Kinetic parameters for the generation of endothelins-1,-2 and -3 by human cathepsin E

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The specific conversion of human endothelin (ET) precursors big ET-1, big ET-2 and big ET-3 into their respective ET by cathepsin E was examined. Comparable pH optima were obtained for ET-1 and ET-2 generation, whereas effective conversion of big ET-3 into ET-3 necessitated a lower pH value. Determination of kinetic parameters (K_m , k_{cat}) for all three conversions indicated that the precursors were efficiently bound by cathepsin E. The significance of the values obtained for the catalytic-centre activities and the effect of a specific inhibitor are discussed.

INTRODUCTION

Genes encoding a family of endothelin (ET) polypeptides (termed ET-1, ET-2 and ET-3) are present in the tissues of a variety of species, including man [1]. In addition to displaying potent vasoconstrictor activities, the derived peptides have been shown to produce a number of other pharmacological actions (depending on the individual endothelin/tissue/species of origin [2,3]). Each ET is produced in the form of a precursor, big ET. A number of reports have demonstrated qualitatively that generation of ET from big ET precursor(s) may be effected (i) by metalloproteinase [4,5] activity (although the conversion rate of big ET-3 → ET-3 is much slower than that observed with big ET-1 [6], or (ii) by the action of a tissue aspartic proteinase [7,8]. We have shown previously [9] that cathepsin E, one of five aspartic proteinases known (so far) to be present in the human body, processes big ET-1 very precisely to generate ET-1 and release the C-terminal fragment and might thus be a likely candidate for the tissue endothelin-converting-enzyme activity.

Here we show that big ET-2 and big ET-3 are processed just as specifically by cathepsin E and, for the first time, present quantitative data for the generation of ET-1, ET-2, and ET-3. The kinetic parameters thus determined indicate that the precursor big ETs bind with high affinity to cathepsin E.

MATERIALS AND METHODS

Human big ET-2, big ET-3, their corresponding ETs and C-terminal fragments were products of the American Peptide Co., Santa Clara, CA, U.S.A. Big ET-1 and human cathepsin E were obtained as described previously [9]. The inhibitor H-77 (D-His-Pro-Phe-His-Leu-ψ[CH₂-NH]-Leu-Val-Tyr) was kindly given by Dr. Peter Charlton, formerly of Glaxo Group Research Ltd., Greenford, Middx., U.K., and now at Xenova Ltd. The peptides His¹⁶-His²⁷ and His¹⁶-Ser³⁸ from the human big ET-1 sequence were synthesized on an ABI model 431A peptide synthesizer; their identities were established by m.s. analysis, and their purities were confirmed by reverse-phase h.p.l.c. and found to be > 95%.

Incubations of each human big ET with cathepsin E were carried out at 37 °C in 0.1 M-sodium formate/0.1 M-sodium

acetate buffers at a variety of pH values and at different precursor/active-proteinase ratios. After appropriate lengths of time the reaction was quenched by the addition of trifluoroacetic acid, as described previously [9]. Each endothelin thus generated was quantified by radioimmunoassay (r.i.a.) using the RPA-555 Kit (Amersham International), which has 100 % cross-reactivities with ET-1, ET-2 and ET-3. Appropriate corrections were made for non-specific reactivity by the inclusion in each assay set of a control sample containing the respective big ET incubated in the absence of cathepsin E. For the determination of the kinetic parameters, K_m and V_{max} , the initial rates (v) were measured with at least six values of the initial big-ET concentration within an appropriate range. Values were derived by using the curve-fitting program described previously [10] and were estimated to have a precision of ±5–10%. Values for k_{cat} were derived from the equation:

$$V_{max} = k_{cat} [E]$$

using cathepsin E of known concentration ([E]) determined by active-site titration with pepstatin [10].

The identity of the products (ET and the C-terminal fragment) derived from human big ET-2 and big ET-3 respectively by the action of cathepsin E was established by reverse-phase h.p.l.c. as described previously [9]. However, a modified acetonitrile (containing 0.1 % trifluoroacetic acid) gradient from 0 to 15 % over 5 min followed by 15–45 % over 13 min was used to improve resolution and to ensure separation of big ET-3 from ET-3.

RESULTS AND DISCUSSION

We have shown previously that cathepsin E cleaves big ET-1 very specifically to generate ET-1 and release the C-terminal fragment [9]. When parallel experiments were performed with big ET-2 and big ET-3 at appropriate pH values (see below), cathepsin E effected equivalent specific conversions. In both cases, (only) two cleavage products were found, which were co-eluted on h.p.l.c. with authentic standard samples of the appropriate component (not shown) and were thus identified as ET-2 (or ET-3) and the respective C-terminal fragments.

Initial experiments were also carried out to establish conditions of time and pH under which more detailed investigations could

Abbreviations used: ET, endothelin; big ET, endothelin precursor (the peptide bond in each big endothelin that is required to be cleaved to generate endothelin is indicated by an asterisk); r.i.a., radioimmunoassay; H-77, D-His-Pro-Phe-His-Leu-ψ[CH₂-NH]-Leu-Val-Tyr.

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Fig. 1. Time courses for the generation of ET-1, -2 and -3 from their respective precursors by cathepsin E

Each big ET (4 μ g) was incubated at pH 4.3 and at 37 °C with cathepsin E (30 ng), and aliquots were withdrawn at the indicated times. The amount of each ET thus generated was determined by r.i.a. $\circ$, Big ET-1; $\bullet$, big ET-2; $\star$, big ET-3.

Fig. 2. pH-dependences for the conversion of big ET-1, big ET-2 and big ET-3 into their respective ETs by cathepsin E

Incubations were performed with each big ET (0.5 μ g; 125 pmol) and cathepsin E (30 ng) at the indicated pH values. Aliquots were withdrawn after 30 min for the determination of the amount of ET generated by r.i.a. as described in the legend to Fig. 1. $\circ$, Big ET-1; $\bullet$, big ET-2; $\star$, big ET-3.

then be performed. Aliquots of each precursor, big ET-1, big ET-2 and big ET-3 (4 μ g; $\sim$ 1000 pmol) were incubated with cathepsin E (30 ng), and samples were withdrawn for analysis by r.i.a. at appropriate times. Linear time courses were observed, at pH 4.3, for big ET-1 and big ET-2 conversions, but big ET-3 was not cleaved (Fig. 1). However, when the big ET-3 incubation was performed at pH 3.3, a time course similar to those depicted for big ET-1 and -2 in Fig. 1 was obtained (results not shown). Thus, in an assay time of 30 min, the extent of conversion into each ET was always < 5% of the respective big ET.

From the pH-dependence of each big-ET cleavage (Fig. 2), it is evident that big ET-1 and big ET-2 have very similar profiles for their hydrolysis, with a plateau of maximal rates observed between approx. pH 3.5 and 4.4. By contrast, big-ET-3 cleavage has a somewhat lower optimum, in the region of pH 3.3 (Fig. 2). The scissile peptide bond which has to be cleaved in both big ET-1 and big ET-2 is -Trp*Val-, whereas that in big ET-3 is

Table 1. Kinetic parameters for the conversion of big ET-1, big ET-2 and big ET-3 into their respective ETs by human cathepsin E

Values for big ET-1 and big ET-2 conversions were measured at pH 4.3, whereas that for big ET-3 was determined at pH 3.3. Conversions were analysed by detection of each ET generated by means of an ET-specific r.i.a.

Substrate	K_m (μ M)	k_{cat} (s^{-1})
Big ET-1	9	0.13
Big ET-2	8	0.10
Big ET-3	20	0.14

-Trp*Ile-. Thus the different optimum observed for big ET-3 conversion by cathepsin E and the 9-fold slower conversion rate observed by others [6] for the cleavage of this precursor by the metalloproteinase component(s) from bovine endothelial cells may be a reflection of this slight change. However, the considerable differences that are present elsewhere in the (big) ET-3 molecule relative to (big) ET-1 and ET-2 are more likely to account for these effects.

A further indication of the significant contribution of the ET structure to precursor cleavage efficiency was derived from studies with synthetic peptides corresponding to residues His¹⁶-His²⁷ and His¹⁶-Ser³⁸ in the human big-ET-1 sequence (i.e. the downstream region of the molecule devoid of residues 1-15 which contribute the two intramolecular -S-S- loops of ET [9]). Despite both of these containing the (potential) -Trp*Val- scissile peptide bond, neither were cleaved, even upon prolonged incubation with cathepsin E.

Kinetic parameters were determined for the processing of big ET-1 and big ET-2 by cathepsin E at pH 4.3, whereas the equivalent measurements were made with big ET-3 at pH 3.3. The data obtained (Table 1) show that big ET-1 and big ET-2 have barely distinguishable values for K_m . Indeed, the value measured for big-ET-3 processing, albeit at a different pH value, was only 2-fold greater than those derived for big ET-1 and big ET-2 (but sufficient to account for the apparently lower rate of conversion of big ET-3 at the optimum pH in Fig. 2, where the measurements were made at a single, constant, concentration of each precursor). The k_{cat} values determined for all three big ETs were virtually identical (Table 1).

Thus all three polypeptides (of approx. 40 residues) bind very effectively to human cathepsin E, with a magnitude (10-20 μ M) directly comparable with the best chromogenic substrates that have been reported for this enzyme [10-13]. Indeed, this was substantiated by using the conversion of big ET by cathepsin E as an assay to quantify inhibition of the enzyme (e.g. by the compound H-77). The K_i derived at pH 4.3 for the interaction of this competitive inhibitor with cathepsin E by this means (approx. 0.5 nM) was essentially identical with values obtained utilizing other (more convenient) types of (chromogenic) substrate. An inhibitor such as H-77 may therefore be valuable for use in identification of enzymes in tissue/cell extracts that are being investigated as potential endothelin-converting-enzyme activities.

Many previous investigations have indicated that mammalian aspartic proteinases can accommodate as many as nine residues in the P₅-P₄' positions within the length of the active-site cleft [14]. By using a series of chromogenic substrates in which the residue in each individual position from P₅ to P₄' was systematically varied, Rao *et al.* [13] have very recently demonstrated that cathepsin E is no exception. The identity of residues in individual positions, even those as remote from the catalytic

apparatus as P_5 and P_4 , had minimal effects on K_m , but substantially influenced k_{cat} . On this basis the low values derived for the catalytic-centre activities with each of the three precursors (Table 1) may be readily rationalized. Whereas a lysine residue was shown to be optimal in P_5 by Rao *et al.* [13], the presence of a leucine in this position lowered k_{cat} from 10 to 0.6 s^{-1} ; similarly, replacement of an optimal proline residue in P_4 with aspartic acid resulted in a 3-fold lowering of k_{cat} [13]. The sequences around the scissile peptide bonds in big ET-1, big ET-2 and big ET-3 are closely similar, but not identical:

	P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'
Big ET-1	-His-Leu-Asp-Ile-Ile-Trp*	Val-Asn-Thr-Pro-Glu-His-										
Big ET-2	-His-Leu-Asp-Ile-Ile-Trp*	Val-Asn-Thr-Pro-Glu-Gln-										
Big ET-3	-His-Leu-Asp-Ile-Ile-Trp*	Ile-Asn-Thr-Pro-Glu-Gln-										

The residues (-Leu-Asp-), which occupy the S_5 and S_4 subsites within the cathepsin E active-site cleft are, however, conserved in all three precursors. It is therefore perhaps not surprising that cathepsin E displays relatively low k_{cat} values when acting upon polypeptide substrates containing residues that appear to be poorly accommodated. Since peptides spanning most of the big ET-1 molecule (residues 16–27 and 16–38) and indeed ET-1 itself [9] were not cleaved by cathepsin E, it is evident that the three-dimensional structure of each big ET is an important parameter that governs presentation of, or accessibility to, the scissile -Trp*Val/Ile- bond. The pH-dependences of cathepsin E acting upon substrates such as big ETs are characteristic of this class of proteinase. However, we have shown previously that cathepsin E is stabilized by physiological concentrations of ATP [15]. Its activity (as reflected in k_{cat}) is thus maintained to pH values of 6 and above [9,15] and, indeed, one recent report has demonstrated a concomitant release of ATP together with ET after deliberate stimulation of endothelial cells [16]. Thus, if these effects should be reflected *in vivo* within the tubulovesicular secretory network, cleavage of the various big ETs, even at the relatively low rates quantified for cathepsin E, would still be capable of generating the low concentrations of endothelin that are necessary for it to manifest its physiological effects. Human endothelial cells in culture secrete 1 fmol of ET/min per 10^6 cells

[17], and circulating levels of ET in normal human plasma range between 0.1 and 5 pM [18].

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Received 9 December 1991/14 January 1992; accepted 20 January 1992