RESEARCH COMMUNICATION

Experimental evidence for the essential role of the C-terminal residue in the strict aminopeptidase activity of the thiol aminopeptidase PepC, a bacterial bleomycin hydrolase

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PepCs isolated from lactic acid bacteria and bleomycin hydrolases of eukaryotic organisms are strict aminopeptidases which belong to the papain family of thiol peptidases. The structural basis of the enzymic specificity of the lactococcal PepC has been investigated by site-directed mutagenesis. The deletion of the C-terminal

residue (Ala-435) abolished the aminopeptidase activity, whereas this deletion led to a new peptidase specificity. The enzymic properties of wild-type and mutant PepCs demonstrate that the terminal α -carboxy group plays a key role in the strict aminopeptidase activity.

INTRODUCTION

Aminopeptidases are a class of peptidases widely distributed in Nature. They release free amino acids from the N-terminus of peptides and proteins. Although most aminopeptidases have been identified as metalloenzymes [1], thiol-dependent aminopeptidases have recently been found in both bacterial and eukaryotic cells.

Lactococcal PepC (Lc-PepC) is a cytosolic cysteine aminopeptidase isolated from *Lactococcus lactis* [2]. This enzyme displays a broad substrate specificity, capable of releasing all types of amino acids from the N-terminus of peptide substrates, with the exception of proline [2]. Amino acid sequence analysis revealed that Lc-PepC belongs to the papain family of cysteine peptidases, but the homologies between Lc-PepC and papain are restricted to catalytic and active-site residues [3]. Several genes coding for proteins which show a significant sequence homology with Lc-PepC have been cloned in lactic acid bacteria [4–6], yeast [7–9] and mammals [10–14]. All of these enzymes are organized as hexamers, composed of identical subunits of molecular mass 50–55 kDa, and display a strict aminopeptidase activity. In mammals, these cytoplasmic enzymes are responsible for the metabolic degradation of bleomycin, a glycopeptide antibiotic used in anti-tumoral therapy, and are called bleomycin hydrolases (BLMases) [15,16].

The X-ray-crystallographic structure of yeast BLMase [17] and Lc-PepC (M. Y. Mistou and D. Housset, unpublished work) has revealed that the C-terminus of both enzymes is projected on to the active-site cleft. This arrangement would permit the α -carboxy group of the enzyme to interact with the α -amino group of the substrate.

In the present paper, we argue that, if the C-terminal residue bearing the α -carboxyl group is responsible for the strict aminopeptidase specificity of Lc-PepC, its deletion should modify the specificity of the enzyme. We have used site-directed mutagenesis

to construct a mutant of Lc-PepC in which the C-terminal residue Ala-435 was deleted, the enzymic characterization of which supported our hypothesis.

EXPERIMENTAL

Site-directed mutagenesis

The *EcoRV/XbaI* (2.6 kb) fragment containing the *pepC* gene [3] was introduced into the *EcoRV/XbaI*-digested pUC19M plasmid. Mutagenesis was performed with the Transformer Mutagenesis kit (Clontech) on the basis of the method described by Deng and Nickoloff [18].

The mutagenic oligonucleotide CAATGGGAGCCTTGTAATTGTAAATTGAAAC was used to delete the last ATT codon coding for Ala-435. The selection oligonucleotide GTGACTGTGAGGCCTCAACCAAGTG suppressed the unique *ScaI* site in the mutagenesis vector and introduced a *StuI* site. The *EcoRV/TthI* fragment (2.2 kb) containing the mutated region was substituted for the non-mutated region in the lactococcal expression vector pTIL25 [19] and subsequently sequenced to confirm the deletion.

Expression and purification of recombinant enzymes

The wild-type and mutant PepC proteins were produced in the strain TIL90 of *L. lactis*. In this strain, the chromosomal *pepC* gene has been inactivated by insertion of a 3 kb gene conferring tetracyclin resistance in the *AatII* site (codon 346) using the pGhostV delivery plasmid [20]. The construction was verified by Southern-blot analysis. The absence of functional PepC was confirmed by Western-blot analysis and the lack of thiol-dependent aminopeptidase activity in cellular extracts.

The cytoplasmic extracts were prepared as previously described [19]. The wild-type and mutant (PepC Δ Ala⁴³⁵) enzymes were purified to homogeneity from cell extracts in three steps. The

Abbreviations used: Arg-pNA, arginine *p*-nitroanilide; Bz-Arg-pNA, α -N-benzoylarginine *p*-nitroanilide; BLMase, bleomycin hydrolase; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Lc-PepC or PepC, PepC isolated from *Lactococcus lactis*; St-PepC, PepC isolated from *Streptococcus thermophilus*; PepC Δ Ala⁴³⁵, mutant of PepC with an Ala-435 deletion.

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cytoplasmic extract was applied to a Resource Q column (Pharmacia) equilibrated with 50 mM Tris/HCl, pH 7.5 (buffer A) and the proteins were eluted at a flow rate of 3 ml/min with buffer A containing 0.2 M NaCl, followed by a linear salt gradient (from 0.2–0.7 M NaCl in buffer A). The fractions displaying activity against arginine *p*-nitroanilide (Arg-*p*NA) or α -*N*-benzoylarginine *p*-nitroanilide (Bz-Arg-*p*NA, determined as described below) were pooled and dialysed against 50 mM Mes/NaOH/0.5 mM EDTA, pH 6.0 containing 1.7 M ammonium sulphate (buffer B). The sample was then loaded on a Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with buffer B. The column was run at a flow rate of 1 ml/min. When the absorbance at 280 nm was negligible, elution was performed with 50% buffer C (buffer B without ammonium sulphate), followed by a linear salt gradient (50–100% buffer C). Active fractions against Arg-*p*NA or Bz-Arg-*p*NA were pooled and dialysed against 20 mM Mes/NaOH/0.2 mM EDTA, pH 6.5 (buffer D). The dialysed sample was applied to a MonoQ HR 5/5 column (Pharmacia) equilibrated with buffer D. The proteins were eluted at a flow rate of 0.5 ml/min with a linear salt gradient (from 0.4–0.7 M NaCl in buffer D). Fractions displaying activity against Arg-*p*NA or Bz-Arg-*p*NA were concentrated by ultra-filtration, dialysed against 50 mM Mes/NaOH/0.1 mM EDTA, pH 5.5 and stored at -80°C . Protein purity was confirmed by SDS/PAGE [21]. The C-terminal end of the mutant protein was sequenced (as described below) to confirm the deletion of the Ala-435 residue.

PepC from *Streptococcus thermophilus* was obtained as previously described [4].

Sequence of the C-terminal end

The purified enzymes were cleaved with a 100-fold molar excess of CNBr in 70% formic acid (v/v). After 24 h at room temperature, the peptides produced by CNBr treatment were injected into a reversed-phase HPLC C-18 column (150 $\times$ 4.6 mm, 5 μm particle size, AIT Kromasil, Interchim, Montluçon, France) and eluted at 40°C with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 1 ml/min. Elution was monitored at both 214 and 280 nm. The first peak to be detected at 214 nm corresponded to the C-terminal peptide. The N-terminal sequencing was performed on a 494-Procise protein sequencer Applied Biosystem (Perkin-Elmer).

Enzyme assays

The enzymic activities against the chromogenic amino-acid derivatives Arg-*p*NA and Bz-Arg-*p*NA (Bachem, Voisins-le-Bretonneux, France) were determined at 37°C in the assay buffer [40 mM sodium phosphate/10 mM EDTA/10 mM dithiothreitol (pH 6.5)] containing DMSO at a final concentration of 10% (v/v). Initial reaction rates at various substrate concentrations were determined by measuring the release of *p*-nitroaniline at 410 nm in a continuous assay (ϵ 9600 $\text{M}^{-1}\cdot\text{cm}^{-1}$). In all experiments, the enzyme concentration was 2×10^{-7} M for wild-type PepC and 1.2×10^{-6} M for PepC ΔAla^{435} . To verify the absence of activity of wild-type PepC against Bz-Arg-*p*NA, the concentration used was 1.2×10^{-6} M. Protein concentrations were determined by the method of Bradford [22], with BSA as a standard. The molar concentration was calculated on the basis of the molecular mass of one monomer (M_r 49 700). The enzymic parameters K_m and $V_{\max}$ were obtained by non-linear regression analysis of the data to the Michaelis-Menten equation. k_{cat} was calculated as $V_{\max}$ divided by enzyme concentration. Non-linear regression analysis was performed with the computer package SigmaPlot 3.0 (Jandel Scientific, San Rafael, CA, U.S.A.).

The activity of PepC ΔAla^{435} was tested against several peptides: GGGG, GGGA, GGFL, GGYR, GGGGG, GGGGGG and YGGFM (metenkephalin), at a substrate concentration of 1 mM, with the conditions described above. Substrate and products were separated by reversed-phase HPLC after derivative formation with fluoren-9-ylmethoxycarbonyl chloride and identified by their retention time and N-terminal sequencing, as described in [23,24].

Kinetic parameters for the inactivation of wild-type PepC and PepC ΔAla^{435} by *trans*-epoxysuccinyl-L-leucylamido-(4-guani-dino)butane inhibitor (E64) (Sigma) were measured on Arg-*p*NA and Bz-Arg-*p*NA. Enzyme and inhibitor stock solutions were separately pre-equilibrated at 37°C , and the reaction was started by mixing both solutions at a final inhibitor concentration of 1×10^{-6} and 6×10^{-6} M for wild-type PepC and PepC ΔAla^{435} respectively. Samples were removed at suitable times, diluted into the assay buffer and the residual activity was measured on Arg-*p*NA (1 mM) and Bz-Arg-*p*NA (5 mM) for wild-type PepC and PepC ΔAla^{435} respectively. In these conditions where $[I] \ll [E]$, the apparent rates of inactivation (k_{obs}) can be obtained from the slope of the semi-logarithmic plots of the activity against time, and the apparent second-order rate constants for inactivation (k'_{+2}) were calculated as $k_{\text{obs}}/[I]$ [25].

For pH activity profiles, we used the following buffers: 100 mM sodium citrate (pH 5.0–5.8), 100 mM sodium phosphate (pH 5.8–8.0) and 100 mM Tris/HCl (pH 8.0–8.6). All buffers contained 10 mM dithiothreitol, 10 mM EDTA and 0.3 M NaCl to minimize the variation in ionic strength. It was found that, during the time course of the experiments, both the wild-type and mutant PepC were stable in the pH range 5.5–8.0. The concentrations of the substrates were kept constant (0.2 mM for Arg-*p*NA and 0.5 mM for Bz-Arg-*p*NA). Under these conditions, $[S] \leq K_m$, and the specificity constant k_{cat}/K_m is obtained from $k_{\text{cat}}/K_m = v_0/[S_0][E_0]$. The pH-activity curves were analysed according to a three-protonation-state model by non-linear regression analysis of the data to the equation:

$$(k_{\text{cat}}/K_m)_{\text{obs}} = (k_{\text{cat}}/K_m)_{\text{lim}} / (1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+]) \quad (1)$$

where $(k_{\text{cat}}/K_m)_{\text{lim}}$ is the upper limiting value of k_{cat}/K_m and K_1 and K_2 are the acid dissociation constants.

RESULTS

The alignment of the C-terminal amino acid sequences of the cytoplasmic thiol aminopeptidases reveals a consensus sequence

The three-dimensional structure of the yeast BLMase protein has revealed that the C-terminus was inserted into the active-site cleft with the carboxylate group of the C-terminal Ala-454 residue being located 0.32 nm (3.2 Å) from the catalytic residue Cys and pointed towards it [17]. This observation suggests that this structural organization could be important for the aminopeptidase specificity of the enzyme. In Figure 1, we aligned the C-terminal residues deduced from the DNA sequences of all the members of this group of enzymes known at present. This alignment reveals the existence of a consensus sequence WDPMG(A/S)LA. With the exception of Lc-PepC and the rat BLMase, all the enzymes show an extension of one to three residues beyond the consensus sequence. The presence of these additional residues is contradictory with the role postulated for the last residue. However, during the refinement of the X-ray-crystallographic model of yeast BLMase, the authors found no electron density corresponding to the lysine residue anticipated from the gene sequence [17]. The cloning and biochemical characterization of the streptococcal PepC (St-PepC) protein in our laboratory [4] prompted us to develop a method for sequencing

Lc-PepC	427	P	W	D	P	M	G	A	L	A	435			
St-PepC	435	P	W	D	P	M	G	S	L	A	S	K	445	
Lbd-PepC	438	P	W	D	P	M	G	A	L	A	F	D	F	449
Lbh-PepC	438	P	W	D	P	M	G	A	L	A	F	K	Y	449
y-BLMase	445	I	W	D	P	M	G	A	L	A	K	454		
r-BLMase	446	A	W	D	P	M	G	A	L	A	454			
h-BLMase	446	A	W	D	P	M	G	A	L	A	E	455		
ch-AP H	446	A	W	D	P	M	G	A	L	A	K	455		

Figure 1 Alignment of bacterial PepCs and BLMase C-terminal sequences

Numbers beside residues indicate their positions in the respective sequences. Abbreviations: Lbh-PepC, PepC from *Lactobacillus helveticus* [5]; Lbd-PepC, PepC from *L. delbrueckii* [6]; y-BLMase, bleomycin hydrolase from *Saccharomyces cerevisiae* [7–9]; r-BLMase, rat bleomycin hydrolase [13]; h-BLMase, human bleomycin hydrolase [11,12]; ch-AP H, chicken aminopeptidase H [14].

the C-terminal peptide of the purified enzymes. We have found that the C-terminal peptide of St-PepC beyond the methionine residue (Met-439) is GSLA. The serine and lysine residues deduced from the gene sequence are absent in the active protein. This finding indicates that the protein probably follows a maturation step which eliminates the supplementary amino acids.

This result, and the X-ray-crystallographic data obtained for yeast BLMase [17] and Lc-PepC (M. Y. Mistou and D. Housset, unpublished work), strengthen the hypothesis of an important role of the C-terminus in this class of enzymes. To confirm this hypothesis, we have constructed a mutant of Lc-PepC, in which the last residue (Ala-435) was deleted. The enzymic properties of this mutant (PepC Δ Ala⁴³⁵) were studied.

Deletion of Ala-435 abolishes the aminopeptidase activity of PepC and creates a new specificity

The chromogenic substrate Arg-*p*NA, classically used to characterize aminopeptidases, is efficiently cleaved by wild-type PepC (K_m 4.79 ± 0.27 mM; k_{cat} 7.41 ± 0.27 s⁻¹). For PepC Δ Ala⁴³⁵, no activity was detected at substrate concentrations below 3 mM. At and around 3 mM substrate concentration, a residual activity was found, albeit 10⁴ times lower than that measured for the wild-type enzyme. The near-absence of aminopeptidase activity of the mutant was confirmed on a wide range of chromogenic derivatives of amino acids, which were all hydrolysed by wild-type PepC (results not shown).

A new specificity was displayed by PepC Δ Ala⁴³⁵ on the N-terminal blocked substrate Bz-Arg-*p*NA (K_m 4.64 ± 1.25 mM; k_{cat} 0.03 ± 0.005 s⁻¹), whereas wild-type PepC did not show any detectable activity on this substrate. From the sensitivity of the spectrophotometric assay, we can state that the hydrolytic potency of wild-type PepC on Bz-Arg-*p*NA (5 mM) is at least 10² times lower than that measured for PepC Δ Ala⁴³⁵. It should be noted that the K_m value of the mutant for Bz-Arg-*p*NA is similar to that of the wild-type enzyme for Arg-*p*NA, whereas the k_{cat} value is 250-fold smaller.

The new specificity of PepC Δ Ala⁴³⁵ was confirmed by examining its hydrolytic activity on peptides. As opposed to wild-type PepC, which degrades all the peptides tested by releasing free amino acids [24], PepC Δ Ala⁴³⁵ was found to produce YG and GFM with metenkephalin, GG and GGG with pentaglycine and GG and GGGG with hexaglycine, whereas no product of hydrolysis was detected for the tetrapeptides (results not shown).

At a substrate concentration of 1 mM, we estimated the initial rate of hydrolysis of metenkephalin ($v = 0.96$ mol hydrolysed/min per mol of PepC Δ Ala⁴³⁵) to be 4 times higher than that measured on Bz-Arg-*p*NA.

The deletion of the C-terminal residue Ala-435 has a strong effect on the catalytic properties of PepC. This result led us to further study the enzymic characteristics of PepC Δ Ala⁴³⁵ on Bz-Arg-*p*NA.

Inactivation of wild-type and mutant PepC by the E-64 inhibitor

E-64 is an irreversible inhibitor specific for cysteine proteases which reacts with the thiol group of the catalytic cysteine residue. Kinetic parameters for the inactivation of wild-type PepC and PepC Δ Ala⁴³⁵ by the E-64 inhibitor were measured on Arg-*p*NA and Bz-Arg-*p*NA respectively. The rate of inactivation (k_{obs}) of PepC Δ Ala⁴³⁵ was 21.2×10^{-3} s⁻¹ ([I] 6 μ M), whereas k_{obs} for wild-type PepC was determined to be 0.68×10^{-3} s⁻¹ ([I] 1 μ M). By assuming a linear relationship between inhibitor concentration and the rate of inactivation [25], the apparent second-order rate constant for inactivation of PepC Δ Ala⁴³⁵ is 5-fold higher than that of the wild-type enzyme. The X-ray-crystallographic structure of the papain complex with E-64 revealed that the inhibitor interacts with the S subsites on papain [26]. In the case of PepC, these subsites are partially occupied by the C-terminal residues. The low reactivity of PepC and BLMase with E-64 [4,11] in comparison with papain [25] could be due to steric hindrance. The deletion of the C-terminal residue of PepC liberates space in the active site, and consequently improves the efficiency of the E-64 inhibitor by favouring its binding. Moreover, this result indicates that the reactivity of the catalytic residue (Cys-73) is not affected by the deletion.

PepC Δ Ala⁴³⁵ is more resistant to thermal denaturation than wild-type PepC

Enzymic assays on Arg-*p*NA and Bz-Arg-*p*NA were used to study the thermal stability of wild-type and mutant PepC, respectively. Figure 2 illustrates that the mutation seems to protect the enzyme against thermal denaturation. We interpret this result as a consequence of the modification of the specificity between the two enzymes: whereas a precise orientation of the C-terminal residue is required for the aminopeptidase activity of the wild-type enzyme, the new activity of PepC Δ Ala⁴³⁵ is

Figure 2 Heat stability of wild-type and mutant PepC

Wild-type PepC (●) and PepC ΔAla⁴³⁵ (○) were held in the assay buffer at indicated temperatures for 15 min. The residual activity was then measured at 37 °C in 40 mM sodium phosphate/10 mM EDTA/10 mM dithiothreitol, pH 6.5, and DMSO 10% (v/v) on Arg-*p*NA (5 mM) and Bz-Arg-*p*NA (5 mM) for wild-type PepC and PepC ΔAla⁴³⁵, respectively.

Figure 3 pH-activity profiles of wild-type and mutant PepC

Arg-*p*NA (0.2 mM) for wild-type PepC (●) and Bz-Arg-*p*NA (0.5 mM) for PepC ΔAla⁴³⁵ (○) were used as substrates, and initial rates of hydrolysis were measured and analysed as described in the Experimental section. The curves are the best-fit of the data to the three-protonation-state model.

probably more tolerant to the structural disorganization of the C-terminus. This interpretation is corroborated by crystallographic data which show that the last four C-terminal residues are not firmly held in the native structure of yeast BLMase [17] or PepC (M. Y. Mistou and D. Housset, unpublished work), and could therefore be mobile.

pH-dependence of wild-type and mutant PepC

The ionizations of the free enzyme and/or the free substrate which influence the catalytic activity have been studied through the pH dependence of $(k_{cat}/K_m)_{obs}$. The mutant and wild-type enzymes show nearly the same pH optimum, about 7.0 (Figure 3), but the pH-activity profile is wider for the mutant enzyme. Apparent pK_a values of 6.0 and 8.2 have been obtained for

mutant PepC, which are significantly different from those obtained for wild-type PepC ($pK_{a1} = 6.6$ and $pK_{a2} = 7.2$).

The observed pK_{a1} value of 6.6 measured for the wild-type is in the range described for the yeast and human BLMases (6.9 and 6.8 respectively) [11]. Although this ionization constant cannot be decisively attributed to a discrete negatively charged group on the enzyme, its absence in the mutant enzyme suggests that it could be assigned to the α -carboxy group of Ala-435.

The value of pK_{a2} of 7.2 for the wild-type PepC could be attributed to the ionization of the α -amino group of the substrate, as has been proposed for the lysosomal thiol aminopeptidase cathepsin H [27]. The basic shift observed for the activity of PepC ΔAla⁴³⁵ on the N-terminal-blocked substrate Bz-Arg-*p*NA reinforces this hypothesis, and the new pK_{a2} value of 8.2 displayed by the mutant is similar to that found for other thiol peptidases. In papain, a pK_{a2} of 8–8.5 ensures the formation of the catalytically competent protonated form of His-159 [28]. Therefore the pK_{a2} value obtained for PepC ΔAla⁴³⁵ may reflect the deprotonation of the catalytic His-355.

DISCUSSION

The thiol peptidases which belong to the group of BLMases are characterized by their hexameric organization, cytoplasmic localization and strict aminopeptidase activity. This work focuses on the structural determinant responsible for the specificity of the lactococcal enzyme. We report that, if the C-terminal alanine residue of Lc-PepC is deleted, the enzyme loses its hydrolytic potency on *p*-nitroanilide-substituted amino acids, whereas it can now hydrolyse an N-terminal-blocked substrate. This result demonstrates that the aminopeptidase activity is dependent on the presence of the well-conserved C-terminal alanine residue in the active site. This hypothesis was initially proposed by Joshua-Tor et al. [17]. The pH-activity profile of PepC ΔAla⁴³⁵ measured on Bz-Arg-*p*NA has revealed a large modification of the ionization constants which drive the aminopeptidase activity. The modification of pK_a values in acidic and basic regions of the pH profile is in accordance with a model in which the protonated α -amino group of the substrate would interact with the negatively charged form of the C-terminal group, establishing a salt bridge. No structural data are available concerning cathepsin H, a close homologue of papain with prominent aminopeptidase activity, but convincing enzymic studies suggest a major kinetic contribution of a carboxylate group in the vicinity of the active site which would interact with the charged α -amino group of the substrate [27].

The K_m measured for Bz-Arg-*p*NA on PepC ΔAla⁴³⁵ is similar to that of the wild-type enzyme for Arg-*p*NA. This indicates that the binding of the substrate is not significantly affected, probably because the deletion creates a more spacious active site which can accommodate the bulky benzoyl group. This hypothesis is supported by the enhanced efficiency of the specific inhibitor E-64 on PepC ΔAla⁴³⁵. The strong decrease of k_{cat} of PepC ΔAla⁴³⁵ on Bz-Arg-*p*NA suggests that the potential interaction between the α -carboxylate group of wild-type PepC and the α -amino group of the substrate is involved in the hydrolytic process. The salt bridge could increase the electrophilicity of the carbon atom of the peptidyl bond, thus favouring the nucleophilic attack by thiolate. Another possibility would be the stabilization of the transition state.

From the new hydrolytic profile observed for PepC ΔAla⁴³⁵ on peptide substrates, it appears that the active site can accept two residues on the N-terminal side of the hydrolysed peptide bond. The absence of activity on tetrapeptides suggest that at least three residues must be present on the C-terminal side of the

hydrolysed bond to obtain an efficient catalytic binding. This hypothesis is in accordance with the recently reported substrate-length-dependency of wild-type PepC, for which it was observed that optimal aminopeptidase activity was obtained on tetrapeptide substrates [24].

These results show that an original solution was found for restricting the specificity of this group of aminopeptidases. To our knowledge, these results constitute the first biochemical demonstration of the structural basis of the specificity of an aminopeptidase. The biochemical properties of PepC Δ Ala⁴³⁵ indicate that the chemical reactivity of the catalytic residues is preserved. This observation opens up the possibility of constructing new deletional mutants which will display new specificities, and consequently of constructing variants of lactic acid bacteria with new technological properties.

Owing to the highly conserved structural and functional properties of the thiol cytoplasmic aminopeptidases, our results can probably be applied to the other members of this group of enzymes. Our data provide the possibility of defining specific inhibitors for these enzymes via the attack of the active-site carboxylate group. Specific inhibitors are highly desirable as they could help to define the biological functions of mammalian enzymes. They could also constitute important therapeutical agents, which could enhance the action of bleomycin by limiting its metabolic degradation.

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