Volume 117, number 1 FEBS LETTERS August 1980

THE 4.5 Å RESOLUTION STRUCTURE ANALYSIS OF THE EXOCELLULAR DD-CARBOXYPEPTIDASE OF *STREPTOMYCES ALBUS* G

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Received 9 June 1980

1. Introduction

On the basis of their mechanistic properties, there exist two classess of DD-carboxypeptidases. The exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61 [1] and the membrane-bound DD-carboxypeptidases of several *Bacilli* [2,3], on the one hand, are serine-enzymes; they catalyse the attack of the sensitive amide bonds via the transitory formation of covalently serine-ester linked acyl-enzyme intermediates. The *Streptomyces* R61 enzyme has been crystallized [4]. The exocellular 18 000 M_r DD-carboxy-peptidase of *Streptomyces albus* G (in short the G enzyme) [5], on the other hand, is probably a metallo (Zn^{2+}) enzyme [6]. The apoprotein binds Zn^{2+} with K_a $\sim 2 \times 10^{14}$ M^{-1} and this Zn^{2+} cofactor is required both for activity on substrate analogues (e.g., Ac_2-L-Lys–D-Ala–D-Ala) and for binding of benzylpenicillin. The functioning of the active center, however, remains unknown. The G enzyme has been crystallized [7] into well-formed prismatic crystals (space group $P2_1$; unit cell dimensions $a = 51.1$ Å, $b = 49.7$ Å, $c = 38.7$ Å, $\beta = 100.6$ Å; 2 enzyme molecules/unit cell). This paper presents the 4.5 Å resolution structure analysis of the G enzyme and studies which have permitted visualization of the enzyme active center and localization of the Zn^{2+} binding site.

2. Materials and methods

Native crystals and heavy atom derivatives. The prismatic crystals used [7] had dimensions of $\sim 0.8 \times 0.5 \times 0.5$ mm. The stability of the crystals was checked under various pH conditions; no change in the diffraction patterns was observed at pH 5.5–8.0. Native crystals were soaked for various times, in solutions (made in the crystallization buffer) containing various concentrations of heavy atom compounds. Precession photographs showed that 5 mM K_2PtCl_4, 25 mM $NaUO_2(CH_3COO)_3$ and 5.2 mM $K_2Pt(C_2O_4)_2$ markedly affected the diffraction patterns.

Data collection and determination of the positions of the heavy atoms. The intensity data were measured using a Hilger and Watts 4 circle diffractometer with a Ni-filtered CuK_α radiation. Two equivalents (hkl and $h\bar{k}\bar{l}$) for the native enzyme and 4 equivalents (hkl, $h\bar{k}l$, $\bar{h}kl$, $\bar{h}\,\bar{k}\,\bar{l}$) for the derivatives were collected in shells of increasing θ values. The decay of the crystals was monitored using four standard reflections; a linear decay correction was applied to the data and an empirical absorption correction, as described in [8], was also used. The $\Sigma \mid\mid F_{PH} \mid - \mid F_P \mid\mid / \Sigma \mid F_P \mid$ ratio values (where F_P = structure factor of the native enzyme and F_{PH} = structure factor of the heavy atom derivatives) were 0.225, 0.119 and 0.158 for the K_2PtCl_4–, $NaUO_2Ac_3$– and $K_2Pt(C_2O_4)_2$–enzyme derivatives, respectively. On the basis of Crick-Magdoff plots, the K_2PtCl_4–enzyme derivative was the only one which showed lack of isomorphism. With the two other derivatives, the lower estimates of the heavy atom structure amplitudes, F_{HLE}, were calculated from the native protein and the derivative structure amplitudes and anomalous differences. The Patterson functions, with the F^2_{HLE} coefficients, were easily interpretable. The protein phases were calculated on the basis of the $K_2Pt(C_2O_4)_2$ derivative and the difference Fourier techniques were used to localize the Pt positions in the $PtCl^{2-}_4$

Elsevier/North-Holland Biomedical Press

derivative and to bring the $UO_2Ac_3^-$ derivative solution to the same origin. In all cases, the heavy atom coordinates, the relative occupancies and the temperature factors were refined by least-square procedures (table 1). Refinement was alternated with computation of difference Fourier synthesis in order to add any new sites if they were indicated. The 1200 reflections phased by the 3 derivatives gave a mean figure of merit of 0.86. The estimated errors of lack of closure E were calculated from the centric refinement and the E' values, for anomalous data, were set at 1/3 of the E values in the same angular shell.

Studies with enzyme inhibitors. The dipeptide *N*-acetyl-D-Ala–D-Glu (a competitive inhibitor of the hydrolysis of the substrate analogue Ac_2-L-Lys–D-Ala–D-Ala, [9]) and the β-lactam compound *para*-iodo-7-β-phenylacetylaminocephalosporanic acid (for a study of the action of the β-lactam compounds on the G enzyme, see [10]), were used. Crystals of the native enzyme were soaked in solutions of either 2.9 mM Ac-D-Ala–D-Glu or 10 mM Δ^3-cephalosporin, and the difference Fourier maps were calculated at 4.5 Å resolution.

3. Results and discussion

The parameters and R_F values of the heavy atoms in the corresponding enzyme derivatives are given in table 1. The electron density map, as calculated by using the best phases and the corresponding figures of merit, was contoured at arbitrary but equal intervals, plotted at a scale of 4 Å/cm and copied into plexiglass sheets which were stacked at the correct intervals. Fig. 1 shows views of the model thus obtained. The Fourier map phased by the 3 heavy atom derivatives, reveals the presence of 2 enzyme molecules/unit cell, surrounded by a very low electron density region. Each enzyme molecule can be inscribed in a 48 Å × 34 Å × 28 Å ellipsoid and consists of a 2 globular domains. The largest domain characterizes itself by a region of very high electron density, most likely the Zn^{2+} cofactor, in the vicinity of which occurs a deep cleft (20 Å × 6 Å × 6 Å) which roughly cuts the domain in two parts, thus allowing the solvent molecules to reach the presumed Zn^{2+} site.

The difference Fourier synthesis for the enzyme–dipeptide inhibitor complex gave one peak which was 3 times higher than any other feature in the map. This site of interaction is well visualized as a 12 Å segment of difference density elongated in the y direction of the map, inside the cavity and close to the Zn^{2+} site (fig. 1). The difference Fourier synthesis for the enzyme–β-lactam complex was much more noisy but the highest peak in the map was also located in the same cavity. All these observations strongly suggest that the active center of the G enzyme, where binding of the dipeptide and Δ^3-cephalosporin inhibitors occurs, is situated in the immediate vicinity of the Zn^{2+} site and support the view proposed [6] that, in contrast to the

Table 1
Heavy atom parameters and R_F values

Heavy-atom derivatives	Site no.	Relative occupancy of the site	x	y	z	R_F
K_2PtCl_4	Pt (1)	0.85	0.8009	0.3660	0.7699	
	(2)	0.50	0.2473	0.1158	0.1716	0.49
	(3)	0.27	0.8815	0.4814	0.5716	
	(4)	0.26	0.7459	0.2012	0.4254	
$NaUO_2(CH_3COO)_3$	U (1)	0.7	0.7140	0.4400	0.1659	
	(2)	0.6	0.6930	0.4800	0.1810	0.48
	(3)	0.2	0.7796	0.6659	0.3721	
$K_2Pt(C_2O_4)_2$	Pt (1)	1.02	0.1126	0	0.4422	0.29

$R_F = \Sigma \mid F_{HLE} - F_{HC} \mid / \Sigma \mid F_{HLE} \mid$

(F_{HLE}, see text; F_{HC}, heavy atom structure factor amplitude)

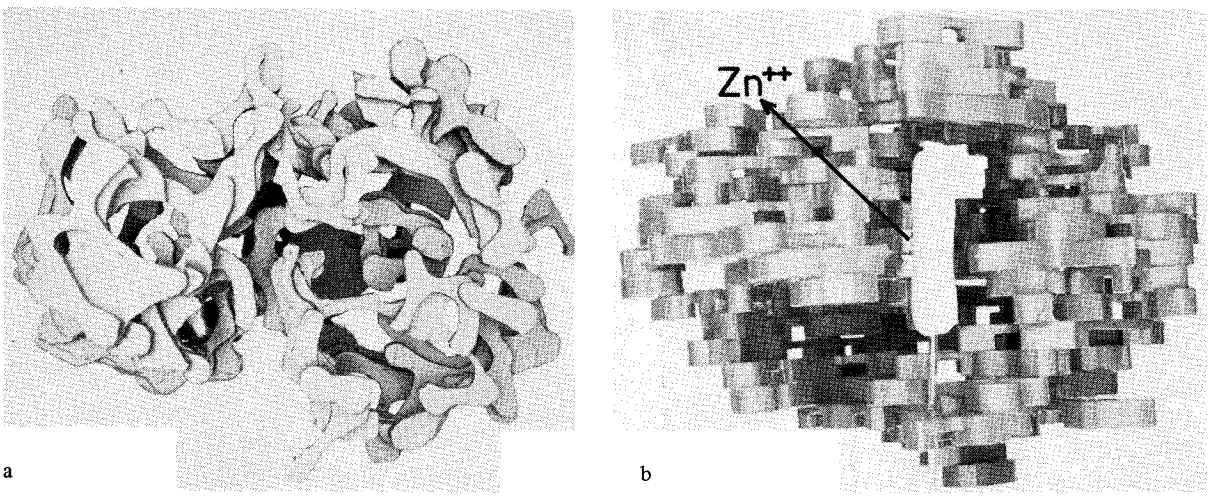

Fig. 1. Views of the model constructed from the electron density map. (a) Along the $\bar{b}$ axis. (b) Along (101). The difference density observed with the peptide inhibitor is represented in white, inside the cleft.

other DD-carboxypeptidases so far characterized, the G DD-carboxypeptidase–endopeptidase is a metallo (Zn^{2+}) enzyme.

Acknowledgements

The authors are grateful to Professor T. L. Blundell, Dr I. Tickle and Professor J. Toussaint for helpful advice. This work has been supported by the National Institutes of Health, USA (contract no. 2 RO1 13364 -04), the Fonds de la Recherche Scientifique Médicale, Brussels (contract no. 3.4501.79), the Actions Concertées (convention no. 79/48-11) and the Fonds National de la Recherche Scientifique, Brussels (contract no. S2/5-FG-E20). O. D. thanks the European Molecular Biology Organization for a research fellowship. P. Ch. is a fellow of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture, Brussels. We also thank UCB, Brussels, for financial support. Finally, the *para*-iodo-7-β-phenylacetylaminocephalosporanic acid was synthesized by Dr L. Christiaens, Service de Chimie organique, University of Liège.

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