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PS3.10 — MO

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## STUDIES ON THE LANTHANIDE COMPLEXES OF SUBTILISINS

A large number of proteins contain strongly bound calcium ions, Ca(II), which are essential for their conformational stability and biological function. The electronic transitions of Ca(II) can not be studied by conventional spectroscopic techniques and this makes it difficult to study the respective binding sites. The trivalent lanthanide ions, Ln(III), possess physico-chemical and spectroscopic properties which make them suitable replacement probes for Ca(II) in calcium-binding proteins.

Subtilisins are a group of extracellular alkaline proteases of bacterial origin. They are stabilized by calcium against autolysis and thermal denaturation. In the present paper circular dichroism (CD) and proteolytic activity determinations were used for studying changes in the conformational and thermal stabilities of four subtilisins; mesentericopeptidase and subtilisins Novo, Carlsberg and DY.

## EXPERIMENTAL

Mesentericopeptidase and subtilisin DY were isolated in homogeneous state as described in [1] and [2], respectively. Subtilisins Novo and Carlsberg were received as a gift from Professor IB SVENDSEN (Carlsberg Laboratory, Denmark). The proteolytic activity was determined with casein as substrate. Circular dichroism was measured with a Rousel Jonan Dichrographe III instrument. The thermostability of the enzymes was measured in the sense of heat inactivation at 50°C. The solutions were incubated at 50°C for 24 h in the presence of 10<sup>-2</sup> M CaCl<sub>2</sub> or TbCl<sub>3</sub> or NdCl<sub>3</sub>.

## RESULTS AND DISCUSSION

The replacement of Ca(II) by Tb(III) or Nd(III) did not affect the dichroic properties and catalytic activity of subtilisins at neutral pH and room temperature, as judged from the CD spectra and proteolytic activity determinations. The retention of the biological activity can serve as an additional evidence that the native conformation has not been appreciably altered due to the substitutions. Fig. 1 shows that the Tb(III) or Nd(III) substitutions have no significant effect on the conformational stability of mesentericopeptidase at acidic pH. The behaviour of the other three subtilisins, after the respective replacements, was similar. The heat inactivation kinetics at 50°C showed that the substitution of the two lanthanides for Ca(II) lowers significantly the stability of all four subtilisins. This is illustrated in the case of mesentericopeptidase in Fig. 2. For example, after 5 h of incubation in the presence of Ca(II) at neutral pH, this enzyme preserves 78% of its caseinolytic activity, but it retains only 15-18% of the initial acti-

vity for the same period after the substitution. Probably, Ca(II) ions are better accommodated at the respective binding site than the lanthanides.

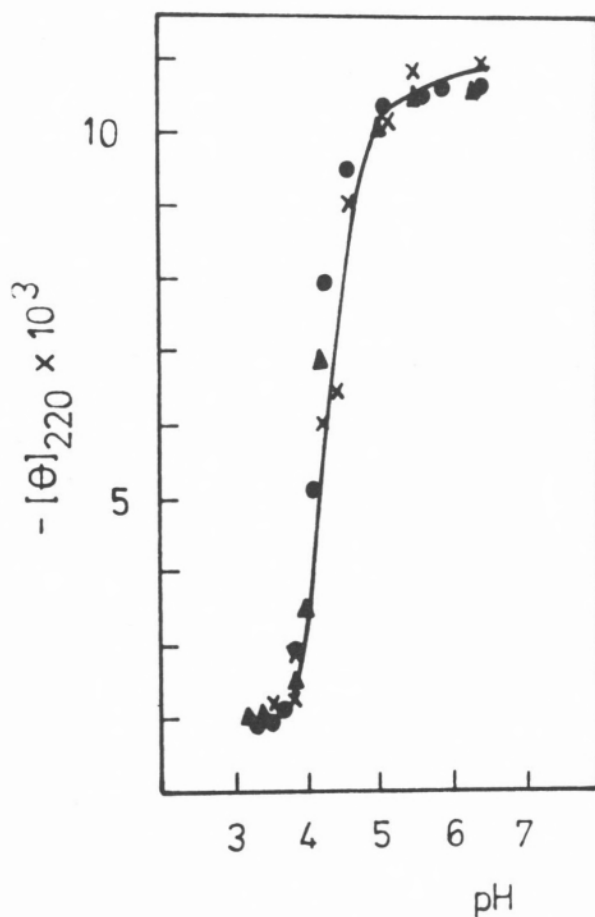


Fig. 1  
Effect of replacement of Tb(III) ( $\times$ — $\times$ ) or Nd(III) ( $\blacktriangle$ — $\blacktriangle$ ) for Ca (II) ( $\bullet$ — $\bullet$ ) on the ellipticity at 220 nm of mesentericopeptidase at room temperature

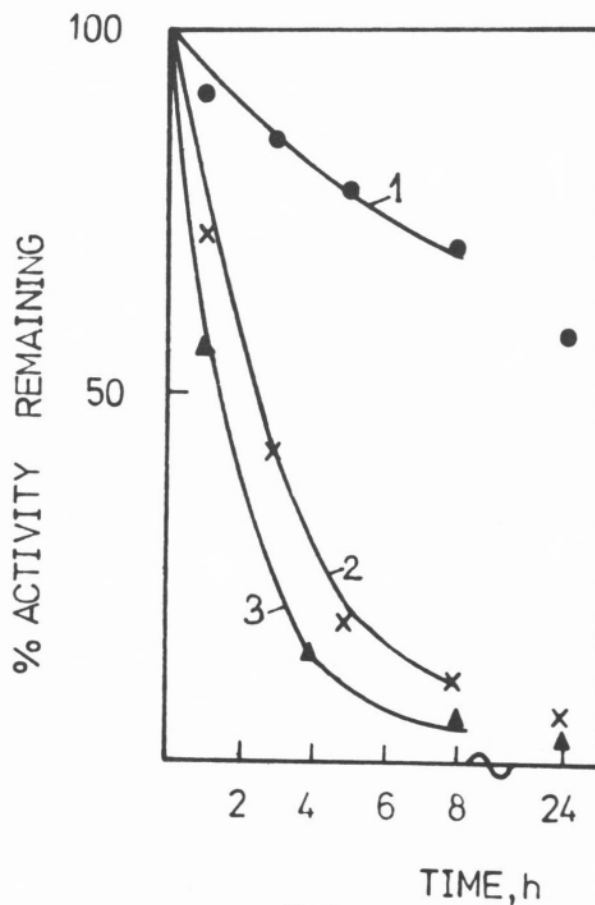


Fig. 2  
Effects of  $10^{-2}$  M  $\text{CaCl}_2$  ( $\bullet$ — $\bullet$ ),  $\text{TbCl}_3$  ( $\times$ — $\times$ ) and  $\text{NdCl}_3$  ( $\blacktriangle$ — $\blacktriangle$ ) on the heat inactivation at  $50^\circ\text{C}$  of mesentericopeptidase. Casein was used as a substrate

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## 4. Models for Metalloproteins



PS4.1 — MO

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### TRANSPORT OF HYDROGEN IONS BY A 4Fe-4S MODEL COMPOUND IN A DIRECTIONAL ELECTRON TRANSPORT SYSTEM.

The ability of  $[\text{Fe}_4\text{S}_4(\text{SC}_6\text{H}_5)_4]^{2-/3-}$  to cotransport electrons and hydrogen ions in a directional electron transport system has been examined. A sequential electron transport system was used, where aqueous Cr(II)edta was the electron donor, and  $(\text{CH}_3\text{N}((\text{CH}_2)_7\text{CH}_3)_3)_2[\text{Fe}_4\text{S}_4(\text{SC}_6\text{H}_5)_4]$  in toluene solution mediated the terminal reduction of methyl viologen in aqueous solution. The iron-sulfur complex,  $(\text{CH}_3\text{N}((\text{CH}_2)_7\text{CH}_3)_3)_2[\text{Fe}_4\text{S}_4(\text{SC}_6\text{H}_5)_4]$  was prepared by the addition of  $(\text{CH}_3\text{N}((\text{CH}_2)_7\text{CH}_3)_3)\text{Cl}$  (Aldrich), to the sodium salt of the cluster in methanol. The complex was isolated after storage at  $-40^\circ\text{C}$  and recrystallized from warm  $\text{CH}_3\text{CN}/\text{MeOH}$ . The complex is freely soluble in toluene, and as such is the first reported  $[\text{Fe}_4\text{S}_4(\text{SR}_4)]^{n-}$  complex soluble in a water immiscible solvent.

Reduction of the Fe-S complex was accomplished by vigorously agitating a mixture of aqueous Cr(II)edta with a toluene solution of the model for several minutes. In a typical experiment, 4.05 ml of 37 mM Cr(II)edta (pH 7.5) and 0.3 ml of 3 mM Fe-S complex were used, although the exact concentrations and volumes used varied between experiments. After waiting 10-20 minutes to allow

the immiscible aqueous and toluene phases to separate, a portion of the toluene phase was carefully transferred to a tube containing 3.00 ml of 10 mM methyl viologen in 0.5 mM tris/100 mM KCl buffer (pH 8). This mixture was agitated for approximately three minutes, and the immiscible phases were allowed to separate. The toluene phase was then removed from the top of the methyl viologen solution. Reduction was evidenced by the appearance of a deep blue color in the methyl viologen phase. The number of moles of reduced methyl viologen generated was measured optically. In all cases a drop in the pH was observed to be coincident with the reduction of methyl viologen. The average molar ratio of reduced methyl viologen to transported hydrogen ions ( $\text{MV}/\text{H}^+$ ) for these eleven experiments was  $1.18 \pm 0.24$ . The reduction potential of methyl viologen is pH independent, so it is assumed that methyl viologen does not bind hydrogen ions on reduction.

Evidence suggesting that electron transport between Cr(II)edta and methyl viologen was actually mediated by the Fe-S complex was obtained in several ways. After exposure of the Fe-S model to the aqueous Cr(II)edta phase, an EPR spectrum characteristic of reduced 4Fe-4S complex was obtained. While the g-values for this approximately axial spectrum ( $g = 2.02$ ,  $g = 1.90$ ) are not identical with reported values obtained in other solvents, some variation between solvents has been previously noted [1]. This observation of reduced Fe-S complex is consistent with optical experiments which show the characteristic bleaching expected for reduction of  $[\text{Fe}_4\text{S}_4(\text{SR})_4]^{2-}$  complexes [2]. After reoxidation by exposure to methyl viologen, the original optical spectrum was regained, with recoveries of the original absorbance typically between 85 and 100%.

Electron transfer experiments were performed in the absence of the Fe-S complex, and these yielded no reduction of methyl viologen. If  $\text{CH}_3\text{N}((\text{CH}_2)_7\text{CH}_3)\text{Cl}$  is included in the toluene phase (as a test of possible solubilization of the negatively charged Cr(II)edta complex), there still is no reduction of methyl viologen observed.

Optical studies were performed to quantitate the number of moles of reduced model compound after exposure to Cr(II)edta, and the number of moles of reduced methyl viologen ultimately pro-