

Investigations into the Enzymology and Biotechnology of the  
Hyperthermophilic Carboxypeptidase (PfuCP) from the Archaeon  
*Pyrococcus furiosus*

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## Abstract

A novel metallocarboxypeptidase (PfuCP) from the hyperthermophilic archaeon *Pyrococcus furiosus* was purified and characterized to investigate its dependence on metal ion cofactors and to evaluate its suitability as a biotechnological tool for protein sequencing at elevated temperatures.

The crystal structure reveals a dimer of primarily  $\alpha$ -helical subunits that bears no resemblance to the  $\alpha/\beta$ -hydrolase morphology of typical carboxypeptidases and which defines a new family of HEXXH metalloproteases (M32) based on primary sequence alignments. A deep active site groove appears to function not only in size-selection of substrates but also in modulating the activity and substrate affinity through complicated allosteric effects involving ambient ligands which may play a role in regulatory metabolism.

Two forms of the enzyme were observed; one which retains stabilizing metal(s) that confer structural thermostability and a remarkable retention of activity to the dimer, and another demetallated form which has lost stability with regards to both dimeric integrity and activity. Difficulties in expressing a properly folded recombinant necessitated refolding of the expressed clone from inclusion bodies and further suggest that *in vivo* the stabilizing metal(s) may participate in folding a metastable enzyme.

The apparent paradox of activation by only  $\text{Co}^{2+}$  and not  $\text{Zn}^{2+}$  is resolved into two issues, uncompetitive inhibition by the latter as seen in steady-state kinetic experiments, and intrinsic, electronic aspects of a catalytic  $\text{Co}^{2+}$ . Several explanations are proposed for the intrinsic rate enhancement of  $\text{Co}^{2+}$  over  $\text{Zn}^{2+}$  including the ability of  $\text{Co}^{2+}$  to modulate

the potential energy surface for both reactants and transition states by virtue of its greater mobility within the protein framework.

The broad amino acid specificity and rapid digestion by PfuCP in peptide sequencing trials show promise, and high-temperature protein sequencing has now been demonstrated for the first time.

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## Abbreviations and Glossary

CP	Carboxypeptidase
ICPMS	Inductively coupled plasma mass spectrometry
IMAC	Immobilized metal affinity chromatography
PES	Potential Energy Surface
Pf	<i>Pyrococcus furiosus</i>
Standard Assay Conditions	0.1 M KMes pH 6.5, 2 mM substrate, 400 $\mu$ M Co <sup>2+</sup> , 4 $\mu$ L enzyme
TSS	Transition State (Hyper)Surface
ZAX	N-Carbobenzoxy-Alanyl-Xaa (X amino acid, N-blocked dipeptide)