 7.5) either as such or supplemented with various bivalent cations. The native enzyme was always assayed in the presence of 5 mM $MgCl_2$ (turnover no. = 150 min^{-1}).

The enzyme-bound Zn^{2+} or Co^{2+} were quantitatively estimated by proton-induced X-ray emission [7,8]. For this purpose, the target samples were prepared by depositing on 4 μm-thick propylene films, mixtures containing 10 μl protein solution (at 5–20 mg/ml), 20 μl 14 mM yttrium nitrate solution (used as internal standard) and 10 μl liposome suspension (in order to ensure a better homogeneity of the sample) [9]. The solvent was evaporated, and the film, stretched on a commercial 24 × 36 mm slide frame, was irradiated under vacuum by a 2.5 MeV proton beam (40 nA intensity) originating from the accelerator of the local Cyclotron Research Center. The beam, having diam. 10 mm, covered the entire sample, thus avoiding possible problems of non-uniformity of the target. After dialysis of the protein solution against the selected buffer, the protein-bound Zn^{2+} or Co^{2+} were estimated by subtracting the amounts found in the dialysis buffer from those found in the dialysed enzyme preparation.

3. Results

3.1. *Occurrence of ~1 Zn^{2+}/molecule of native enzyme and preparation of the apoenzyme*

Analysis of the native enzyme (pre-dialysed against the 10 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$) by proton-induced X-ray emission revealed the presence of 0.75–0.85 Zn^{2+} equiv. bound/18 000 M_r protein molecule. This result was surprising since neither the culture medium used for the *Streptomyces* growth nor the buffers used at the various stages of the

enzyme purification had been supplemented deliberately with any zinc salt.

Two procedures were used to obtain preparations of the G protein with both low DD-carboxypeptidases activity and low Zn^{2+} content:

Apoenzyme 1: The native enzyme (1.8 mg protein in 300 μl of 10 mM Tris–HCl buffer (pH 7.5) + 5 mM $MgCl_2$) was supplemented with 30 μl 0.25 M EDTA and the mixture incubated for 20 min at 20°C (note that at 37°C, the protein underwent precipitation). Filtration of the solution through a 1.2 × 15 cm column of Sephadex G-25 (fine) pre-equilibrated against 10 mM Tris–HCl buffer (pH 7.5) (made in bidistilled water), yielded a protein fraction well separated from EDTA. This protein preparation contained 0.18 equiv. bound Zn^{2+} and had a residual activity of 23% (as determined in 10 mM Tris–HCl buffer (pH 7.5) in the absence of $MgCl_2$).

Apoenzyme 2: The native enzyme (10 mg protein in 400 μl 10 mM Tris–HCl buffer (pH 7.5) + 5 mM $MgCl_2$) was dialysed against 100 ml 100 μM EDTA in 10 mM Tris–HCl buffer (pH 7.5), for 40 h at 4°C; the dialysis was repeated twice (total time: 120 h). This protein preparation contained 0.05 equiv. bound Zn^{2+} and had a residual activity of 10% (as determined in 10 mM Tris–HCl buffer (pH 7.5) containing 5 μM EDTA final conc.).

3.2. *Effects of divalent cations on the DD-carboxypeptidase activity of the apoprotein*

Samples of apoenzyme 1 (2.5 μM final conc.) were supplemented with various cations (25 μM final conc.) in 10 mM Tris–HCl buffer (pH 7.5). The DD-carboxypeptidase activities thus measured in the presence of $ZnSO_4$, $CoCl_2$, $MnCl_2$, $CuSO_4$ and $MgCl_2$ were 120, 140, 30, 26 and 36%, respectively, of that of the native enzyme. In turn, 50 μl samples of apoenzyme 2 were dialysed for 24 h at 4°C against 100 ml solutions of various cations, made in 10 mM Tris–HCl buffer (pH 7.5). Aliquots (0.1 μl) were then supplemented with 50 nmol Ac_2-L-Lys–D-Ala–D-Ala (5 μl) and 30 μl 10 mM Tris–HCl buffer (pH 7.5) and incubated for 15 min at 37°C. The enzyme activities thus measured after dialysis against $ZnSO_4$, $CoCl_2$, $FeCl_2$, $NiCl_2$, $CdCl_2$ and $HgCl_2$ were 100, 120, 100, 19, 19 and 33%, respectively, of that of the native enzyme. Considering that the residual activities of the apoenzymes 1 and 2 were 23% and 10%, respectively, it follows that only the Zn^{2+}, Co^{2+} and Fe^{2+} were able to activate the apoprotein. Finally, 100 μl samples containing 0.5 mg apoenzymes 1 and 2 were dialysed against 100 ml 10 mM Tris–HCl buffer (pH 7.5) containing 10 μM $ZnCl_2$ and 10 μM $CoCl_2$, respectively. Proton-induced X-ray emission analyses showed that the reactivated apoenzyme 1 contained 0.8–0.9 equiv. bound Zn^{2+} (and no bound Co^{2+}), and that the reactivated apoenzyme 2 contained 0.15 equiv. bound Zn^{2+} and 0.89 equiv. bound Co^{2+}. In this latter case, the amount of bound Zn^{2+} was higher than that present in the apoenzyme 2 itself (0.05 equiv.), suggesting that the apoprotein had an especially high propensity to bind traces of Zn^{2+}.

3.3. *Partition of Zn^{2+} between enzyme and EDTA*

A 9 μM solution of the native enzyme (200 μl in 10 mM Tris–HCl buffer (pH 7.5) + 5 mM $MgCl_2$) was dialysed for 18 h at 4°C against 100 ml 100 mM Tris–HCl buffer (pH 8.0) containing 5 μM $ZnSO_4$. Samples of the dialysed solution were then supplemented with EDTA so that the final enzyme concentration was 4.5 μM, the final concentration in exogenously added Zn^{2+} was 2.5 μM, and the final EDTA concentrations were 3.5, 7.0, 10.5, 14.0, 17.5, 35, 70 and 170 μM, respectively. Note that even at the lowest EDTA concentration used (3.5 μM), the total concentration of Zn^{2+} (7 μM) was smaller than the sum of enzyme concentration + EDTA concentration (8 μM). Immediately after the addition of EDTA, the progressive decreases in enzyme activity were followed as a function of time at 37°C (fig. 1). These measurements showed that:

(i) At low EDTA concentrations, the enzyme effectively competed with the complexing agent for the limited amount of Zn^{2+} available;

(ii) The exchange reaction was rather slow (as shown by the shapes of the time versus activity curves);

(iii) At <20 μM EDTA, the enzyme activity stabilized itself at constant values (depending upon the EDTA concentrations) after ~30 min incubation.

From the residual enzyme activities thus measured (and assuming that the concentration of free Zn^{2+} was negligible), the ratio of the dissociation constant of the Zn^{2+}–apoenzyme complex (K_E) to that of the Zn^{2+}

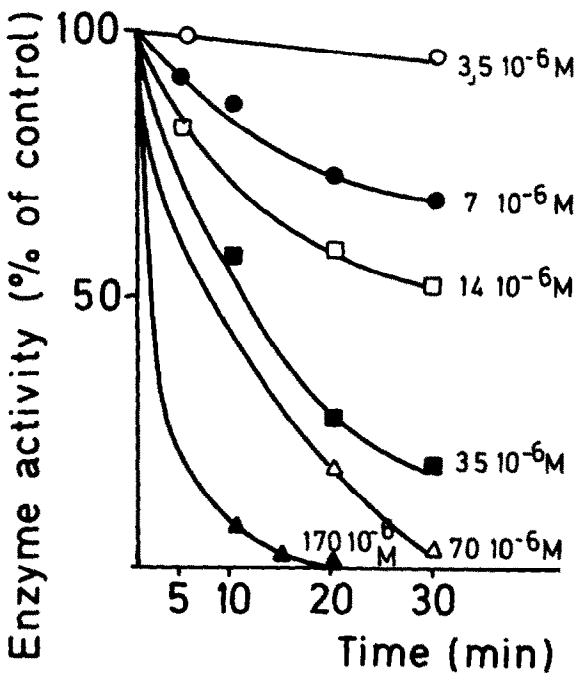

Fig.1. Competition between apoenzyme and EDTA for a limited amount Zn^{2+}. For conditions, see text. The enzyme activities were determined on 10 μl samples which where supplemented with 50 nmol Ac_2-L-Lys–D-Ala–D-Ala in 20 μl 10 mM Tris–HCl buffer (pH 8.0) and incubated for 5 min. The indicated concentrations are the initial EDTA concentrations.

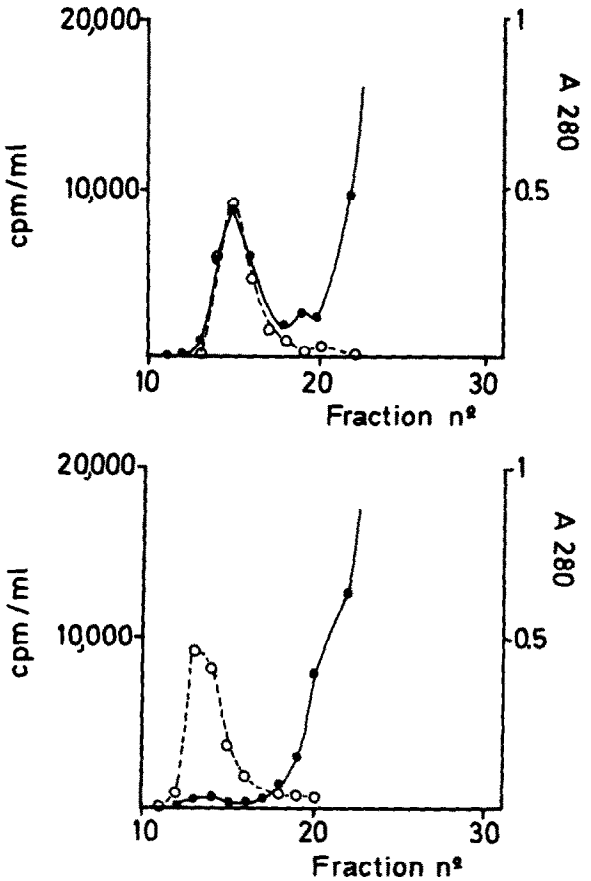

Fig.2. Influence of Zn^{2+} on the stable binding of [^{14}C]-benzylpenicillin by the enzyme. For conditions, see text. Fractions (0.65 ml) were collected. Protein concentration (○– –○) was estimated by measuring the A_{280}. The radioactivity (●– –●) was determined on 100 μl samples. (A) control without EDTA; (B) after preincubation in the presence of EDTA.

Zn^{2+}–apoenzyme complex (K_E) to that of the Zn^{2+}–EDTA complex (K_C) was estimated to be 0.60 ± 0.25. On the basis that the K_C value (at pH 8.0 and 37°C) is 0.8×10^{-14} M [10], the K_E value is $0.48 \pm 0.2 \times 10^{14}$ M.

3.5. *Effect of the Zn^{2+} on the ability of the enzyme to bind [^{14}C]benzylpenicillin*

The native enzyme binds benzylpenicillin to form a stable inactive complex. The process however is very slow so that even at high antibiotic concentrations and prolonged incubation times, a fraction of the enzyme remains non-combined with the antibiotic (detailed [11,12]). A 1.5 μM solution of the native enzyme (40 μl in 10 mM Tris–HCl buffer (pH 7.5) + 5 mM $MgCl_2$) was supplemented with 20 μl 50 mM EDTA solution and maintained at 22°C for 30 min. The solution was then supplemented with 20 μl of an aqueous solution of 30 mM [^{14}C]-benzylpenicillin (spec. act. 0.7 mCi/mmol) and maintained at 22°C for 5 h. A control sample was treated under the same conditions as above except that 20 μl water was used instead of EDTA. Filtration of the samples on a 1.2 × 15 cm column of Sephadex G-25 pre-equilibrated against 10 mM Tris–HCl buffer (pH 7.5) showed that in the presence of EDTA, binding of [^{14}C]benzylpenicillin to the protein was decreased by >90% (fig.2).

4. Conclusion

The native G DD-carboxypeptidase contains 1 equiv. bound Zn^{2+}/18 000 M_r protein molecule. The apoprotein binds the Zn^{2+} with $K_a \sim 2 \times 10^{14}$ M^{-1} and this Zn^{2+} cofactor is required for both DD-carboxypeptidase activity and binding of benzylpenicillin. Binding of benzylpenicillin to the apoenzyme, which is <10% of that measured with the

Zn^{2+} enzyme, is close to that observed with insulin and lysozyme [12,15]. The affinity and selectivity of the apoenzyme for the Zn^{2+} make it able to scavenge them from other competing cations present in the culture medium during growth of the *Streptomyces*, and to retain most of its zinc during the purification procedure (although Zn^{2+} are not deliberately added at any of the steps involved). It thus appears that the role played by $MgCl_2$, which was seemingly required for optimal enzyme activity and stability (see section 1), is only to provide enough Zn^{2+} to saturate the enzyme active center. The Co^{2+} also binds stoichiometrically to the apoenzyme, a feature which will permit NMR studies of the amino acid residues that may serve as ligands of the cofactor ion. Activation of the apoenzyme by Fe^{2+} has also been observed but whether or not this cation binds stoichiometrically to the protein is unknown. The above properties strongly support the view that the G DD-carboxypeptidase is a metallo (Zn^{2+} or Co^{2+}) enzyme, perhaps the counterpart of the β-lactamase II of *Bacillus cereus* [13,14]. The localization of the Zn^{2+} binding site in the close vicinity of the active site of the G enzyme, has been investigated by X-ray crystallographic studies. These results are presented in [16].

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