Use of Ln^{3+} Optical Probes in the Study of Two Zinc-Metallo Peptidases that Hydrolyse the Enkephalins

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Received: August 28, 1989; November 26, 1989

A dipeptidilaminopeptidase (DAP) e a endopeptidase neutra 24.11 ou "encefalinase", são metalopeptidase que contêm zinco e que hidrolisam os pentapeptídios "opióides" encefalinas. A atividade enzimática, fortemente diminuída após a retirada do íon Zn^{2+}, pode ser recuperada entre 60 a 90% pela adição do mesmo íon, e entre 30 a 50% pelo acréscimo dos íons lantanídios trivalentes Eu^{3+} ou Tb^{3+}. A atividade enzimática aumenta com a concentração do íon adicionado, atinge um máximo e cai em seguida nas concentrações mais elevadas, tanto para o Zn^{2+} como para os lantanídios. Isto sugere fortemente que os Ln^{3+} substituem o Zn^{2+} no centro ativo das enzimas, o que é confirmado pelo estudo espectroscópico das soluções das enzimas ligadas ao Eu^{3+}. A complexação do Eu^{3+} é verificada para todas as concentrações estudadas desse íon. Aspectos distintos são observados nos espectros nas concentrações mais altas, correspondentes à faixa de queda da atividade enzimática, provavelmente associados à degradação das enzimas.

The dipeptidylaminopeptidase (DAP) and the neutral endopeptidase 24.11 (NEP) or "enkephalinase", are zinc metallopeptidases able to hydrolyse the opioid pentapeptides enkephalins. The enzymatic activity, strongly lowered after displacing the Zn^{2+} ion, may be recovered up to 60-90% by adding again the Zn^{2+} native ion or up to 30-50% by adding the lanthanide ions Eu^{3+} or Tb^{3+}. The activity is observed to reach a maximum before falling again at higher metallic concentrations in the same way for Zn^{2+} and the two trivalent cations. This strongly suggests that Ln^{3+} substitutes Zn^{2+} at the enzymes active sites, which is confirmed by the spectroscopic study of enzyme — Eu^{3+} solutions. The complexation of Eu^{3+} is evidenced at all europium concentrations studied. Specific features are observed at the higher metallic concentration, corresponding to the observed lowering in enzymatic activity, and probably to the degradation of the enzyme.

Key words: *endopeptidase, dipeptidylaminopeptidase, enkephalins, zinc metallopeptidase.*

Introduction

This study deals with two zinc metallopeptidases, the neutral endopeptidase 24.11 and the dipeptidylaminopeptidase. The neutral endopeptidase 24.11, NEP or "enkephalinase", is a zinc metallopeptidase initially isolated from the kidney brush border[1] and later found in the brain[2]. The physiological implication of this enzyme in the *in vivo* catabolism of the "opioid" endogenous pentapeptides Met-enkephalin and Leu-enkephalin, was demonstrated by the analgesia produced by NEP inhibitors, such as thiorphan[3]. The NEP hydrolyses the enkephalins at the Gly^3 - Phe^4 bond. The dipeptidylaminopeptidase, DAP, hydrolyses the Gly^2-Gly^3 bond of the enkephalins[4] but its physiological role is still obscure[5].

These two metallopeptidases contain zinc cation in their catalytic site, that probably participates in their mechanism of action, as indicated by comparison with the well known zincmetallopeptidases carboxypeptidase A[6] and thermolysin[7]. The NEP holds important analogies with the active site of thermolysin as confirmed recently by the cloning of the NEP c DNA and the determination of the enzymme primary sequence[8]. On the other hand, the DAP structure remains yet unknown.

The possibility of substitution of trivalent lanthanide ions Ln^{3+} for Ca^{2+} in calcium proteins is well known, owing to the similarity of their ionic radii (ionic radii = Ca^{2+} 0.99 Å, Ln^{3+} between 0.85 and 1.06 Å). Many investigations have been performed with Ln^{3+} (Eu^{3+} or Tb^{3+}) acting as an optical probe for the chemical and structural characteristics of Ca^{2+} containg targets[9-15]. In many cases, the *in vitro*, function of the biological molecule remains unaltered. To our knowledge, the sub-

stitution of Zn^{2+} for Ln^{3+} (ionic radius = 0.74 Å) in zinc proteins has not been described till now. There are examples of zinc-calcium proteins, like thermolysin[10,11,16] and insulin[13], in which only substitution of the Ca^{2+} has been performed.

In this way, the effect of substitution of Zn^{2+} for Ln^{3+} in the enzymes NEP and DAP was studied. Enzyme activities being maintained, spectroscopic study of these lanthanide substituted enzymes was undertaken. The experiments have been performed on frozen aqueous solutions of enzymes in presence of Ln^{3+}, allowing us to work on very small quantities of dilute solutions and to take profit of high resolution experimental methods usually employed for the investigation of inorganic solids.

Experimental

Chemicals (tyrosyl-3,5-^{3}H) D.Ala^2 Leu^5 - enkephalin (49 Ci/mmol) and (tyrosyl-3,5-^{3}H) Leu^5 - enkephalin (60 Ci/mmol), respectivelvy substrates for NEP and DAP, were purchased from Commissariat à l'Energie Atomique, Gif-Sur-Yvette, France. Porapak Q from Waters Associates, Milford, MA, USA. Terbium and europium chlorides (99.99% pure) from Johnson Matthey. The other reagents were from Prolabo, Merck and Sigma.

Enzymes preparation: the NEP was prepared from the rabbit kidney, by an already described method[17].

The DAP was purified from albino rat (*Ratus norvegicus*) brain, using a slightly modified literature procedure[4]. After decapitation the rats brains were rapidly placed in a cooled saccharose 0.32M solution and homogenized. The homogenate was centrifuged at 3200 rpm for 10 min, and the precipitate discarded. The supernatant was centrifuged again at 19600 rpm for 30 min and the pellet resuspended in 50 mM Tris pH 7.5 then centrifuged at the same rate. The resultant pellet was resuspended, washed and centrifuged two additional times. The membrane pellet was solubilized by resuspension in 25mM Tris pH 7.5 with 2% of Triton X-100, and incubation at 35 °C for 40 min, with agitation. Solubilized brain zinc-peptidases were obtained after centrifugation at 3600 rpm for 60 min.

DEAE — cellulose chromatography: the supernatant of the Triton soluble extract was applied to a DEAE — Sepharose CL6B column, previously equilibrated in 25 mM Tris pH 7.5. Enzymes were eluted with a linear salt gradient from 0 to 0.4 M NaCℓ.

The protein concentration and |^{3}H| Leu^5 - enkephalin hydrolysis were measured in the fractions of chromatography. In the tubes exhibiting the greatest enzymatic activity, NEP and DAP activities were measured, using |^{3}H| D. Ala^2 - Leu^5 - enkephalin or |^{3}H| . Leu^5 - enkephalin, in the presence or the absence of specific inhibitors: Triorphan (inhibitor of NEP), Captopril (inhibitor of ACE) and Bestatin (inhibitor of aminopeptidase). The fractions enriched in DAP were joined, concentrated by ultrafiltration, desalinized by gel filtration in Sephadex G-25 and chromatographed on AH - Sepharose 4B.

AH - Sepharose 4B chromatography: the DAP-rich fraction was applied to a AH-Sepharose column equilibrated with 25mM Tris pH 7.0 and 25 mM TRIS pH 7.7, and eluted with a linear salt gradient, from 0.15 to 1.0M NaCℓ in 25mM TRIS pH 7.0. On each fraction the protein concentration was measured. The fraction which produced only Tyr-Gly as hydrolysis product, in the presence of Bestatin, were considered as solution of DAP.

HPLC analysis: the HPLC analysis of the reaction products of enkephalin hydrolysis was performed as described[18]. Degradation products, obtained by incubation of the enzyme with Leu-enkephalin, at 25 °C for 3 hours, were identified by developing with 0.15 M sodium phosphate buffer pH 2.85, and comparison with the standards Tyr, Tyr-Gly and Tyr-Gly-Gly.

Enzyme assays: NEP was assayed by reaction with (tyrosyl-3,5-^{3}H) D.Ala^2. Leu^5- enkephalin, and the DAP with (tyrosyl-3,5-^{3}H) Leu^5- enkephalin. The reaction products were dosed by a method[19] in which the reaction mixture is eluted with water through a Porapak-Q column, and the eluate counted in a scintillation counter.

Preparation of Zn^{2+} free DAP and NEP: the enzyme solution was mixed with EDTA disodium salt, until a final concentration of 10^{-2}M. After a 2 hours incubation at 25 °C, the solution was dialysed first towards 10^{-2}M EDTA solution for 3 hours and then towards water or buffer solution for 16 hours.

Reactivation of Zn^{2+} free enzymes with metallic ions: the metallic ions were added to the Zn^{2+} free enzymes solutions, at different concentrations, and the enzymatic activity was measured by the method already described. The reactivation assays were performed with Zn^{2+} and the trivalent lanthanides Tb^{3+} and Eu^{3+}.

Spectroscopy: Zn - free solutions of NEP or DAP kept congelated at -20 °C, were reheated just before use. Fresh solutions of Eu^{3+} made by dissolution of europium chloride in adequate proportion (for final experimental concentration of 5.10^{-5} to 5.10^{-3}M) in each of the three solvents: 0.15M NaCℓ in distilled water, TRIS 50 mM pH 6.5 or piperazine 50 mM pH 6.5, were added to 10^{-6}M enzyme solutions in the same solvent. Although some measurements have been made on the liquid solutions, most of the observations have been performed on frozen drops of the solutions at 77 K. Observations at low temperature are possible on a small volume (aprox. 5μℓ) and show a higher fluorescence efficiency with respect to the liquid.

The emission of Eu^{3+} linked enzyme was excited by continuous or pulsed sources. The Eu^{3+} emission was analysed by a Coderg double monochromator spectrometer, and detected by a photomultiplier. The excitation was achieved by a high pressure mercury lamp, the filtered UV emission of which was mainly 260 and 360 nm, or by a pulsed nitrogen laser emiting at 337nm. Selective excitation in the Eu^{3+} $^5D_2$ level was done by a continuous argon ion laser (line at 465.8 nm), or a pulsed dye laser. The continuous signal, measured by a Keithley multimeter, as well as the pulsed signal, analysed via a Tektronix oscilloscope, were treated in a BFM microcomputer. By appropriate data processing, the excitation and emission spectra, as well as the emission transients may be recorded. For each experiment we have investigated in the same conditions the sample (enzyme + buffer + Eu^{3+}) and a blank without the enzyme.

Results and Discussion

The Zn^{2+} depleted DAP and NEP show a significant decrease in enzymatic activity, especially in the case of NEP. When we add Zn^{2+} solution, the activity is recovered until a maximum with 10^{-5}M metal concentration, and then falls another time for greater Zn^{2+} concentrations (Figure 1). While essential to the activity of DAP

and NEP, zinc can also inhibit the enzymes. There may be an additional site to which Zn^{2+} binds and cause inhibition. Similar effects have been observed with carboxypeptidase A[20] and thermolysin[21,22].

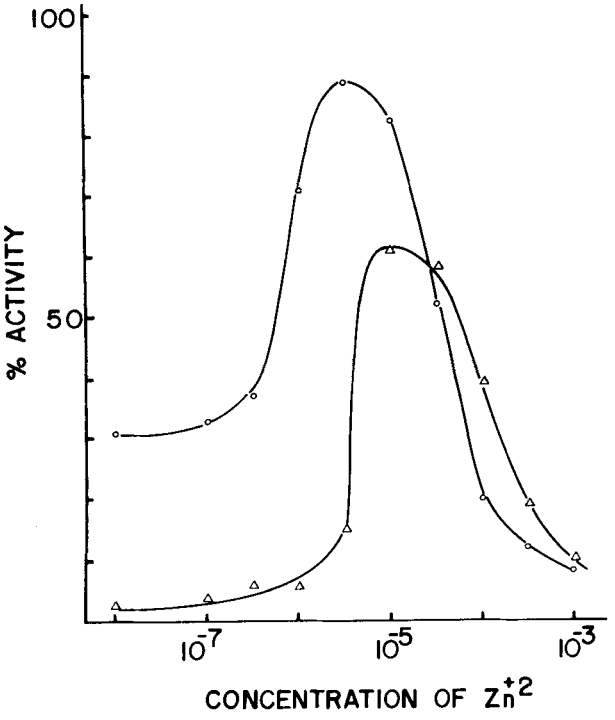

Figure 1. *Zn^{2+} depleted DAP and NEP reactivation by Zn^{2+} addition, in TRIS 50 mM pH 7.4 medium.*
o DAP activity curve
Δ NEP activity curve

A partial enzymatic activity of DAP and NEP may also be recovered by adding the trivalent lanthanide ions Eu^{3+} and Tb^{3+} (Figure 2). Note that both optimal ion concentration and reactivation curve profile, are similar to those of Zn^{2+}. This seems to prove that these trivalent lanthanides actually substitute Zn^{2+} at the enzymes active sites. The enzyme concentration is estimated to be 10^{-8}M, so that the optimal concentration corresponds to a large excess of metal ions.

The spectroscopic investigation has been perfomed on solutions approximately 10^{-6}M in NEP and DAP. Experimental evidences for the complexation of Eu^{3+} at concentrations ranging from 5.10^{-5} to 5.10^{-4}M in the low temperature experiments are of two kinds: the enhancement of the fluorescence intensity under nitrogen laser and more efficiently under U.V. lamp excitation, and the lengthening of the $^5D_0$ emitting level lifetime. In the example shown in Fig.3, the enhancement estimated with respect to the diffused light background, is about 20 times by addition of NEP. The observed emitting level decays are always non exponential. An average lifetime τ_{av} has nevertheless been calculated by linear regression in the range 300-600μs. The values reported in Table I for 5.10^{-4} Eu^{3+} show a increase of 20-30% by addition of the enzyme except in the case NEP/Eu in TRIS.

The observation of these two phenomena suffices to prove the complexation of part of the Eu^{3+} ions by the enzymes. In first approximation the longer $^5D_0$ average

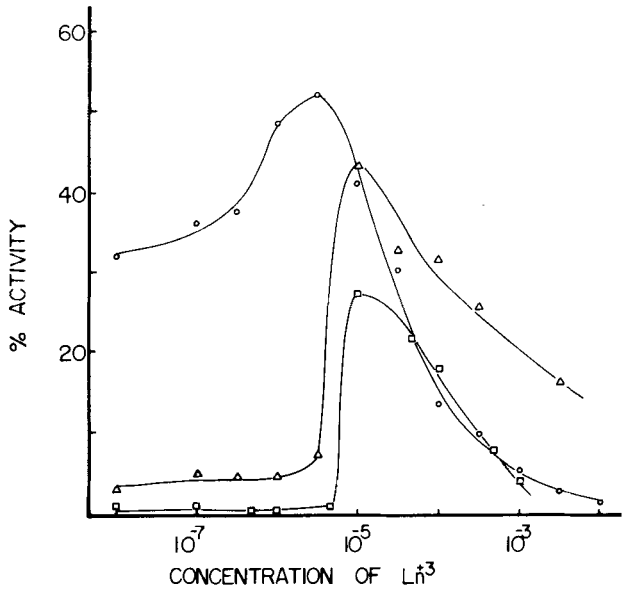

Figure 2. *Zn^{2+} depleted DAP and NEP reactivation by Tb^{3+} and Eu^{3+}, in TRIS 50 mM pH 7.4 medium.*
o DAP activity curve. Reactivation by Tb^{3+}.
Δ NEP activity curve. Reactivation by Tb^{3+}.
□ NEP activity curve. Reactivation by Eu^{3+}.

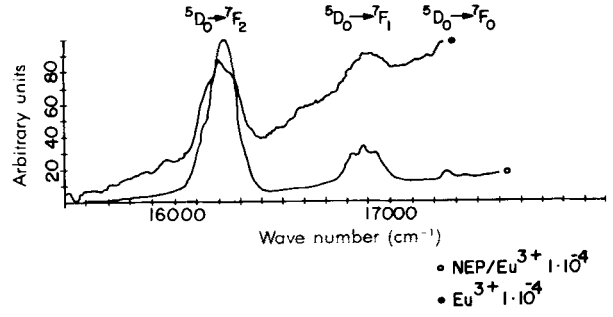

Figure 3. *Emission spectra of 1.10^{-4} M Eu^{3+} with and without NEP, in 0.15 M NaCl. Excitation by mercury lamp; T = 77 K.*

lifetime has to be attributed to less effective quenching by water molecules[10]. The fluorescence enhancement resulting from this effect would be the same for the different excitations. As we observe different enhancement factors associated with the presence of the enzyme (typically × 1 or 2 for nitrogem laser and × 10 or 20 for UV lamp excitations), this proves that differences in absorption efficiencies have to be considered. The high enhancement observed under UV lamp excitation may be due to a better watching of the $4f^n$ or CTS (charge transfer state) levels with the mercury lines for the enzyme-linked Eu^{3+} ions or to an efficient UV absorption by the protein (mainly the tryptophan residue followed by energy transfer to europium). In a study of parvalbumin the authors[23] have shown that very efficient tryptophan-Eu^{3+} CTS energy transfer does not induce fluorescent enhancement because of non radiative deexcitation from CTS. The non radiative processes are much less effective in the frozen solutions at 77 K than in liquid at room tempera-

ture (the experimental conditions for the study of parvalbumin) so that the protein-Eu^{3+} energy transfer may be thought to produce an emission enhancement in our particular experimental conditions.

At 77 K and for the lower Eu^{3+} concentrations studied (5.10^{-5} tp 5.10^{-4}M), besides the two effects described here above, no distinct feature in the decomposition and relative intensities of $^5D_0 \rightarrow {}^7F_{0,1,2}$ transitions may be attributed to the enzyme-linked europpium ions. The spectrum observed at room temperature under 465.8 nm excitation (argon ion laser) on a NEP/Eu 5.10^{-4}M solution shows an enhancement of the $^5D_0 \rightarrow {}^7F_2$ versus $^5D_0 \rightarrow {}^7F_1$ emissions (Fig. 4). This effect qualitatively traduces a lowering in the symmetry of the europium immediate environment associated with the complexation by the enzyme.

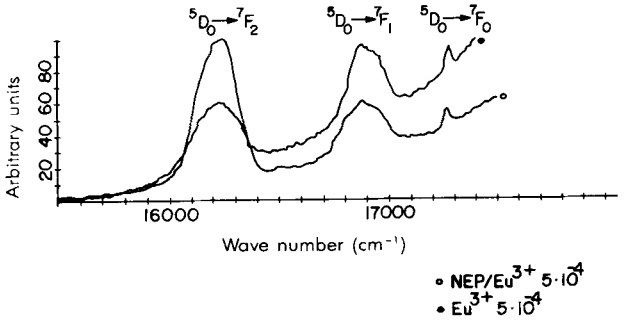

Figure 4. *Emission spectra of 5.10^{-4} M Eu^{3+}, with and without NEP in 0.15 M NaCl. Excitation by ion argon laser at 4658 Å; T=300 K.*

In the series of experiments performed at 77 K, attempts to correlate quantitatively the lengthening of τ_{av} or the fluorescence enhancement with the Eu^{3+} concentration were unsuccessful. Besides the fact that all the measurements have been made with an excess of Eu^{3+} and that both the enzyme linked and aquo-species participated in the emission spectra, it appears that different reactions occur when the metal ion, the enzyme and the buffer are mixed. One of these phenomena is evidenced at higher Eu^{3+} concentration (5.10^{-3}M) by the appearance of a distinct line in the $^5D_0 \rightarrow {}^7F_2$ trasition (line n° 3 in Fig. 5). The $^7F_0 \rightarrow {}^5D_2$ excitation spectrum recorded when monitoring this wavelength shows a very distinct component (line n° 3' in Fig. 6). Reciprocally the emission spectrum observed when selectively exciting this particular n° 3' wavenumber shows an enhancement of n° 3 with respect to n° 1 and 2. These complementary excitation/emission experiments confirm each other and permit to differenciate the spectroscopic characteristics due to a new species appearing at these europium concentrations in our samples. The average $^5D_0$ lifetime corresponding to this species is of 130 μs. Besides numerous examples in inorganic materials, the utility of high resolution spectroscopic techniques had been proven previously in the study of europium substituted thermolysin[24], but to our knowledge such techniques have not been applied to the study of dilute protein solutions. In the systems investigated here effective selectivity has not been observed for the lower Eu concentrations. This may be attributed part to the weakness of the signals that hinders to valuable experimental

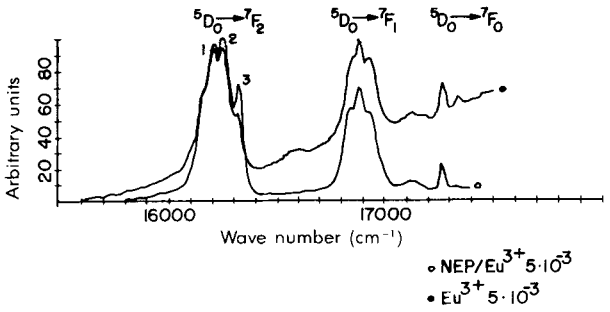

Figure 5. *Emission spectra of 5.10^{-3} M Eu^{3+}, with and without NEP in 0.15 M NaCl. Excitation by mercury lamp; T=77 K.*

results under selective excitation, but also to accidental degeneracies in the $4f^n$ patterns of linked and non linked Eu^{3+} ions.

The nature of the particular site for Eu^{3+} leading to the isolated n° 3 and 3' lines cannot be deduced from the only consideration of spectroscopic results. We may only notice that 10^{-3}M Eu^{3+} correspond to about 10^4 times the enzyme concentration in the solution. An equivalent excess in lanthanide ions added to the enzyme solutions had resulted in a lowering of the enzymatic activity (points at 10^{-4}M Ln^{3+} in Fig. 2), and the new features observed at this high Eu concentration may well be linked to the degradation of the enzyme.

Some additional comments may be put forward. First, the Eu^{3+} concentrations are even much greater than the enzyme concentration, and those high values were suggested by the reactivation experiments. Consequently, the spectra are the averages of the enzyme-linked Eu^{3+} and the aqueous Eu^{3+} with a greater weight for the last. Thus, the differences between the spectra of the solutions, with and without enzymes, are subtle, but mensurable.

We have observed the same behaviour for the two enzymes studied. All the experiments on enzyme-containing solutions have been compared with a corresponding "blank" without the enzyme since we have detected some extent of europium complexation even by the reputed non complexing buffers TRIS and Piperazine. In water + NaCl solutions we have observed two different species, one predominant at low europium concentration and the other one appearing at higher contents (5.10^{-3}M) although this had not been reported previously in the literature. Further investigation of europium aqueous solutions thus appears to be necessary. Among the possibilities of contamination in the enzyme solutions we have checked for Eu-EDTA and hydroxylated Eu^{3+} species. Eu-EDTA characteristics have been reported elsewhere [25]. We have investigated the fluorescence of a gel Eu^{3+} 5.10^{-3} in NaOH, pH=8: the main $^5D_0 \rightarrow {}^7F_2$ line of this gel appears at the same wavelength that line n° 3 in the enzyme-Eu samples, but the excitation spectrum is quite different from those in Fig. 6. These two possibilities of contamination may then be ruled out. Finally we found it an advantage to perform the spectroscopic investigation on frozen solutions rather than on liquids for several reasons: first the signal/noise ratio is generally better, due to reduced radiationless deactivation of Eu^{3+}; the intermolecular processes are much less effective, which increases the chances to record site selective informations

Table I. *Measured $^5D_0$ (Eu^{3+}) average lifetimes (in μs ± 5μs) for 5.10^{-4} M Eu^{3+} concentration and 77 K. Excitation: 337 nm.*

in water + NaCℓ			in TRIS			in Piperazine		
with enzyme		without	with enzyme		without	with enzyme		without
DAP	NEP	enzyme	DAP	NEP	enzyme	DAP	NEP	enzyme
265	253	205	300	180	220	210	245	200

as had been evidenced also in the Eu-EDTA system[25]; the third very important advantage is that only very small volumes (5μl) of dilute enzyme solutions are necessary in our experimental set up.

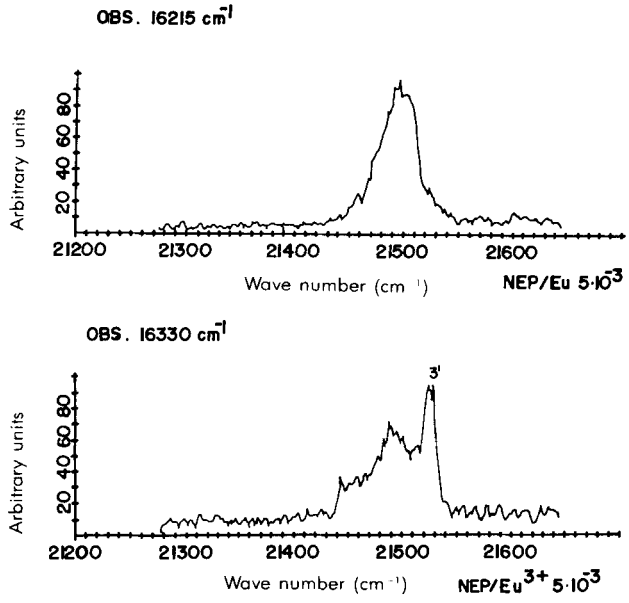

Figure 6. *Excitation spectra of Eu^{3+} linked to the NEP monitored at different wavenumbers in the emission band $^5D_0 \rightarrow {}^7F_2$. Eu^{3+} 5.10^{-3} M with NEP in 0.15M NaCℓ; T=77 K.*
Monitoring wavenumbers correspond to lines n.º 1 or 2 (top) or n.º 3 (bottom) in the emission spectrum.

Conclusions

After displacement of the zinc ions of the zinc metallopeptidases DAP and NEP, by high concentration of the chelating agent EDTA, a strong lowering of the enzymatic activity has been observed. Part of the initial activity may be recovered by adding Zn^{2+} until reaching a maximum for a given concentration (aprox. 10^{-5}M) and then falls again. A partial enzymatic activity may also be recovered by adding the trivalent lanthanide ions Eu^{3+} and Tb^{3+}, suggesting that these ions very likely substitute the Zn^{2+} in the enzymes active sites. The presence of the enzymes linked to Eu^{3+} enhances the europium fluorescence and lengthens the average $^5D_0$ lifetime at all the Eu^{3+} concentrations examined. But only for the highest tested Eu^{3+} concentration (5.10^{-3}M) it was observed a change in emission band profiles: $^5D_0 \rightarrow {}^7F_2$ component, associated with a component in the $^7F_0 \rightarrow {}^5D_2$ excitation spectrum. These specific features have been isolated by means of time resolved site selective excitation techniques on frozen enzyme-Eu^{3+} solutions at 77 K. Two different regimes are then evidenced in the DAP or NEP-Eu^{3+} complexation which may well traduce the two different positive and negative slopes observed in the reactivation curves. This study shows that lanthanide optical probes may be used in the study of metal proteins other than the well know Ca-proteins.

Aknowledgment

One of us (S.R.A.L.) is grateful to FAPESP (Brazil) for a grant (87/2155-5).

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