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Interaction of europium and curium with alpha-amylase

Astrid Barkleit,*[a] Anne Heller,†[a] Atsushi Ikeda-Ohno[a] and Gert Bernhard[a]

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The complexation of Eu(III) and Cm(III) with the protein α-amylase (Amy), a major enzyme in saliva and pancreatic juice, was investigated over wide ranges of pH and concentration at both ambient and physiological temperatures. Macroscopic sorption experiments demonstrated a strong and fast binding of Eu(III) to Amy between pH 5 and 8. The protein provides three independent, non-cooperative binding sites for Eu(III). The overall association constant of these three binding sites on the protein was calculated to be log K = 6.4 ± 0.1 at ambient temperature. With potentiometric titration the averaged deprotonation constant of the carboxyl groups (the aspartic and glutamic acid residues) of Amy was determined to be pK_a = 5.23 ± 0.14 at 25 °C and 5.11 ± 0.24 at 37 °C. Time-resolved laser-induced fluorescence spectroscopy (TRLFS) revealed two different species for both Eu(III) and Cm(III) with Amy. In the case of the Eu(III) species, the stability constants were determined to be log β_{11} = 4.7 ± 0.2 and log β_{13} = 12.0 ± 0.4 for Eu:Amy = 1:1 and 1:3 complexes, respectively, while the values for the respective Cm(III) species were log β_{11} = 4.8 ± 0.1 and log β_{13} = 12.1 ± 0.1. Furthermore, the obtained stability constants were extrapolated to infinite dilution to make our data compatible with the existing thermodynamic database.

Introduction

In case of the incorporation of radioactive heavy metals, these elements represent a serious health risk to humans due to their chemo- and/or radiotoxicity. Trivalent actinides (An(III)), such as Am(III) or Cm(III), are man-made radioactive elements exclusively generated in nuclear reactors, while lanthanides (Ln(III)), which are commonly used as their non-radioactive analogs, are naturally occurring elements having wide technological and medical applications.[1-3] Via different pathways, all these heavy metals can potentially be taken up into the organism exposing a serious health threat to humans.[4-6] Since An(III) and Ln(III) are considered to have no essential function in the human body, little is known about their biochemical behavior from the uptake, *in vivo* transport and "metabolism" to the final accumulation or excretion. Our recent investigations on the *in vitro* speciation of U(VI), Cm(III), and Eu(III) in various body fluids (saliva, urine and sweat) proved experimentally that organic bio-molecules interact with metal ions strongly and dominate the speciation of the metal ions in the body fluids under certain conditions.[7-9] These studies supposed that, in addition to small organic molecules (*e.g.* lactate or citrate), bio-macromolecules like proteins or enzymes are also potential binding partners of these elements under bio-relevant conditions.

For the last decades, the speciation of An(III) and Ln(III) has been studied extensively in blood media with the aim to understand their complexation behavior with blood proteins (*e.g.* albumin and transferrin).[4,10-18] However, only little is known about their speciation in the gastrointestinal tract and their complexation behavior with digestive proteins.[4,16-18] There are several attempts to simulate the speciation of An(III) and Ln(III) in the gastrointestinal tract based on thermodynamic data.[19,20] However, the accuracy of such calculations strongly depends on the model applied and the database used.[21] In case the chemical species existing in the real system are not described correctly (or missing in the worst case) in the thermodynamic database, the resultant speciation could be misleading and/or contradicting experimental results. As a matter of fact, such inconsistency between thermodynamically modeled and experimentally determined speciation has been recently reported for the Eu(III), Cm(III) and U(VI) speciation in several biological media.[7,8,22]

In order to improve the reliability of thermodynamic database for bio-macromolecules, this study aims to experimentally investigate the complexation behavior of Eu(III) and Cm(III) with α-amylase (Amy), one of the most important digestive proteins. The enzyme Amy (α-1,4-glucan-4-glucanhydrolase; EC 3.2.1.1.) is one of the major enzymes in salivary and pancreatic secretions of mammals and catalyzes the hydrolysis of the α-1,4-glycosidic linkages of polysaccharides such as starch or glycogen.[23,24] Human salivary and pancreatic Amys, as well as porcine pancreatic Amys, show considerable similarities in sequence and three-dimensional structures.[25-27] They consist of 496 amino acid residues and have a molecular mass of ~55 kDa.[28-31] The protein binds one calcium and one

[a] *Helmholtz-Zentrum Dresden-Rossendorf, Institute of Resource Ecology, P.O. Box 510119, 01314 Dresden, Germany.*
† Present address: Technische Universität Dresden, Department of Biology, Institute of Zoology, Professorship of Molecular Cell Physiology and Endocrinology, 01062 Dresden, Germany.
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chloride ions per molecule and provides 50 carboxyl groups from both aspartic acid (Asp) and glutamic acid (Glu) residues,[30,31] which can act as major binding sites for metal ions. The Amy monomers are composed of three domains: domain A (residues 1-99 and 169-404) contains a central α/β-barrel of eight parallel strands, domain B (residues 100-168) consists of several helices and β-strands forming an open loop structure, and domain C is composed of eight β-strands which form a compact Greek-key β-barrel. The active site, comprising the catalytic residues Asp197, Glu233 and Asp300, is found in domain A. The chloride ion is placed near the active site and is coordinated by the side chains of arginine (Arg195 and Arg337) and asparagine (Asn298) residues, which could increase the enzyme activity. Calcium is located between domains A and B and is coordinated by a histidine residue (His201) from domain A and Asn100, Arg158 and Asp167 from domain B. The presence of calcium would be necessary for the activity and the structural stability of the protein.[25-27,32-35]

In this paper, we report the complexation of Eu(III) and Cm(III) with Amy over a wide pH range at ambient and physiological temperatures. The deprotonation constants of the protein's functional groups were determined by potentiometric titrations, while the stability constants of the metal-protein complexes were determined by macroscopic sorption experiments combined with time-resolved laser-induced fluorescence spectroscopy (TRLFS). TRLFS is a very sensitive method to study the complexation behavior of luminescent ions at trace concentrations relevant for *in vivo* conditions.[36,37] In this study, the luminescent metal ions Eu(III) and Cm(III) have been employed as representatives for Ln(III) and An(III), respectively.

Experimental section

Sorption experiments

Batch experiments were performed to study the sorption behavior of Eu(III) on Amy (porcine pancreas Amy from Sigma) both in the absence and presence of calcium. The experiments were performed at room temperature as functions of pH, metal- and enzyme concentrations, and sorption time. The pH was varied between 3.0 and 8.0, while the Eu(III)- and Amy concentrations were varied between 10^{-6} and 10^{-4} M and between 0.2 and 3.0 g/L (3.6×10^{-6} to 5.5×10^{-5} M), respectively. Samples with 1 g/L Amy (1.8×10^{-5} M) and 1×10^{-5} to 5×10^{-5} M Eu(III) were additionally spiked with 1×10^{-5} to 5×10^{-5} M Ca. The solubility of Amy is ~0.1 g/L, meaning that the Amy used in the batch experiments was in the form of a suspension. The ionic strength was kept constant at 0.1 M with NaCl for all the experiments.

A Eu(III) stock solution was prepared from $EuCl_3 \times 6\,H_2O$ (Sigma). Aliquots were diluted with a 0.1 M NaCl solution to give the desired Eu concentrations, and the pH was adjusted with HCl and NaOH. Then, Amy were added and the pH was adjusted again, if necessary. In case of the experiments in the presence of calcium, aliquots of a $CaCl_2$ stock solution were also added to the solution. For pH- and concentration-dependent experiments, the mixture was shaken for 24 hours at ambient temperature and the pH was adjusted as necessary. Afterwards, the solution was centrifuged for 20 min at 4000 rpm and filtrated with 150 µm membrane filters. The pH of the filtrate was measured and the Eu and Ca concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS ELAN 9000, Perkin-Elmer). Time-dependent experiments were performed at pH = 6.0. Aliquots of the sample solution were collected at 2 min intervals for the first 10 min, and then at 5 min intervals until 60 min. The collected aliquots were filtrated swiftly with 150 µm membrane filters, and the Eu concentration in the filtrate was determined with ICP-MS.

The Ca content of pure Amy was detected randomly with ICP-MS, resulting in 1 mol Ca per mol Amy.

Potentiometric titration

Potentiometric titration experiments were performed to determine the deprotonation constants (pK_a) of Amy and the stability constants of Eu(III)-Amy complexes ($\log \beta$). All sample preparation and measurements were carried out under N_2 atmosphere. For the determination of pK_a, 3 mg Amy were dissolved in 30 mL deionized carbonate-free H_2O. The resulting solution had a protein concentration of 0.1 g/L (1.8×10^{-6} M), which is equivalent to 9×10^{-5} M carboxyl groups (from Asp and Glu). The solution was then adjusted to I = 0.1 M with NaCl and to pH = 3 with HCl. For the determination of $\log \beta$, the Eu(III) stock solution was added to the protein solution resulting in a final Eu concentration of 10^{-4} M. The solutions were automatically titrated in a thermostatic vessel at both 25.0 ± 0.1 °C and 37.0 ± 0.1 °C with 736 GP Titrino/TiNet 2.50 (Metrohm) using 0.1 M NaOH (carbonate-free, Titrisol, Merck). Dynamic titration was performed using a BlueLine11 electrode (Schott) with a minimum drift of 0.5 mV/min and a delay time of at least 60 s at each pH measurement. Prior to each titration experiment, the electrode was calibrated with standard buffers of pH = 4.008, 6.865 and 9.180 (Schott). Each experiment was performed in triplicate. The experiments for the pure Amy system were carried out in the pH range between 3 and 11, while those with Eu(III) were stopped at pH = 7 in order to avoid the precipitation of Eu(III) hydroxides.

Time-resolved laser-induced fluorescence spectroscopy

TRLFS is another method to determine $\log \beta$ values of metal-Amy complexes. For spectrophotometric titration experiments, Eu(III) samples were prepared from the same Eu(III) stock solution described above, while Cm(III) samples were prepared from a stock solution of ^{248}Cm (supplied from Oak Ridge National Laboratory, U.S. Department of Energy Office of Basic Energy Sciences) in 1 M $HClO_4$. The sample solutions were prepared with metal concentrations of 1×10^{-5} M and 3×10^{-7} M for Eu(III) and Cm(III), respectively. The pH of the solutions was adjusted to 5.5, and the sample solutions were titrated stepwise with 1, 2, or 10 g/L Amy in 0.1 M NaCl until the Amy concentration in the sample reached approximately 1 g/L (1.8×10^{-5} M protein, which is equivalent to 9×10^{-4} M carboxyl groups).

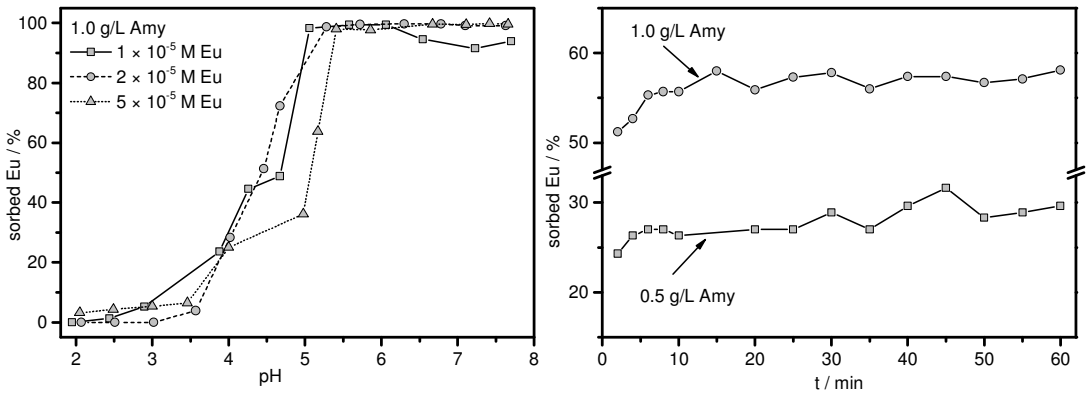

Figure 1. Sorption of Eu(III) on Amy as a function of pH at different constant metal concentrations (left; I = 0.1 M with NaCl, T = 25 °C) and as a function of the sorption time (right; 1×10^{-4} M Eu^{3+}, pH 6.0, I = 0.1 M with NaCl, T = 25 °C).

The ionic strength was kept constant at 0.1 M with NaCl. Another series of pH titration experiments was carried out under the same solution conditions but by raising the pH with NaOH up to 8. At each titration step (0.2 – 0.3 pH values), the sample solution was equilibrated for at least 10 min and TRLFS measurements were performed. Two Eu(III) samples with 1 g/L Amy at pH 5.5 and 7.0 were back titrated with 1×10^{-3} M Ca.

TRLFS spectra were recorded at both 25.0 ± 1 °C and 37.0 ± 1 °C using a pulsed flash lamp pumped Nd:YAG-OPO laser system (Powerlite Precision II 9020 laser equipped with a Green PANTHER EX OPO, Continuum, USA). The temperature was maintained using a temperature-controlled cuvette holder (Flash 300™, Quantum Northwest, USA). The laser pulse energy, which was in the range of 1 – 2 mJ, was monitored with a photodiode. The fluorescence emission spectra were recorded on an optical multi-channel analyzer-system, consisting of an Oriel MS 257 monochromator, a spectrograph with a 300 or 1200 lines per mm grating, and an Andor iStar ICCD camera (Lot-Oriel, Germany). The emission spectra were recorded in the ranges of 440 – 780 nm (300 lines per mm grating) and 570 – 650 nm (1200 lines per mm grating) for time-resolved and single measurements, respectively. A constant time window of 1 ms was applied. The excitation wavelengths were 394 nm and 398 nm for Eu(III) and Cm(III), respectively. For time-resolved measurements, 40 to 60 spectra were collected at delay time intervals between 10 and 50 µs.

Data analysis

The data from the batch sorption experiments at constant pH were fitted with OriginPro9.0 (OriginLab, USA) using the Hill equation (Eq. 1)[38] to calculate the number of binding sites (g), the association equilibrium constant (K_H) and the Hill coefficient (r) from the experimentally determined saturation function, v, with the unit of "bound M^{3+} (mol) / Amy (mol)", and the free metal concentration.

$$v = \frac{g(K_H[M^{3+}])^r}{1+(K_H[M^{3+}])^r} \quad (1)$$

The calculation of pK_a and log β_{ML} was done by applying the following equations:

$$R{-}LH \leftrightarrows R{-}L^- + H^+$$

$$K_a = [R{-}L^-]\,[H^+] / [R{-}LH] \quad (2),$$

$$M^{3+} + xR{-}L^- \leftrightarrows M(R{-}L)_x^{(3-x)+}$$

$$\beta_{1x} = [M(R{-}L)_x^{(3-x)+}] / [M^{3+}]\,[R{-}L^-]^x \quad (3),$$

where M is Eu(III) or Cm(III), R–LH is the protein Amy with the dissociable functional groups LH (–COOH, $-NH_3^+$ and –OH), and x = 1 – 3.

The data from potentiometric titration were treated using the program HYPERQUAD2008 (Protonic Software)[39] to obtain the pK_a and log β values.

The TRLFS data were analyzed with OriginPro9.0. The Eu(III) spectra were normalized to the peak area of the $^5D_0 \rightarrow {}^7F_1$ transition peak (585 – 600 nm), while the Cm(III) spectra were normalized to the whole peak area.

The lifetimes of luminescent species were determined according to the following equation:

$$E(t) = \Sigma_i E_i \times \exp(-t/\tau_i) \quad (4),$$

where $E(t)$ is the total luminescence intensity at the time t, E_i is the luminescence intensity of the species i at t = 0, and τ_i is the corresponding lifetime.

Using the lifetimes τ (in ms), the number of water molecules in the first coordination shell of the heavy metal ions was estimated using the following empirical equations:[40,41]

$$n(H_2O) \pm 0.5 = (1.07 / \tau) - 0.62 \quad \text{for Eu(III)} \quad (5), \text{ and}$$

$$n(H_2O) \pm 0.5 = (0.65 / \tau) - 0.88 \quad \text{for Cm(III)} \quad (6).$$

The luminescence lifetime of pure aquo species in H_2O is 110 ± 4 µs and 65 ± 2 µs for Eu(III) and Cm(III), respectively.[42] These values correspond to each nine water molecules in the first coordination sphere.

The data from spectrophotometric titration were further analyzed using the program SPECFIT[43] to calculate the log β values and the spectra of individual species.

The obtained pK_a and log β values were extrapolated to infinite dilution by applying SIT (Specific Interaction Theory) using the IUPAC software for Ionic Strength Corrections.[44]

Results and discussion

Sorption of europium onto α-amylase

Batch sorption experiments were performed to get a macroscopic overview of the binding behavior of Eu(III) towards Amy. The left figure in Figure 1 shows the sorption behavior of Eu(III) to Amy as a function of pH at three different Eu(III) concentrations. Independent from the Eu(III) concentration, the sorption of Eu(III) began at around pH = 4, attained ~100 % of sorption at pH = 5 – 5.5, and, then, reached a plateau. Based on these results, the pH value for the subsequent time-dependent sorption experiments was set to 6. At this pH, not only the sorption of Eu(III) reaches ~100 % but also the precipitation of Eu(III) hydroxide can be avoided and Amy keeps a high enzyme activity. In fact, it has been reported that Amy shows the highest enzyme activity between pH 5.5 – 8.0 with the optimum at pH = 7[29] but at this pH starting Eu(III) precipitation may interfere with the sorption process.

The right figure in Figure 1 shows the sorption of Eu(III) at two different Amy concentrations as a function of contact time. The metal ion was sorbed within very short time. More specifically, the sorption of Eu(III) started within the first minutes and reached a plateau after 5 minutes. With regard to a potential ingestion of Eu(III), this time span might be relevant for the contact time with saliva in the mouth where the transit time of the ingested substances can last from seconds up to few minutes.[45,46] However, as Amy can also be found in pancreatic juice, one of the digestive fluids,[24] this time frame would be particularly relevant to the gastrointestinal tract where the retention time of substances is much longer (up to several hours).[45,46]

When the equimolar amount of Eu(III) was sorbed on Amy, one molar of Amy was found to release one molar of Ca which was initially retained on Amy. This indicates that Eu(III) replaces the Ca on Amy selectively, which is a well-known process for proteins and other bio-macromolecules.[1,47] This replacement happens regardless of the amount of excess Ca up to the 5-fold excess compared to the Eu concentration suggesting a stronger interaction of Eu(III) on Amy than that of Ca.

Figure 2 shows the binding isotherm of Amy as a function of Eu(III) concentration. By fitting the experimental data with the Hill equation (Eq. 1),[38] which is a modification of the Langmuir sorption isotherm, we obtained information about the macroscopic interaction between metal ions and enzyme. In equation (1), $r = 1$ stands for non-cooperative systems (*i.e.*, identical or non-identical but independent binding sites), $r > 1$ for positively cooperative- and $r < 1$ for negatively cooperative systems (*i.e.*, interacting binding sites in both cases). Based on the data for Eu(III) in Figure 2, the r value was calculated to be 1.1 ± 0.2 with $g = 3.1 \pm 0.1$ suggesting that Amy provides three

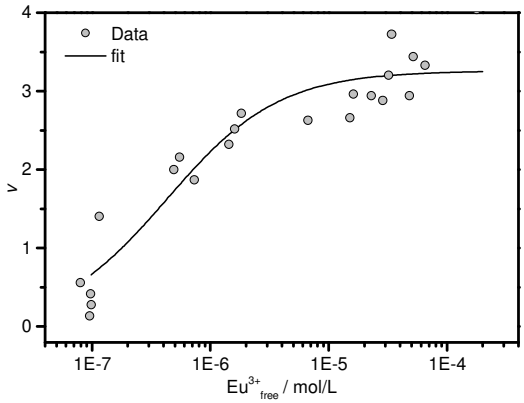

Figure 2. Binding isotherm of Amy (0.2 – 3.0 g/L) as a function of Eu(III) concentration at pH = 6.0, I = 0.1 M with NaCl and T = 25 °C. The fitting curve was obtained according to Eq. (1).

binding sites for Eu(III) with very weak cooperativity, which may be considered as non-cooperative according to the definition of Saboury and Karbassi.[48,49]

The association constant was calculated to be log $K_H = 6.4 \pm 0.1$ and is an averaged overall value for the three binding sites of Amy. It has been reported that the Amy from *Bacillus subtilis* provides two binding sites to Gd(III) with log $K_H = 4.6$.[50] Another study on the crystalline Amy from *Aspergillus oryzae* demonstrated six binding sites for Gd(III).[51] Although the number of binding sites estimated for the present Eu(III) system is within these reported values, the association constants differ considerably. These variations could be caused by the structural differences of the different Amys. Depending on the origin, Amys vary in number, composition and sequence of the amino acids.[31] For instance, the Amys from *Aspergillus oryzae* and *Bacillus subtilis* keep 2 and 3 calcium ions at different binding sites[52,53] and provide more Asp/Glu residues (54 and 66, respectively), compared to the Amy from porcine pancreas (1 bound calcium with 50 Asp/Glu residues), which can consequently cause varying numbers on potential Ln(III) binding sites with varying binding strengths.

Potentiometric titration

Deprotonation of Amy. Prior to the determination of stability constants of metal-Amy complexes, the pK_a values of Amy were determined by potentiometric titration at both ambient (25 °C) and body temperature (37 °C). Each two deprotonation constants were derived and the data is summarized in Table 1. Within the range of error, similar values were obtained for pK_{a1} and pK_{a2}, respectively, at both temperatures (with the tendency to slightly lower pK_a at body temperature). This suggests that the dissociation behavior of Amy remains unaltered at least in this temperature range.

According to the amino acid composition of Amy by ProtParam Tool on ExPASy,[30,31] pK_{a1} can be assigned to the deprotonation of carboxylic groups of Glu and Asp. The pK_a values of pure Glu and Asp are reported to be 4.15[54] and 3.71,[54] respectively, being slightly lower than the pK_{a1} values obtained for Amy. However, due to the concentration of charged functional groups on the protein molecules, a significant electrostatic

Table 1. Conditional deprotonation constants of Amy at I = 0.1 M (NaCl) determined by potentiometric titration. The pK_a^0 values were obtained by extrapolating the $pK_a^{0.1}$ values to infinite dilution applying SIT.[44]

Species	T (°C)	$pK_a^{0.1}$	pK_a^0
Amy–COOH	25	5.23 ± 0.14	5.43
	37	5.11 ± 0.24	5.31
Amy–NH_3^+/Amy–OH	25	10.22 ± 0.10	10.42
	37	10.14 ± 0.21	10.34

effect influencing the deprotonation of functional groups on Amy can occur.[55] This could explain the higher pK_a values of Amy in comparison to those of pure Glu and Asp. In fact, such an enhancement of deprotonation constants was observed for oligopeptides containing Glu and Asp residues.[56] Furthermore, the average pK_a value of Asp and Glu residues in the protein trypsin is 5.2[57] being in line with our results.

The second deprotonation constant obtained for Amy can be associated with the deprotonation of amino groups from arginine, asparagine, glutamine, and lysine and/or the hydroxyl groups from serine, threonine, and tyrosine residues. In fact, the obtained pK_{a2} values are in very good agreement with the pK_a values of amino and hydroxyl groups in bio-macromolecules[58,59] and oligopeptides.[60] However, based on the obtained pK_a values, these functional groups are expected to be protonated under *in vivo* conditions, suggesting that they have none or less contribution to the complexation of Amy with Eu(III) and Cm(III). Furthermore, it was shown that Ln(III) preferably bind to carboxylate groups of Asp and Glu residues rather than to amino or hydroxyl groups.[61]

Complexation of europium with Amy. The conditional stability constants for the complexation of Eu(III) with Amy were calculated by applying equation (3) to the relevant potentiometric titration data obtained at ambient and physiological pH. In the present case, the L in equation (3) represents the carboxylate groups of Glu and Asp. The derived data is summarized in Table 2 and reveals the formation of a 1:1 complex in which Eu(III) is bound via one carboxyl group of a Glu or Asp residue of Amy. Within the range of error, no significant temperature effect was observed suggesting that the complexation behavior is unaltered in this temperature range. The averaged value is $\log \beta_{11}$ = 4.56 ± 0.13. A similar stability constant of $\log \beta_{11}$ = 4.18 ± 0.05 was reported for the complexation of Tb(III) with the protein chymotrypsin[62] supporting the validity of our results.

Time-resolved laser-induced fluorescence spectroscopy

Europium. The left figure in Figure 3 depicts the spectrophotometric titration of Eu(III) with Amy at constant pH 5.5, while the right figure shows the pH titration at constant metal and Amy concentrations. In both systems, due to the complex formation between Eu(III) and Amy, the $^5D_0 \rightarrow {}^7F_0$ transition appeared at ~580 nm. Additionally, the intensity of the hypersensitive $^5D_0 \rightarrow {}^7F_2$ transition at 610 – 625 nm was enhanced with increasing Amy concentration or pH. Furthermore, in both systems, the $^7F_2$ peak was split significantly and the $^7F_1$ peak at 585 – 600 nm became asymmetric as the titration progressed. These spectral changes indicate a strong complex formation between Eu(III) and Amy.

The results from time-resolved measurements revealed a bi-exponential decay, suggesting that at least two luminescent species are formed in both systems (see Tables S1 and S2 in the Electronic Supplementary Information, ESI). The luminescence lifetimes prolonged with increasing protein concentration or pH. This indicates a continuous replacement of water molecules by coordinative functional groups of the protein in the first coordination sphere of Eu(III). The direct excitation of the $^7F_0 \rightarrow {}^5D_0$ transition, which is a non-degenerate transition and consequently yields a single emission line for every non-equivalent Eu(III) species, also supports the formation of two Eu(III)-Amy species (Figure S1 and experimental details see ESI).

The obtained TRLFS data were further fitted by applying equations (2) and (3) to calculate the conditional stability constants for Eu(III)-Amy complexes. That way, two different Eu(III)-Amy complex species could be identified. The obtained values are summarized in Table 2. The stability constant for the first Eu(III)-Amy species was calculated to be $\log \beta_{11}$ = 4.8 ± 0.4 at 25 °C, suggesting the presence of a "Eu(III):L = 1:1" complex where L is the binding carboxylate group of Amy. This stability constant agrees well with that previously determined with potentiometric titration. The stability constant for the second Eu(III)-Amy species was calculated to be $\log \beta_{13}$ = 12.0 ± 0.4, indicating the formation of a "1:3" complex. Attempts to fit the data by assuming the "1:2" complex failed. The spectra calculated for the individual Eu(III) species are shown in Figure S2 in ESI. In fact, the formation of 1:1 and 1:3 complexes was also observed in the crystal structure of the *Aspergillus oryzae*-originated Amy complexing Gd(III).[51] Hence, this study supports our results. Furthermore, in the reported Gd(III)-Amy complex, some

Table 2. Conditional stability constants of M(III)-Amy complexes at I = 0.1 M (NaCl). The $\log \beta^0$ values were obtained by extrapolating the $\log \beta^{0.1}$ values to infinite dilution applying SIT.[44]

Species	T (°C)	$\log \beta^{0.1}$	$\log \beta^0$	Method
$Eu(Amy\text{-}COO)^{2+}$	25	4.54 ± 0.13	5.20	Potentiometry
	37	4.57 ± 0.12	5.24	Potentiometry
	25	4.83 ± 0.43	5.49	TRLFS
	37	4.51 ± 0.15	5.18	TRLFS
$Eu(Amy\text{-}COO)_3$	25	12.04 ± 0.36	13.31	TRLFS
	37	12.13 ± 0.14	13.43	TRLFS
$Cm(Amy\text{-}COO)^{2+}$	25	4.76 ± 0.11	5.42	TRLFS
$Cm(Amy\text{-}COO)_3$	25	12.13 ± 0.12	13.40	TRLFS

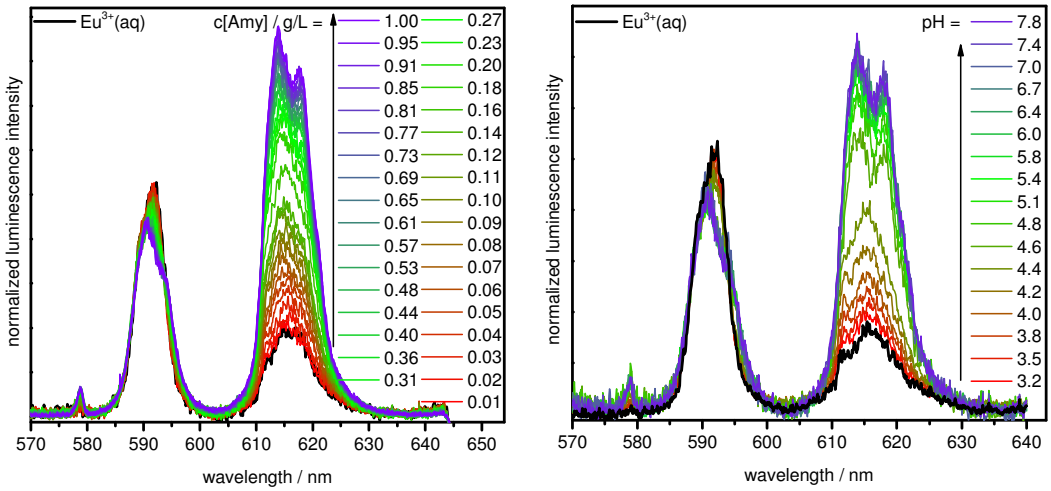

Figure 3. Spectrophotometric titration of 1×10^{-5} M Eu(III) (I = 0.1 M with NaCl, T = 25 °C). Left; as a function of Amy concentration at pH = 5.5, right; as a function of pH with 1.0 g/L Amy.

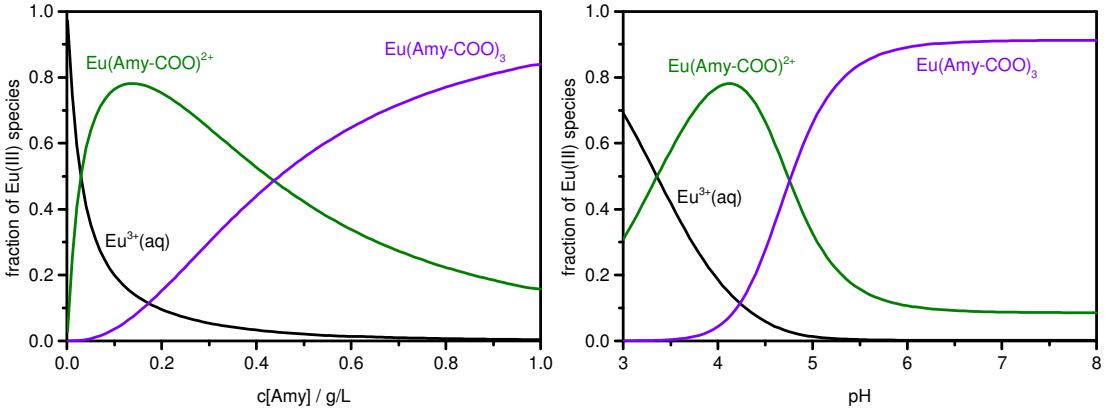

Figure 4. Species distribution of Eu(III)-Amy complexes at 1×10^{-5} M Eu(III) (I = 0.1 M and T = 25 °C). Left; as a function of Amy concentration at pH = 5.5, right; as a function of pH with 1.0 g/L Amy.

Gd(III) act as a linker to connect different protein molecules, resulting in the oligomerization of the proteins.[51] This would suggest that the carboxylate groups of the 1:3 complex identified for our Eu(III)-Amy system may originate from different protein molecules, not from a single molecule.

Based on the complex stability constants obtained from TRLFS measurements, the distribution of Eu(III)-Amy species was calculated as a function of both Amy concentration and pH, respectively. The results are shown in Figure 4. According to this speciation, the measured luminescence lifetimes can be assigned either to one predominant species or to a mixture of several different species. For instance, in the right figure of Figure 4, the Eu(III):Amy = 1:1 complex ($Eu(Amy\text{-}COO)^{2+}$) becomes dominant between pH 4 and 5. Hence, the longer major lifetime observed in this pH range can be assigned to this complex, whereas the shorter minor one of ~110 µs corresponds to the free aquo ion (see Table S1 in ESI). When the pH is raised above 5, the second Eu(III)-Amy complex ($Eu(Amy\text{-}COO)_3$) becomes dominant with more than 90 % fraction. Consequently, the longer major lifetimes obtained in this pH range can be assigned to this species. However, the minor lifetimes observed at pH > 5 are longer than that of the free Eu^{3+} aquo ion but shorter than that of $Eu(Amy\text{-}COO)^{2+}$. This suggests that these shorter minor lifetimes probably originate from mixtures of these two species and/or other minor species (see Table S1 in ESI). The same approach can be applied to the concentration-dependent series at pH 5.5, where the Eu^{3+} aquo ion and the 1:1 complex dominate the species distribution for Amy concentrations below 0.1 g/L but the 1:3 complex becomes dominant for Amy concentrations exceeding 0.4 g/L (left figure in Figure 4). Consequently, considering both TRLFS series, the luminescence lifetimes of the Eu(III)-Amy species can be estimated to be 380 ± 40 µs for $Eu(Amy\text{-}COO)^{2+}$ and 630 ± 50 µs for $Eu(Amy\text{-}COO)_3$ (Tables S1 and S2 in ESI). These luminescence lifetimes correspond to the replacement of 7 and 8 water molecules, respectively, from the first coordination sphere of Eu(III) within species 1 and 2.

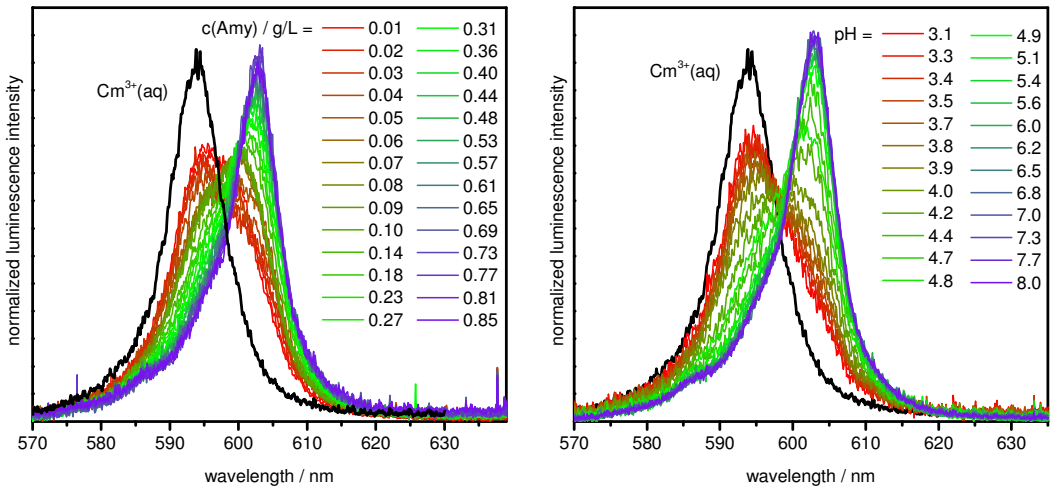

Figure 5. Spectrophotometric titration of 3×10^{-7} M Cm(III) (I = 0.1 M with NaCl, T = 25 °C). Left; as a function of Amy concentration at pH = 5.5, right; as a function of the pH with 1.0 g/L Amy.

Assuming that the carboxylate groups of Amy interact with Eu(III) in a bidentate coordination mode, the complexation by Amy should only exclude 2 and 6 water molecules, respectively, from the Eu(III) coordination sphere for species 1 and 2. This suggests that, in addition to the coordination of the carboxylate groups of Amy, Eu(III) must be surrounded by other ligands (*e.g.*, Cl^-) and/or other functional groups of Amy, which further exclude water molecules from the first coordination sphere of Eu(III). Another possibility is a decline in total coordination number of Eu(III) to a value smaller than 9 in Amy complexes due to sterical hindrance caused by the protein structure.

Sorption experiments indicated that Eu(III) selectively replaces the binding sites of Ca on Amy. On Amy molecules, the calcium ion is coordinated by the carboxyl oxygens of Asp167 in a bidentate manner, the carbonyl oxygens of Asn100, His201 and Arg158 and additional two or three water molecules.[26,32,33] This could explain the replacement of 7 water molecules from the first coordination sphere of species 1 (*i.e.*, $Eu(Amy\text{-}COO)^{2+}$) when Eu(III) is assumed to be sorbed on the Ca binding site on Amy. On the other hand, the Eu(III) in species 2 is surrounded by three carboxyl groups. This cannot be explained by the simple replacement of Ca binding sites, but could be due to the rearrangement and/or aggregation of protein molecules.

The back titration of the Eu-saturated Amy solution with calcium showed no significant changes in the luminescence spectra, indicating that the binding of Eu(III) in the protein is stronger than that of Ca(II).

In general, similar trends were observed for TRLFS series at physiological temperature of 37 °C. The recorded spectra are given in Figure S3 in ESI. Within the error range, the conditional stability constants obtained at this temperature are in good agreement with those at room temperature (Table 2). This further supports and fits to the results from the previous potentiometric titration experiments.

Curium. Figure 5 shows the spectrophotometric titrations of Cm(III) with Amy at constant pH 5.5 (left) and with varying pH at a constant Amy concentration (right). In general, increasing the Amy concentration or the pH, resulted in a red-shift of the $^6D_{7/2} \rightarrow {}^8S_{7/2}$ transition of Cm(III) from 593.8 nm up to 603.0 nm, which corresponds to the speciation change of Cm(III) from the pure aquo complex to those with organic ligands.[13,63,64] The emission band at 603 nm is extremely sharp, which is characteristic of the complexes with chelating ligands (e.g., EDTA).[13] Additionally, the time-resolved spectra always revealed a bi-exponential decay (Tables S3 and S4 in ESI), indicating the presence of at least two independent species in the system. In a similar manner applied like to the Eu(III) data, the obtained Cm(III) TLRFS spectra were analyzed to calculate the stability constants of Cm(III)-Amy complexes. Within the range of error, this resulted in $\log \beta$ values similar to those obtained for the Eu(III)-Amy complexes (Table 2). The spectra calculated for the individual Cm(III) species are shown in Figure S4 in ESI.

Based on these $\log \beta$ values, the distribution of Cm(III)-Amy species was calculated as a function of both Amy concentration and pH. The results are given in Figure 6. According to the Eu(III) system, the measured luminescence lifetimes can be interpreted as either one dominating species or a mixture of several species based on the speciation information in Figure 6. The results are summarized in Tables S3 and S4 in ESI. The luminescence lifetimes for species 1 ($Cm(Amy\text{-}COO)^{2+}$) and species 2 ($Cm(Amy\text{-}COO)_3$) are calculated to be 120 ± 10 and 240 ± 40 µs, respectively. This corresponds to the replacement of 5 and 7 water molecules, respectively, in the Cm first coordination sphere of species 1 and 2. Interestingly, this differs from the previous Eu(III) system indicating that the interaction of Cm(III) with Amy could be unequal to that of Eu(III). However, we also have to consider the difference in metal concentrations (1×10^{-5} M for the Eu(III) system and 3×10^{-7} M for the Cm(III) system), which

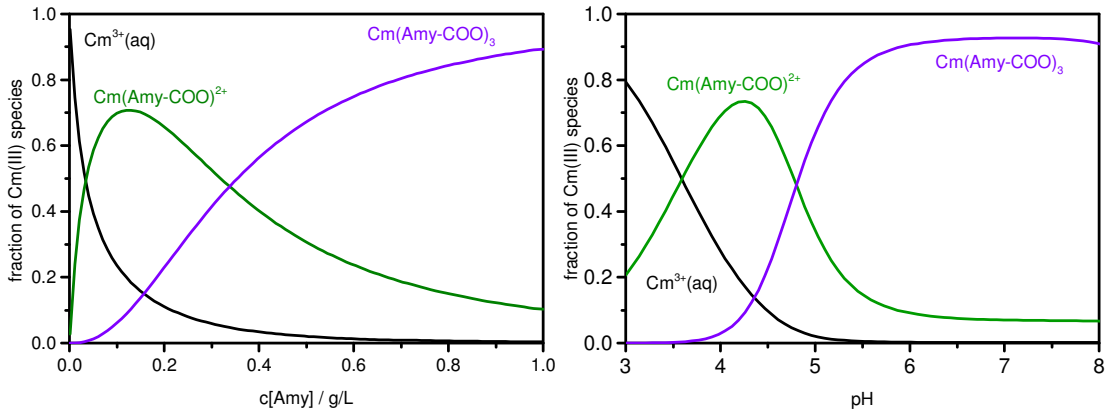

Figure 6. Species distribution of Cm(III)-Amy complexes at 3×10^{-7} M Cm(III) (I = 0.1 M and T = 25 °C). Left; as a function of Amy concentration at pH = 5.5, right; as a function of the pH with 1.0 g/L Amy.

also affect the interaction of the metals with Amy and may be the cause for the differences observed.

Conclusions

The interaction of Eu(III) and Cm(III) with the protein α-amylase, an important digestive enzyme in saliva and pancreatic juice, has been studied over wide pH- and concentration ranges. Batch sorption experiments showed a strong and fast interaction of Eu(III) to Amy over a wide pH-range covering also the *in vivo* conditions in the human gastrointestinal tract. This points to the potential influence of Amy on the biochemical behavior of these trivalent metal cations within the gastrointestinal tract. Potentiometric titrations and TRLFS investigations were performed to successfully calculate the stability constants of M(III)-Amy complexes. These constants are essential for the reliable modelling of chemical speciation and, consequently, for the improvement of the transport modelling of Ln(III) and An(III) in the digestive system. This may also allow us to perform more reliable risk assessment after accidental oral incorporation of radioactive heavy metals and eventually to propose the effective decorporation procedures. The similarity of the stability constants for Eu(III) and Cm(III) confirms the chemical analogy of trivalent lanthanides and actinides due to similar ionic radii. Nevertheless, while Eu(III) offers the benefit to be non-radioactive, Cm(III) provides the possibility to investigate the complexation behavior at much lower concentrations, which is consequently closer to environmental conditions.

Based on the results obtained in this study, the chemical speciation of Eu(III) and Cm(III) in human body fluids associated with the digestive system (*e.g.*, saliva, gastric, pancreatic and bile juice) are currently under investigation. Preliminary results from these on-going studies demonstrate a significant contribution of Amy to the chemical speciation of Eu(III) and Cm(III) in the digestive body fluids, such as saliva. Nevertheless, in order to obtain a comprehensive overview about the potential metabolic pathways of An(III) and Ln(III) in the human body after oral ingestion, we also have to consider other essential proteins and bio-macromolecules in the gastrointestinal tract, such as mucin, in future investigations.

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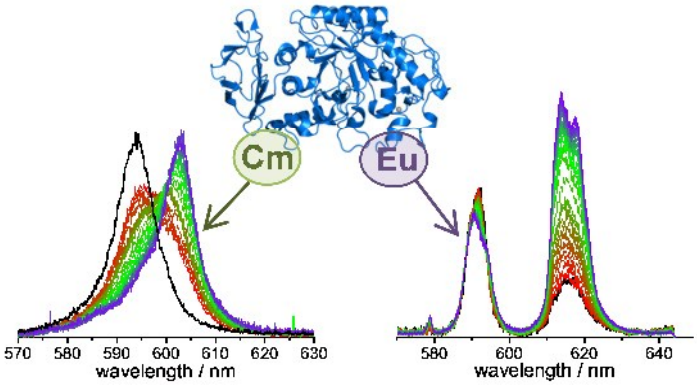

Batch sorption experiments, potentiometric and spectroscopic titration investigations revealed a fast and strong interaction of Eu(III) and Cm(III) with the digestive enzyme α-amylase.