Analysis of the conformation and ligand-binding properties of the activation segment of pig procarboxypeptidase A

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The isolated activation segment of pig procarboxypeptidase A binds two Tb^{3+} ions in a strong and specific way. In contrast, the binding of Ca^{2+} , Cd^{2+} and Mg^{2+} is weak. The binding of Tb^{3+} increases the resistance of the isolated activation segment against proteolysis and competes for the binding of the carbocyanine dye Stains-All. This dye forms complexes with the activation segment showing spectral properties similar to those observed with EF-hand structures. The presented results support a previous hypothesis on the existence of two regions in the activation segment of pancreatic procarboxypeptidases structurally related to Ca^{2+} -binding domains of the EF-hand protein family.

INTRODUCTION

The activation of pancreatic procarboxypeptidases A (PCPAs) is promoted by the proteolytic release and degradation of a 94-residue-long *N*-terminal activation segment (asA) [1–6]. The complete activation segment is a folded globular protein that competitively inhibits carboxypeptidase A (EC 3.4.17.1) in a strong ($K_i \approx 2 \text{ nM}$) and specific way [4,6,7]. Structural studies carried out in our laboratory have revealed that this activation segment is a highly acidic thermostable phenylalanine-rich protein, devoid of disulphide bridges, with a high content of α -helix [4,6,7]. It also shows a two-state reversible transition in both urea and thermal denaturation [8,9].

We have previously reported the primary structure of this activation segment from pig PCPA [6], and it was found to contain two internally homologous regions of about 30 residues each, which present a high propensity to fold in an α -helix-loop- α -helix motif [10]. This characteristic plus all the above-mentioned structural properties are also observed in the Ca^{2+} -binding domains of the proteins of the EF-hand family (EFCaBPs) [11]. These structural coincidences, together with some failure in the sequential fitting, allowed us to propose that the activation segment of pancreatic procarboxypeptidases contains two regions with a distorted EF-hand conformation [10].

In the experiments here presented we have tested the ability of the isolated activation segment from pig PCPA to bind different inorganic (Ca^{2+} , Tb^{3+} , Cd^{2+} and Mg^{2+}) and organic (Stains-All) ligands that show a specific affinity for the EFCaBPs. We have also examined the relative competition of these ligands for the binding to the activation segment and their effect on the proteolysis of the protein. The results obtained confirm the previous hypothesis on the structural relationship between the activation region of pancreatic procarboxypeptidases and the EFCaBPs [10].

MATERIALS AND METHODS

Chemicals and materials

$\text{TbCl}_3 \cdot 6\text{H}_2\text{O}$ (99.99%) was obtained from Aldrich Chemical Co. and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (9%) from Merck. The carbocyanine dye Stains-All (analytical grade) was supplied by Sigma Chemical Co. Distilled water, glassware and plastic tubes were freed from Ca^{2+} and other metal ion contaminants in accordance with ref. [12]. All reagents were of the highest analytical grade. Pig monomeric PCPA and asA derived from it were prepared, and their purity was verified, as previously described [4,13]. Molar absorption coefficients at 280 nm of $74980 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [14] and $15937 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [4] respectively were used to calculate the concentration of each protein. To remove traces of Ca^{2+} , the protein solutions prepared in the appropriate metal-ion-free buffers were slowly passed through a Chelex-X100 column.

Fluorescence spectroscopy

Fluorescence measurements were made on a Perkin-Elmer 650-40 spectrofluorimeter, in the quantum-corrected mode at a spectral bandwidth of 5 nm for both excitation and emission. All the experiments were performed at 25 °C in a 10 mm × 10 mm quartz cell. Binding or release of Tb^{3+} to or from the protein was monitored by measurement of the fluorescence emission at 488 nm (excitation wavelength 260 or 280 nm) and 545 nm (excitation wavelength 235 nm). Perturbation of the fluorescence emission by the second-order Rayleigh scattering was thereby eliminated. In order to avoid inner-filter artifacts, protein solutions were maintained at constant concentration with absorbance below 0.15 (at the excitation wavelength) during each experiment. Analysis of the fluorescence binding data was carried out by using the Adair-Klotz equation, and a curve-fitting programme based on the Gauss-Newton method [15], in

Abbreviations used: PCPA, procarboxypeptidase A; asA, activation segment of procarboxypeptidase A; EFCaBP; EF-hand family Ca^{2+} -binding protein; Stains-All, {1-ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide.

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accordance with Haiech *et al.* [16] and Chiba *et al.* [17].

Stains-All binding

The interaction of Stains-All with isolated asA, bovine calmodulin, chicken troponin C and bovine serum albumin was studied in solution in 10 mM-Tris/HCl buffer, pH 8.8, containing 0.001% Stains-All and 0.1% formamide as described by Campbell *et al.* [18]. The studies were also carried out by the procedure of Caday & Steiner [12] at 7.9 μ M-Stains-All in 30% (v/v) ethylene glycol in 10 mM-Tris/HCl buffer, pH 8.8, instead of the original Mops buffer.

Resistance to trypsin digestion

The effects of EDTA, Ca^{2+} and Tb^{3+} on the rate of digestion of isolated asA by trypsin were studied in 10 mM-Bistris/HCl buffer, pH 6.4, at 37 °C. The asA dissolved in this buffer was freed from Ca^{2+} , and adjusted to 70 μ M-protein (0.75 mg/ml) and to 1 mM-EDTA, - CaCl_2 or - TbCl_3 . Pig trypsin [tosylphenylalanylchloromethane ('TPCK')-treated; Sigma Chemical Co.] was added to reach a 1:25 (w/w) trypsin/asA ratio, and the digestion was allowed to proceed for 4 h and then stopped by boiling the samples in 1.5% SDS. Electrophoresis was carried out by the procedure of Hashimoto *et al.* [19].

RESULTS AND DISCUSSION

Binding of Ca^{2+} and Tb^{3+}

Ca^{2+} binding to isolated asA was assayed by several methods: intrinsic fluorescence, circular dichroism in the u.v. (222 nm) and batch microcalorimetry. No strong binding was found. Only a weak binding of Ca^{2+} to the isolated asA was detected by an increase (up to 65%) of the fluorescence emission band (340 nm) of the protein when the cation was progressively added at molar ratios in the range 100–10000-fold the concentration of asA (7 μ M). From this a dissociation constant, $K_d \approx 5 \times 10^{-3}$ M, was estimated, either at pH 6.4 or at pH 8.3, in 10 mM-Bistris/HCl or -Tris/HCl buffer. This estimated K_d value is much greater than those calculated for the binding of Ca^{2+} to EF-CaBPs, which range from 10^{-7} to 10^{-4} M [11,16,20]. This result was not surprising, as we now know from sequence and conformational analysis [10] that the two putative Ca^{2+} -binding regions of the asA present a distorted structure with regard to the normal EF-hand Ca^{2+} -binding sites.

However, these distorted sites are still probably able to bind Tb^{3+} , a frequently used fluorescent probe for Ca^{2+} -binding sites. It is well known that the discrete fluorescence emission bands of Tb^{3+} at 488 and 545 nm are greatly enhanced when this cation forms specific complexes with the EF-CaBPs [17,21,22]. When isolated asA, at 7 μ M concentration in 10 mM-Bistris/HCl buffer, pH 6.4, was titrated with Tb^{3+} , from 0 to 70 μ M, an increase in the fluorescence bands of this cation appeared up to a molar ratio of 2–2.25:1 (Tb^{3+} /asA) (see Fig. 1). At this low ionic strength the titration curve exhibits two steps, which are detected when Tb^{3+} is excited at 235 nm (shown in Fig. 1) or at 260 or 280 nm (results not shown). These two steps could be assigned to the sequential binding of two Tb^{3+} ions to the protein. When the titration proceeded beyond the molar ratio 3:1 (Tb^{3+} /asA), a new enhancement in fluorescence was observed.

Fig. 1. Titration of asA with Tb^{3+} as monitored by fluorescence

The increase in the fluorescence of Tb^{3+} at 545 nm at different Tb^{3+} /asA ratios was studied in 10 mM-Bistris/HCl buffer, pH 6.4, in metal-ion-free solution (●), in the presence of 1 mM- Ca^{2+} (■) and in 0.1 M-KCl (▲). The points are experimental data. The continuous lines represent the computed best-fit curves assuming two different and independent binding sites. The intrinsic fluorescence of the added Tb^{3+} is also indicated (----).

This could be due to the binding of a third Tb^{3+} ion. However, the large variability observed from sample to sample for the intensity changes of fluorescence in this third enhancement, and the parallel increase in the first-order Rayleigh scattering, suggest that the binding of a third Tb^{3+} ion is accompanied by self-association of the protein.

When addition of Tb^{3+} to the isolated asA was carried out at intermediate ionic strength, in the presence of 0.1 M-KCl, a less-intense but still clearly detectable titration fluorescence-enhancement curve was obtained (Fig. 1). This observation indicates the specific character of the Tb^{3+} -asA associations. Assuming, as a first approach, the existence of two different and independent Tb^{3+} -binding sites in asA, analysis of the binding data (see the Materials and methods section for details) gave the following estimated apparent dissociation constants for the binding of the first two Tb^{3+} ions; $K'_{d1} = 0.16 \pm 0.04 \mu\text{M}$ and $K'_{d2} = 5.9 \pm 1.4 \mu\text{M}$ at low ionic strength, and $K'_{d1} = 0.21 \pm 0.06 \mu\text{M}$ and $K'_{d2} = 15 \pm 5.0 \mu\text{M}$ at intermediate ionic strength. It can be seen from these values that the affinity of Tb^{3+} for asA is within the range found for the binding of Tb^{3+} to the high-affinity and low-affinity Ca^{2+} -binding regions in EF-CaBPs [17,21,22].

Ca^{2+} apparently competes weakly with Tb^{3+} for the same sites in asA. Thus a concentration of 4 mM- Ca^{2+} (corresponding to a Ca^{2+} /asA molar ratio of 800:1) was required to produce a 50% decrease in the fluorescence of the 2Tb^{3+} -asA complex. The same effect was observed on the addition of 4 mM- Cd^{2+} or 16 mM- Mg^{2+} , two cations that can substitute for Ca^{2+} in certain sites of EF-CaBPs [16,23]. It is also of interest to note that in the presence of 1 mM- Ca^{2+} the binding curve of Tb^{3+} to asA loses its third fluorescence-enhancement step (Fig. 1), and the fluorescence scattering of the complexes remains low even at high Tb^{3+} /asA ratio. This suggests that Ca^{2+}

protects asA from self-association promoted by Tb^{3+} binding.

The above results indicate that the isolated asA contains two sites that specifically bind Tb^{3+} . Thus asA appears related to several EFCaBPs, particularly to those containing two specific Tb^{3+} (substituting for Ca^{2+})-binding regions, such as the intestinal Ca^{2+} -binding protein [17,22]. A third Tb^{3+} -binding site, which would promote protein self-association, could also be present in asA. In this respect it is important to note that the existence of one high-affinity site for Tb^{3+} , different from the EF-hand Ca^{2+} -binding sites [24], and frequently responsible for the self-association of the protein, has been reported for typical EFCaBPs such as intestinal Ca^{2+} -binding protein [22,25], calmodulin [21] and parvalbumin [24,26]. The Tb^{3+} -promoted self-association of some of these EFCaBPs is also prevented by the presence of Ca^{2+} in the medium [25,27].

Stains-All binding

A remarkable characteristic of EFCaBPs is the generation of a spectral band (the J band) at 610–650 nm in the absorption spectrum of the Stains-All dye after complex-formation [12,18,28]. This property has been proposed to identify potential EFCaBPs [18]. The same spectral phenomenon was also observed for asA. Thus when isolated asA was added to a 0.001% solution of Stains-All (in 0.1% formamide in 10 mM-Tris/HCl buffer, pH 8.8) the complex formed had a difference spectrum (against Stains-All as reference) with a band centred at 640 nm (Fig. 2). The intensity of this band increased linearly with addition of asA up to a concentration of 4 μg of asA/ml. At higher protein concentration this band fades and is replaced by another band centred at 490 nm. Similar behaviour was observed for several EFCaBPs, such as bovine calmodulin and chicken troponin C, whereas bovine serum albumin, a non- Ca^{2+} -binding protein used as a reference, exhibited only the 490 nm band (Fig. 2).

Addition of 1 mM- Tb^{3+} or 10 mM- Ca^{2+} to the complexes of asA or calmodulin with Stains-All induced a complete disappearance of the band at 640 nm and its replacement by the band at 490 nm. This experiment was carried out in the presence of 30% ethylene glycol instead of 0.1% formamide in order to avoid pre-

Fig. 2. Spectroscopic behaviour of a Stains-All-asA complex

Absorption difference spectra of the complexes of Stains-All (0.001%) with asA (—), bovine calmodulin (---) and bovine serum albumin (·····) at 4 $\mu\text{g}/\text{ml}$ in 10 mM-Tris/HCl buffer, pH 8.8, are shown.

Fig. 3. Effect of added Tb^{3+} and Ca^{2+} on the tryptic degradation of asA

Isolated pig asA (0.75 mg/ml) and 10 mM-Bis-Tris/HCl buffer, pH 6.4, was digested with pig trypsin (30 $\mu\text{g}/\text{ml}$) for 4 h at 37 °C in the presence and in the absence of the above bivalent cations and subsequently analysed by SDS/polyacrylamide-gel electrophoresis: lane a, trypsin-untreated (and metal-ion-free) asA; lanes b, d and e, digestion in the presence of 1 mM- Tb^{3+} , 1 mM- Ca^{2+} and 1 mM-EDTA respectively; lane c, digestion of the Tb^{3+} -asA complex at a molar ratio of 2:1. See the text for more details. Positions of M_r markers are indicated.

cipitation of the dye by the added cations. The above behaviour strongly suggests that Stains-All binds to the EF-distorted sites in isolated asA, as previously deduced for several EFCaBPs [12,18,28], and that Tb^{3+} ions compete with the dye for binding to those sites. The latter property eliminates the possibility that the formation of the J state could be due to the binding of Stains-All to undetected sialic acids or phospho groups in the protein [18].

Resistance to trypsin digestion

Also significant is the resistance, induced by the presence of Tb^{3+} , to the degradation of the isolated asA by trypsin. When different samples of isolated asA in the presence of EDTA, Ca^{2+} or Tb^{3+} , at 1 mM each, were submitted to the action of pig trypsin, a much greater resistance to protein hydrolysis was observed in the Tb^{3+} -containing samples (Fig. 3). The same protection against the action of trypsin was also afforded at a Tb^{3+} /asA molar ratio as low as 2.0:1. This fact, and the use of experimental conditions similar to those of the fluorescence studies, exclude the possibility that protection against proteolysis arises from non-specific aggregation of asA caused by the presence of Tb^{3+} .

The above result suggests that Tb^{3+} stabilizes the tertiary structure of the isolated asA, probably favouring more compact conformations. Conformational changes

of diverse character and importance have also been detected in several EFCaBPs after the binding of Ca^{2+} , Tb^{3+} or other cations. These changes, among other things, clearly influence the spectral [22,26] and electrophoretic [21,29] properties of these proteins, as well as their susceptibility to denaturation [29–31]. It should also be noted that several well-established EFCaBPs, such as bovine intestinal Ca^{2+} -binding protein [32], bovine calmodulin [33], rabbit skeletal-muscle troponin C [34] and bovine cardiac-muscle troponin C [35], also show a greatly increased resistance to trypsin hydrolysis in the presence of Ca^{2+} .

GENERAL DISCUSSION

On the whole, the results presented here confirm our previous work where we found, by means of comparisons of primary and secondary structure, that the activation segment of pig pancreatic PCPA contains two distorted EF-hand Ca^{2+} -binding sites [10]. These sites, located in the regions of residues 3–34 and 55–84, could be responsible for the binding of the ligands analysed here. The conformational deviation of asA from a typical EF-hand structure is probably responsible for the decrease in its capability to bind Ca^{2+} . However, the asA retains some structural traits of the EF-hand structure that permit the high-affinity binding of Tb^{3+} . In addition, the formation of complexes with the dye Stains-All with similar spectroscopic properties to those presented by a EF-hand protein is also consistent with the proposed relationship between asA and the EFCaBPs.

Ca^{2+} strongly affects the kinetics and extent of the activation of several pancreatic procarboxypeptidases, such as those of dogfish [36], lungfish [37] and cattle [38]. Although this could be achieved by the binding of Ca^{2+} to their active regions, we have found no evidence for the influence of Ca^{2+} in the proteolytic activation of pig PCPA at cation concentrations around those found in pancreatic juice (J. Vendrell & F. X. Avilés, unpublished work). Consequently, no clear functional role can be at present attributed to the distorted EF-hand Ca^{2+} -binding sites in pig asA.

The present knowledge on the confirmation of the activation regions of pancreatic procarboxypeptidases is very limited. The results reported here and in a previous paper [10] allow the first insight into the above question. In this respect attention is drawn to the evidence for the flexible conformation of the activation segment as shown by its different susceptibilities to denaturation and to the action of proteinases when within the proenzyme and when in the isolated state [3,4,8,9]. Such flexibility is an important characteristic of proteins such as the ECaBPs, formed by packing of α -helices, in which it plays an important functional role [39]. The very high percentage of α -helix found in the activation segment of procarboxypeptidases [7,10] is also in favour of similar conformational properties.

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