Biochimica et Biophysica Acta, 452 (1976) 161—164

BBA 67951

THE EFFECT OF CALCIUM IONS ON THE HYDROLYSIS OF BENZOYLARGININE ETHYL ESTER BY PORCINE ENTEROPEPTIDASE

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(Received May 10th, 1976)

Summary

Calcium ions are shown to have a marked pH-dependent effect on the kinetics of benzoyllarginine ethyl ester hydrolysis by porcine enteropeptidase (EC 3.4.21.9). Below pH 6.0, calcium ions stimulate benzoylarginine ethyl ester hydrolysis but inhibit this activity above pH 6.0. This effect is mainly on the K_m for benzoylarginine ethyl ester. At pH 5.3, 2 mM calcium ions reduce the K_m for benzoylarginine ethyl ester from 0.31 mM to 0.26 mM while at pH 6.5 the K_m increases four-fold from 0.035 mM to 0.12 mM in the presence of calcium ions. Enteropeptidase activity is not inhibited by ethylenediaminetetraacetate indicating that calcium ions are a non-essential cofactor for benzoylarginine ethyl ester hydrolysis.

Introduction

It has been shown that porcine enteropeptidase (EC 3.4.21.9) has a requirement for Ca^{2+} for the full expression of enzymic activity [1]. Although Baratti et al. [2] have confirmed this requirement, they were unable to detect any direct association of Ca^{2+} with enteropeptidase or any influence of Ca^{2+} on the esterase activity of enteropeptidase towards benzoylarginine ethyl ester (Bz-Arg-OEt). They therefore proposed that the Ca^{2+} stimulation of the peptidase activity towards trypsinogen was brought about by the well characterised ability of Ca^{2+} to inhibit the formation of inert protein during the trypsinogen activation [3—5].

However the marked stabilising effect of Ca^{2+} on enteropeptidase activity indicated an avid binding of the metal by the enzyme and preliminary results had suggested that Ca^{2+} did influence the hydrolysis of Bz-Arg-OEt by enteropeptidase [1]. Since these results are contrary to those of Baratti et al. [2], further studies on the relationship between Ca^{2+} and enteropeptidase were neces-

Abbreviation: Bz-Arg-OEt, benzoyllarginine ethyl ester.

sary. It is shown that Ca^{2+} has a marked pH-dependent effect on the kinetics of Bz-Arg-OEt hydrolysis by porcine enteropeptidase.

Materials and Methods

The source of all reagents was as previously described [1,6]. Enteropeptidase was isolated from acetone powders of fresh pig duodenal fluid by a method involving chromatography on DEAE-cellulose, pH 6.0, Sephadex G-200, pH 6.0, DEAE-Sephadex, pH 8.0, and finally again Sephadex G-200, pH 6.0. The acetone powder (80 g/l) was extracted with 0.01 M sodium acetate, pH 5.0, at 0° C for 30 min. The extract was fractionated on a 4.8 × 16-cm DEAE-cellulose column (Whatman DE-23) equilibrated with 0.02 M Tris acetate/0.05 M NaCl/0.1 mM $CaCl_2$, pH 6.0, using a 1500 ml concave gradient to 0.3 M NaCl. The 5 × 80-cm Sephadex G-200 column was equilibrated with 0.02 M Tris acetate/0.5 M NaCl/0.1 mM $CaCl_2$, pH 6.0. Further fractionation was achieved on a 2 × 12-cm DEAE-Sephadex (A50) column with a convex 500-ml gradient from 0.075 M to 0.2 M NaCl in 0.02 M Tris chloride, pH 8.0. Finally, enteropeptidase was again passed through the Sephadex G-200 column above. In later purifications, a Con-A Sepharose (concanavalin-A Sepharose) column was used after the first Sephadex G-200 column and DEAE-cellulose (Whatman DE-32) replaced the DEAE-Sephadex [7].

The peptidase activity of enteropeptidase was determined as previously described using bovine trypsinogen as substrate [1]. The esterase activity towards Bz-Arg-OEt was determined titrimetrically as previously described [6]. This activity could also be determined spectrophotometrically using a procedure based on the corresponding trypsin assay [8,9]. Reaction mixtures (1.0 ml) contained 0.05 M sodium 3,3'-dimethylglutarate, pH 5.8, and 0.5 mM Bz-Arg-OEt (neutralised) and were incubated at 30° C in the sample cuvette contained in the thermostatically temperature-controlled holder of the spectrophotometer. The absorbance of the substrate solution was balanced with the zero offset facility (if available) or with Bz-Arg-OEt in the reference position. The reaction was started by the addition of aliquots of enteropeptidase and the increase in absorbance at 253 nm was recorded.

Results

Ca^{2+} was found to have a marked pH-dependent effect on the esterase activity of enteropeptidase, stimulating the activity below pH 5.8 but inhibiting it above this value. In the absence of Ca^{2+}, the esterase activity increased from pH 4.5 to 7.0 to a plateau level above pH 7.0. In the presence of 2 mM Ca^{2+}, the pH profile was moved towards the acid region so that up to pH 5.8 activities showed stimulation (a maximum of 25% around pH 5.3) but the plateau level which was now reached at pH 6.0 was only 75% of that shown in the absence of Ca^{2+}. Under the conditions used in the present assay, the two profiles crossed at pH 5.8. Since at this pH the esterase activity was independent of the presence of Ca^{2+}, pH 5.8 was selected for the standard esterase assay. As was the case with the peptidase activity, unless enzyme which had been depleted of Ca^{2+} was used the response to added Ca^{2+} was variable [1]. Ba^{2+} and Sr^{2+} also enhanced

the esterase activity below pH 6.0 but inhibited it above this value.

Because the response to Ca^{2+} was pH-dependent its effect on the kinetics with respect to Bz-Arg-OEt of the esterase activity was determined at the pH values 5.3, 5.8 and 6.5. In each case a small but consistent increase in V was shown although the dominant effect was on K_m (Table I). At pH 5.3, the Ca^{2+} stimulation was brought about by a small decrease in K_m from 0.31 mM to 0.26 mM together with the slight increase in V, whereas at pH 6.5 the Ca^{2+} inhibition resulted from a fourfold increase in K_m. This change in K_m from 0.035 mM to 0.12 mM more than cancelled the small increase in V. At pH 5.8, where under the standard assay conditions Ca^{2+} shows little or no effect on the esterase activity, Ca^{2+} has an intermediate effect on K_m with a twofold increase from 0.07 mM to 0.143 mM. At pH 5.8, the Lineweaver-Burk plots intersect to the right of the y-axis so that at a particular Bz-Arg-OEt concentration (the concentration in the standard titrimetric assay) Ca^{2+} will be without any effect on the observed activity and at Bz-Arg-OEt concentrations above and below this value Ca^{2+} will have opposite effects. Thus the point of intersection of the two curves of the pH profile will be dependent on the Bz-Arg-OEt concentration.

Despite the influence of Ca^{2+} on the esterase activity of enteropeptidase this activity was not susceptible to inhibition by EDTA in agreement with the results of Baratti et al. [2]. This non-essential requirement for Ca^{2+} is best shown using the spectrophotometric Bz-Arg-OEt assay which, above pH 5.0, can be used to determine the esterase activity of enteropeptidase (pK_a of benzoylarginine = 3.4 [8]). It is the preferred procedure when EDTA is present since in the titrimetric assay EDTA buffers strongly around pH 6.0 and below, thereby drastically reducing its sensitivity. Use of the spectrophotometric assay confirmed the non-essential requirement for Ca^{2+} and furthermore showed that enteropeptidase which had undergone prior incubation with 10 mM EDTA still responded to the presence of excess Ca^{2+}. In fact in those cases where the enteropeptidase has not or cannot be made Ca^{2+}-free the control level of the esterase activity is given by assaying the enzyme in the presence of 1 mM EDTA. Addition of excess Ca^{2+} then produces results comparable to those described above. It has previously been shown that Ca^{2+} is a non-essential cofac-

TABLE I

EFFECT OF Ca^{2+} ON THE KINETICS WITH RESPECT TO Bz-Arg-OEt OF ESTERASE ACTIVITY OF PORCINE ENTEROPEPTIDASE

Data obtained from double reciprocal plots of initial velocity against Bz-Arg-OEt concentration. Reaction mixtures (4.2 ml) contained 0.1 mM sodium 3,3'-dimethylglutarate, pH as specified, 2 mM NaCl or $CaCl_2$ and varying concentrations of Bz-Arg-OEt. The reaction was initiated with enteropeptidase and the reaction was followed titrimetrically with a Radiometer Type TTT1d pH-stat. Enteropeptidase used was 0.038 units at pH 5.3, 0.035 units at pH 5.8 and 0.029 units at pH 6.5.

pH	With Ca^{2+}		Without Ca^{2+}	
	K_m (mM)	V (μmol · min^{-1})	K_m (mM)	V (μmol · min^{-1})
5.3	0.26	0.036	0.31	0.034
5.8	0.143	0.039	0.07	0.035
6.5	0.12	0.038	0.035	0.037

tor for the peptidase activity of enteropeptidase (trypsinogen activation) stimulating that activity below pH 6.0 [1].

Discussion

Ca^{2+} was found to have a complex pH-dependent effect on the esterase activity of enteropeptidase in addition to its previously reported effects on trypsinogen activation and stability of enteropeptidase [1]. This Ca^{2+} effect was independent of ionic strength since it was found at both low (titrimetric) and high (spectrophotometric) ionic strengths and has been confirmed with enzyme isolated from the duodenal mucosa by the method of Baratti et al. [10] except that 1% (v/v) Triton X-100 was used instead of sodium deoxycholate as the solubilising agent. Because of their failure to observe any influence of Ca^{2+} on Bz-Arg-OEt hydrolysis, Baratti et al. [2] considered that the stimulation of trypsinogen activation by Ca^{2+} was an indirect one on the stability of the trypsin formed. However it is also possible that with the prolonged incubations used by these and other workers for the determination of enteropeptidase activity, the enteropeptidase, which itself is inherently labile [1], is stabilised by the Ca^{2+} thereby enhancing the rate of trypsin formation. However with the short incubations used in this and previous studies [1] these considerations are of little consequence. Therefore it does appear that Ca^{2+} directly influences the catalytic activity of the enzyme (both the esterase and peptidase activities) and the stability of the enzyme protein.

The active site of enteropeptidase has been shown to consist of two subsites with the recognition of the lysine (residue 6 in bovine trypsinogen) and the subsequent Lys-Ile bond hydrolysis being the responsibility of the heat-stable catalytic site, and the recognition and binding of the polyaspartyl cluster (residues 2—5) the responsibility of the heat-labile specificity site [6]. Ca^{2+} would appear to be associated with both of these sites since Ca^{2+} specifically stabilises the specificity site [6] as well as influencing the enzyme-catalysed bond hydrolyses in trypsinogen [1] and Bz-Arg-OEt which would occur within the catalytic site. It would therefore appear that two moles of Ca^{2+} are associated with porcine enteropeptidase in the full expression of its enzymic activity below pH 6.0.

Acknowledgements

This work was supported in part by the National Health and Medical Research Council and the Clive and Vera Ramaciotti Foundations.

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