Regulation of carboxypeptidase E

Effect of pH, temperature and Co^{2+} on kinetic parameters of substrate hydrolysis

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Carboxypeptidase E is a member of the carboxypeptidase A and B gene family, with many of the putative active-site and substrate-binding residues conserved between these enzymes. However, the pH optimum of carboxypeptidase E is substantially lower than that of carboxypeptidases A and B. To evaluate whether the difference in the pH optima of these carboxypeptidases reflects fundamental differences in the ionization behaviour of active-site residues, the influence of pH on carboxypeptidase E activity was examined. The V_{max} for hydrolysis of dansyl-Phe-Ala-Arg is pH-independent between 5 and 7, but decreases at pH values below 5. The $\text{p}K_a$ for the group the protonation of which leads to the loss of activity is approximately 4.8, and the slope of the V_{max}/pH profile suggests that only a single ionizable group is involved. In contrast, K_m and V_{max}/K_m are dramatically influenced by pH over the range 5–7, with multiple ionizable groups detected in this pH range. The $\text{p}K_a$ of the group the protonation of which decreases the V_{max} of substrate hydrolysis is lower (4.5) for carboxypeptidase E which had been reconstituted with Co^{2+} . The enthalpy of ionization of the group observed in the V_{max} profile for carboxypeptidase E is approx. 28.9 kJ/mol. These results are compatible with the active-site model of the homologous carboxypeptidase A: in this model the ionization of a metal-bound water molecule is responsible for the observed decrease in V_{max} .

INTRODUCTION

Carboxypeptidase E (CPE) is a prohormone-processing enzyme which is present in brain and many endocrine tissues (Fricker, 1988, 1991). This enzyme has been designated EC 3.4.17.10, and is also known as carboxypeptidase H and enkephalin convertase. CPE removes C-terminal basic amino acids from a wide range of peptide hormone precursors (Fricker & Snyder, 1982; Hook & Loh, 1984; Davidson & Hutton, 1987; Fricker, 1988, 1991; Smyth *et al.*, 1989). CPE has been purified to apparent homogeneity from bovine brain, pituitary and adrenal medulla (Fricker & Snyder, 1983). The amino acid sequences of bovine, rat and human CPE, which have been deduced from the nucleotide sequences of cDNA clones, show homologies of greater than 90% between these species (Fricker *et al.*, 1986, 1989; Rodriguez *et al.*, 1989; Manser *et al.*, 1990). The gene encoding rat CPE has recently been isolated and partially sequenced (Jung *et al.*, 1991). One intron in the CPE gene is located in a position identical with an intron within the rat carboxypeptidase A (CPA) and carboxypeptidase B (CPB) genes, suggesting that these different enzymes evolved from the same ancestral protein (Jung *et al.*, 1991).

The amino acid sequences of human, rat and bovine CPE show some homology with other metallocarboxypeptidases, such as pancreatic CPA and CPB, mast-cell CPA, serum CPB, serum carboxypeptidase N (CPN), placental carboxypeptidase M (CPM), and carboxypeptidase SG (CPSG), a bacterial carboxypeptidase (Bradshaw *et al.*, 1969; Titani *et al.*, 1975; Fricker *et al.*, 1986; Rodriguez *et al.*, 1989; Reynolds *et al.*, 1989b; Gebhard *et al.*, 1989; Tan *et al.*, 1989; Manser *et al.*, 1990; Narahashi, 1990; Vallee & Auld, 1990; Eaton *et al.*, 1991; Fricker, 1991). Analysis of the sequence similarity suggests that there are three subgroups of metallocarboxypeptidase: two for the mammalian enzymes and a third for the bacterial enzyme. Pancreatic CPA,

pancreatic CPB, plasma CPB and mast-cell CPA show approx. 40–50% sequence identity with each other, but only 20% identity with CPE, CPM and CPN (Bradshaw *et al.*, 1969; Titani *et al.*, 1975; Fricker *et al.*, 1986; Reynolds *et al.*, 1989b; Gebhard *et al.*, 1989; Tan *et al.*, 1989; Eaton *et al.*, 1991). Similarly, CPE, CPM and CPN are approx. 40–50% identical with each other, and only 20% identical with the other carboxypeptidases (Bradshaw *et al.*, 1969; Titani *et al.*, 1975; Fricker *et al.*, 1986; Reynolds *et al.*, 1989b; Gebhard *et al.*, 1989; Tan *et al.*, 1989; Eaton *et al.*, 1991). In addition, the sizes of these two subgroups of carboxypeptidases are different: pancreatic CPA and CPB, serum CPB, and mast-cell CPA are all 30–35 kDa, whereas CPE, CPM, and CPN are 50–60 kDa (Bradshaw *et al.*, 1969; Titani *et al.*, 1975; Fricker *et al.*, 1986; Reynolds *et al.*, 1989b; Gebhard *et al.*, 1989; Tan *et al.*, 1989; Eaton *et al.*, 1991). The bacterial carboxypeptidase, CPSG, represents a third group of this gene family, with approx. 20–30% sequence identity between CPSG and either the CPA/B subfamily or the CPE/M/N subfamily. The size of the bacterial carboxypeptidase is approximately 34 kDa, and the substrate specificity appears to represent a combination of the specificities of CPA and CPB (Narahashi & Yoda, 1979).

Although the overall homology between the three subgroups of carboxypeptidases is low, all of the amino acids thought to be essential for metal binding and catalytic activity in CPA and CPB are present in comparable positions in the other carboxypeptidases (Fig. 1). These residues include His-69, Glu-72 and His-196, which bind the Zn^{2+} in CPA, and Glu-270, which is thought to participate in the cleavage of the substrate (Vallee & Auld, 1990). (Note: the numbering of CPA is used; the position of these residues is slightly different in the other carboxypeptidases.) Some of the amino acids that are thought to bind to the substrate in CPA, such as Arg-145 and Tyr-248 are also conserved in all carboxypeptidases (Vallee & Auld, 1990). Other substrate-binding amino acids in CPA, such as Arg-71, Arg-127,

Abbreviations used: CPE, CPA, CPB, CPM and CPN, carboxypeptidases E, A, B, M and N; CPSG, carboxypeptidase SG.

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Fig. 1. Comparison of the putative active-site regions of metallo-carboxypeptidase

Putative active-site and/or substrate-binding residues of CPA/B are marked with asterisks. Number above the asterisk indicates the position within bovine CPA. #, Residue discussed in text. Abbreviations are: Bovine E, bovine CPE (Fricker *et al.*, 1986); Human M, human CPM (Tan *et al.*, 1989); Human N, human CPN (Gebhard *et al.*, 1989); Hum.MC-A, human mast-cell CPA (Reynolds *et al.*, 1989b); Bovine A, bovine CPA (Bradshaw *et al.*, 1969); Bovine B, bovine CPB (Titani *et al.*, 1975); Hum.PL-B, human plasma CPB (Eaton *et al.*, 1991); and Bact.SG, bacterial (*Streptomyces griseus*) carboxypeptidase SG (Narahashi, 1990).

Tyr-198, Gly-207 and Ile-255 are replaced by an Asn, Lys, Gly, Asp, and Gln (respectively) in CPE, as well as in CPN (Fig. 1). CPM contains most of these substitutions, but with an Asp, rather than an Asn, for the Arg-71 of CPA (Fig. 1).

Except for CPE, all of the metallocarboxypeptidases are maximally active in the neutral pH range (Hartsuck & Lipscomb, 1971; Folk, 1971; Narahashi & Yoda, 1979; Plummer & Erdos, 1981; Skidgel *et al.*, 1989; Reynolds *et al.*, 1989b). In contrast, CPE is maximally active in the pH 5.0–5.5 range (Fricker & Snyder, 1982), corresponding to the intragranular pH of secretory granules in which CPE presumably functions (Russell, 1984; Johnson & Scarpa, 1976). The reported pH optimum of CPE was determined using a single substrate concentration, and no information regarding the effect of pH on K_m or V_{max} was provided. The influence of pH on the kinetic parameters for substrate hydrolysis can provide an indication of the involvement of active-site residues in binding and catalysis (Fersht, 1983). The analysis of the pH-dependence of V_{max} of the reaction catalysed by CPA has demonstrated that two ionizable groups, with pK_a approx. 6 and approx. 9, are involved in catalysis (Auld & Vallee, 1970; Kaiser & Kaiser, 1972; Makinen *et al.*, 1985; Mock & Tsay, 1988). Although there are conflicting reports regarding the participation of specific groups in the active site of CPA, the consensus is that the decrease in CPA activity (k_{cat}/K_m) at lower pH is due to protonation of the metal-bound hydroxide ion (pK_a approx. 6). The decrease in CPA activity at high pH is less clear; the conventional assignment of Tyr-248 to this ionization is not

supported by recent data, and other active-site residues have been proposed (Makinen *et al.*, 1985; Mock & Tsay, 1988). Likely candidates include His-69 or His-196, both of which bind Zn^{2+} and thus would perturb pK values (Mock & Tsay, 1988).

To investigate whether the active site of CPE is similar to that of CPA and other metallo-carboxypeptidases, the effect of pH on the $V_{\max}$ and $V_{\max}/K_m$ of substrate hydrolysis by CPE has been determined. In addition, the influence of temperature and metal substitution on the kinetic parameters has been examined. Some interesting similarities and differences between CPE and CPA are apparent from this analysis.

MATERIALS AND METHODS

Purification of CPE and preparation of Co^{2+} -substituted CPE

Enzyme was purified from 0.1 M-sodium acetate, pH 5.5, extracts of bovine pituitary glands (from Pel-Frez), as previously described (Fricker & Snyder, 1983), with minor modifications in the conditions for washing and eluting the affinity column (Fricker *et al.*, 1990). Basically, the 3000–5000-fold purification of CPE from the pituitary requires a single-step on a Sepharose-*p*-aminobenzoyl-Arg substrate affinity resin. CPE activity is specifically eluted from the column by raising the pH of the buffer from 5.5 to 8 (Fricker *et al.*, 1990). Denaturing PAGE of the purified CPE shows a single band with an apparent M_r of approx. 50000. Protein was determined from the Bradford (1976) assay with BSA as standard.

Co²⁺-substituted CPE was prepared by a modification of the procedures of Davies *et al.* (1968). First, apoenzyme was prepared by treating a solution of 100 μ g of CPE (2 nmol), 200 μ g of BSA and 0.1% Triton X-100 in 10 mM-sodium acetate, pH 5.5, with 5 mM-1,10-phenanthroline for 1 h at 4 °C (final volume 2 ml). The enzyme solution was then dialysed against 2 litres of 10 μ M-1,10-phenanthroline for 2 h, and then against 4 litres of 1 mM-CoCl₂ (99.999% pure, Aldrich) overnight. This CoCl₂ solution and all subsequent buffers were made in glass-distilled deionized water that had been additionally treated with Chelex 100 (Bio-Rad). For the assay of Co²⁺-substituted CPE activity, all buffers contained a final concentration of 1 mM-CoCl₂, as previously described for the assay of Co²⁺-substituted CPA (Davies *et al.*, 1968).

CPE assay

CPE activity was assayed using the substrate dansyl-Phe-Ala-Arg. This peptide was synthesized from dansyl-Phe and Ala-Arg as previously described (Fricker & Snyder, 1983). The purity of the substrate was more than 99%, on the basis of t.l.c. in two different solvent systems and amino acid analysis (Fricker & Snyder, 1983). To remove traces of Zn^{2+} from the substrate, stock solutions were treated with dithizone dissolved in carbon tetrachloride before use (Davies *et al.*, 1968). The final substrate concentrations used for kinetic analysis over the pH range 3.8–6 were 200, 100, 50, 25, 12.5 and 6.25 μM . Over the pH range 6.2–6.4, additional substrate concentrations of 800 and 400 μM were used; for pH values above 6.5, the highest substrate concentration was 1.6 mM.

The assay was performed by adding 125 μ l of enzyme/buffer mixture to 125 μ l of substrate in borosilicate culture tubes (washed with HNO₃ for the studies with Co²⁺-substituted CPE). All points were measured in triplicate. The enzyme/buffer mixture was typically composed of purified CPE, 0.2 mg of BSA/ml, 0.02% Triton X-100 and 0.1 M-Tris/acetate buffer of various pH values. The actual pH of each sample was determined from a duplicate mixture of BSA, Triton and buffer, but without enzyme, at the appropriate temperature (usually 37 °C). The

amount of CPE varied from 25 ng/125 μ l over the pH range 4.8–6.0, with an increase in the amount of CPE for the other pH values, up to a maximum of 250 ng/125 μ l for pH values below 4.4 and above 6.6. For Co^{2+} -substituted CPE, the amount of CPE, and the pH range over which the amount was increased, was slightly different. For all pH values, the amount of CPE or Co^{2+} -substituted CPE that was used produced 10–30% hydrolysis of the substrate over the length of the incubation. The incubations were typically for 20 min at 37 °C. For the study on the enthalpy of the pK_a values, incubation times of 30 min at 25 °C, 1 h at 15 °C and 1 h at 4 °C were used, and a sodium acetate buffer was used in place of the Tris/acetate buffer. After the incubation, 100 μ l of 0.5 M-HCl was added to each tube to quench the reaction. Then 2 ml of chloroform was added, the tubes were mixed on a vortex mixer, centrifuged at 1000 g for 1 min, and then the fluorescence in the chloroform phase was determined by directly inserting the tubes into a Perkin–Elmer LS3 spectrofluorimeter. The aqueous phase is above the path of the light beam, and does not interfere with the fluorescence measurement. Substrate is virtually insoluble in chloroform, whereas the product of carboxypeptidase activity, dansyl-Phe-Ala, quantitatively partitions into this solvent (Fricker & Snyder, 1983). Blanks, containing substrate and buffer, but no enzyme, were similarly analysed. The fluorescent readings were converted into pmol of product using a standard curve generated with dansyl-Phe-Ala.

Data analysis

Reciprocal initial rates were plotted against reciprocal substrate concentrations, and the data were fitted to the appropriate equations using the Fortran programs of Cleland (1979). The individual saturation curves used to obtain pH profiles were fitted to eqn. (1), where A is the variable substrate concentration. Data for pH profiles that showed a decrease in $\log V_{\text{max}}$ with a slope of +1 as the pH was decreased were fitted to eqn. (2). Data for pH profiles of $\log V_{\text{max}}/K$ were more complicated, and the data were analysed using several equations. The entire dataset was fitted to eqn. (3) which describes a bell-shaped curve. Then the subset of the data obtained between pH 3.8 and 5.4 was fitted to either eqn. (2) or eqn. (4), and another subset of the data obtained between pH 4.5 and 6.7 was fitted to either eqn. (5) or eqn. (6). In eqns. (2)–(6), y is the parameter the pH-dependence of which is being determined (V_{max}/K for peptide hydrolysis), C is the pH-independent value of y , K_1 and K'_1 are the dissociation constants of the groups the protonation of which causes y to decrease from its pH-independent value, and K_2 and K'_2 are the dissociation constants of the groups the deprotonation of which causes y to decrease from its pH-independent value.

$$v = V_{\text{max}} \cdot A / (K + A) \quad (1)$$

$$\log y = \log \{C / (1 + [\text{H}^+] / K_1)\} \quad (2)$$

$$\log y = \log \{C / (1 + [\text{H}^+] / K_1 + K_2 / [\text{H}^+])\} \quad (3)$$

$$\log y = \log \{C / (1 + [\text{H}^+] / K_1 + [\text{H}^+]^2 / K_1 K'_1)\} \quad (4)$$

$$\log y = \log \{C / (1 + K_2 / [\text{H}^+])\} \quad (5)$$

$$\log y = \log \{C / (1 + K_2 / [\text{H}^+] + K_2 K'_2 / [\text{H}^+]^2)\} \quad (6)$$

RESULTS

Double-reciprocal plots for the hydrolysis of dansyl-Phe-Ala-Arg by CPE are linear over a 32-fold range of substrate concentration spanning the K_m for all pH values examined. No evidence of substrate inhibition is observed for the hydrolysis of

dansyl-Phe-Ala-Arg by CPE at any pH examined; this differs from the marked substrate inhibition found for the hydrolysis of small peptide substrates by CPA (Davies *et al.*, 1968).

The pH-dependencies of V_{max} , K_m , and V_{max}/K_m are shown in Fig. 2. V_{max} is pH-independent at pH values between 5 and 6.6; it could not be accurately determined at higher pH because of the need for substrate concentrations exceeding the solubility of the peptide. For pH values below 5, the V_{max} decreases with a slope of 1 as a single ionizable group exhibiting a pK_a value of 4.84 ± 0.08 (S.E.M.) is protonated. The changes in V_{max}/K_m with pH are substantially more complicated than the changes in V_{max} , and graphical analysis suggests that multiple ionizations which influence V_{max}/K_m are occurring over the pH range examined (Fig. 2). From the graphs, it appears that V_{max}/K_m decreases below pH 5 with a slope of 2, suggesting that two groups with pK_a approx. 4–5 are protonated. Similarly, V_{max}/K_m decreases with a slope of 2 above pH 5 as two groups with pK_a approx. 5–6 are deprotonated. The decrease in V_{max}/K_m above pH 5 is mirrored by a large change in the K_m values of the peptide substrate. Since V_{max} is pH-independent in this range, the pH-dependence of V_{max}/K_m and K_m suggests that two ionizing groups on free enzyme or free substrate with pK_a approx. 5–6 are involved in substrate binding.

Analysis of the V_{max}/K_m data was performed in several ways. The entire dataset was first analysed by using eqn. (3), which assumes two ionizing groups: a single group on the acidic side the deprotonation of which leads to an increase in V_{max}/K , and another single group on the basic side the deprotonation of which leads to a decrease in V_{max}/K . Further analysis was performed using subsets of the data, corresponding to either the acidic range (3.8–5.4) or basic range (4.5–6.7). For the acidic range, computer analysis using eqn. (2), which assumes a single ionization, yielded a pK_a value of 5.0 ± 0.2 , with a sigma value of 0.47. Analysis of the same data with eqn. (4), which assumes two ionizations, yielded a statistically better fit with identical pK_a values of 4.40 ± 0.03 and a sigma value of 0.15. The analysis using the basic pH value subset of data also gave a better fit when the equation which assumes two ionizations [eqn. (6)] was used. This analysis yielded identical pK_b values of 5.59 ± 0.02 and a sigma value of 0.15. In contrast, analysis of these data with eqn. (5), which assumes only a single ionization, yielded a pK_b value

Fig. 2. Effect of pH on kinetic parameters for dansyl-Phe-Ala-Arg hydrolysis

Enzyme activity was determined with six concentrations of substrate, as described in the Materials and methods section, with an incubation of 20 min at 37 °C. All points were measured in triplicate, with less than 5% variation between replicates. The pH values were determined from a duplicate set of samples, without enzyme, also at 37 °C. □, V_{max}/K_m ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \cdot \mu\text{M}^{-1}$); △, K_m (μM); O, V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$).

Fig. 3. Comparison of the effect of pH on $V_{\max}$ for substrate hydrolysis of native CPE (O) and Co^{2+} -substituted CPE (□)

The assay was performed as described in the Materials and methods section and legend to Fig. 2. Co^{2+} -substituted CPE was prepared by dialysis of the enzyme first with 1,10-phenanthroline, and then with 1 mM- CoCl_2 , as described in the Materials and methods section. The native CPE was also dialysed, but without phenanthroline or CoCl_2 . Both activities were assayed at the same time, and with the same dilutions of substrate.

Fig. 4. Effect of temperature on the pH-dependence of $V_{\max}$ for substrate hydrolysis by native CPE

The $V_{\max}$ values shown are relative, and are normalized to 10 for the value at pH 5.8. The actual $V_{\max}$ values at pH 5.8 are 6.8 (37 °C; Δ), 4.4 (25 °C; $\circ$) and 1.9 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (13 °C; $\square$). Inset: Calculated pK_a values versus reciprocal inverse absolute temperature. Error bars show S.E.M.

of 4.9 ± 0.2 with a sigma value of 0.61. These computer-generated pK values for the $V_{\max}/K_m$ profile should be interpreted with caution since the two acidic and two basic ionizations are not widely separated, and are therefore likely to be shifted from their intrinsic values (Cleland, 1977).

In an attempt to assign the pK_a values determined from the pH-dependence of $V_{\max}$ to a specific chemical entity, the pH profile was examined using CPE in which the native metal (Zn^{2+}) was replaced with Co^{2+} (Fig. 3). Double-reciprocal plots of the hydrolysis of dansyl-Phe-Ala-Arg by Co^{2+} -substituted CPE are linear over the substrate range investigated (results not shown). The $V_{\max}$ for substrate hydrolysis by Co^{2+} -substituted CPE at pH 5.4 ($40 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) is 6-fold higher than the $V_{\max}$ for native CPE at this pH. The K_m value for peptide substrate using

Co^{2+} -substituted CPE ($27 \mu\text{M}$) is also higher than the K_m for native CPE ($16 \mu\text{M}$), but the difference is much smaller than the difference in $V_{\max}$. The pH-dependence of the $V_{\max}$ of substrate hydrolysis catalysed by Co^{2+} -substituted CPE decreases as a group with a pK_a value of 4.46 ± 0.11 (S.E.M.) is protonated. This pK_a value is approximately 0.4 pH unit lower than the corresponding pK_a values for native CPE. This shift in the pK_a is slightly smaller, but in the same direction as the 0.7–0.9 pH unit shift observed on substitution of Co^{2+} for the Zn^{2+} of CPA (Auld & Vallee, 1970).

The temperature-dependence of the pK value observed in $V_{\max}/\text{pH}$ profiles catalysed by native CPE was determined (Fig. 4). The pK and the reciprocal absolute temperature show a linear relationship over the temperature range examined (inset, Fig. 4). The enthalpy of ionization calculated from the slope of pK_a versus inverse temperature is 28.9 kJ/mol for the group the protonation of which decreases $V_{\max}$.

DISCUSSION

The major findings of the kinetic analysis of CPE are (1) enzyme activity is markedly influenced by pH over the physiological range 5–7, and (2) the decrease in CPE activity caused by increasing pH above 5.5 is due to a change in K_m for substrate binding, and not a change in $V_{\max}$. This result and the results with Co^{2+} -substituted CPE and with native CPE at various temperatures are consistent with a catalytic mechanism similar to that proposed for CPA and CPB.

Although the exact mechanisms of action of CPA and CPB have not been unequivocally identified, there is general agreement that both Glu-270 and the metal-bound water molecule play important roles (Auld & Vallee, 1970, 1971; Kaiser & Kaiser, 1972; Schmid & Herriott, 1976; Makinen *et al.*, 1985; Mock & Tsay, 1988). Recent evidence supports the proposal that the metal-bound water molecule in CPA is the ionizing group exhibiting the pK_a value of approx. 6 which decreases both $V_{\max}$ and $V_{\max}/K_m$. The key points that support this assignment are the enthalpy of ionization (Auld & Vallee, 1971), the shift to lower pK_a values by substitution of Co^{2+} for the native Zn^{2+} in CPA (Auld & Vallee, 1970) and the pH-dependence of K_i for several active-site-directed inhibitors (Mock & Tsay, 1988). In the present study, the apparent shift in pK_a of $V_{\max}$ for the Co^{2+} -substituted CPE is consistent with the ionization of a metal-bound water molecule. Furthermore, the enthalpy of ionization of the group in CPE the protonation of which decreases $V_{\max}$ (28.9 kJ/mol) is also consistent with the ionization of a metal-bound water; this enthalpy is considerably higher than the enthalpies expected for the carboxylate group of Glu or Asp residues (approx. 4.2 kJ/mol). Although kinetic analysis cannot distinguish between metal-bound water (pK_a approx. 6; ΔH approx. 25–29 kJ/mol) and histidine residues (pK_a approx. 6; ΔH approx. 29 kJ/mol) (Makinen *et al.*, 1985), the most likely assignment of this ionization is that of a metal-bound water molecule, based on the sequence identity between CPA and CPE, and the shift in pK_a observed for Co^{2+} -substituted CPE.

The change in CPE activity observed in the pH range 5–7 appears to be due to a change in substrate affinity, and not a change in $V_{\max}$. The ionizations in this pH range are due to changes in the free enzyme, and not the free substrate (dansyl-Phe-Ala-Arg) which has pK_a values approx. 2 and approx. 12 (results not shown). Assignment of the groups responsible for the ionization of CPE in this pH range is difficult. The separation between the pK_a values for the acidic and basic limbs of $V_{\max}/K_m$ are too close to permit accurate measurements (Cleland, 1977; Fersht, 1983). Attempts to determine the enthalpy of these ionizations were not reproducible (results not shown). Without

this information, it is not possible to distinguish the ionization of a histidine residue from an aspartate, glutamate or even a highly perturbed lysine; all of these residues can have pK_a values in the approx. 5–6 range (Fersht, 1983).

Analysis of the homologies between CPE and the other metallocarboxypeptidases can provide some clues as to the groups responsible for the pH-dependent substrate binding. Assuming that CPE has a similar, but not necessarily identical, three-dimensional structure to that of CPA and the other enzymes, there are a relatively small number of His, Glu and Asp residues in CPE that are not found in comparable positions in any of the other enzymes. For this analysis, only those residues conserved between human, bovine, rat and angler-fish CPE were considered (Manser *et al.*, 1990; Fricker *et al.*, 1986, 1989; Rodriguez *et al.*, 1989; Roth *et al.*, 1991). The sequences of CPE were compared with human CPM (Tan *et al.*, 1989), human CPN (Gebhard *et al.*, 1989), human and mouse mast-cell CPA (Reynolds *et al.*, 1989a,b), bovine and rat CPA (Bradshaw *et al.*, 1969; Quinto *et al.*, 1982; Gardell *et al.*, 1988), bovine, rat and crayfish CPB (Titani *et al.*, 1975, 1984; Clauser *et al.*, 1988), human plasma CPB (Eaton *et al.*, 1991) and bacterial CPSG (Narahashi, 1990). Only three histidine residues (positions 105, 192 and 225 of bovine CPE) are conserved in CPE but not in any of the other enzymes. If the three-dimensional structure of CPE is similar to that of CPA and CPB, these histidines would not be located in appropriate positions to interact with the substrate: His-105 would be present near the exterior portion of α -helix C [using the numbering system of Schmid & Herriott (1976)], His-192 would be present in the middle of α -helix E, and His-225 would be located in an exterior loop between β -sheet 6 and α -helix F. All of these positions are on the opposite side of the protein from the substrate-binding region (of CPA). The possibility that an aspartic acid or a glutamic acid is involved with the substrate binding is more likely from this analysis. There are five glutamic acid and seven aspartic acid residues in CPE that are not in the other carboxypeptidases. Although this analysis is quite speculative, there are two acidic residues that deserve to be mentioned. Asp-132 and Asp-209 in CPE (Fig. 1) are presumably near the substrate-binding region since these residues are near other residues predicted to be in the active site by homology with CPA and CPB. In CPA, CPB, plasma CPB, mast-cell CPA and CPSG, the residues in a position comparable with these Asp residues are usually protonated at neutral pH (Lys, Ser). Thus only the protonated form of Asp-132 and Asp-209 could be functionally equivalent to the corresponding residues in CPA, CPB, plasma CPB, mast-cell CPA and CPSG. If the protonated form of these groups are involved with substrate binding, the ionization of the Asp residues in CPE could account for the observed decrease in K_m for pH values above 5.5. It is also possible that other residues within CPE are responsible for the observed pH-dependence of enzyme activity. Residues located at a distance from the substrate-binding region may play a role in the conformation of CPE, which may in turn influence substrate binding. Studies examining the structure of CPE are needed to resolve this possibility, and to elucidate the mechanism of the pH-dependent regulation of CPE.

The kinetic behaviour of CPE over the pH range 5–7 is important since this range corresponds to the physiological pH values encountered by CPE within the cell. CPE presumably functions in the processing of bioactive peptides within secretory granules (Fricker, 1988). Before the packaging of CPE into the secretory granules, CPE is initially produced in the endoplasmic reticulum, and then transported through the Golgi apparatus. The internal pH of the endoplasmic reticulum and Golgi apparatus is near neutrality, and CPE would have extremely low activity in these compartments. As CPE is packaged into the

secretory granules in the trans Golgi network, the slightly acidic pH of this compartment (Anderson & Pathak, 1985) would cause a substantial increase in CPE activity. This is consistent with the findings that processing of proinsulin (Orci *et al.*, 1987), pro-opiomelanocortin (Schnabel *et al.*, 1989) and prosomatostatin (Bourdais *et al.*, 1990) does not occur in the cis or medial Golgi, but only in the trans Golgi network and newly formed secretory granules. At the intragranular pH of the mature secretory granules, which is approximately 5.0–5.5 for granules from bovine pituitary and adrenal medulla (Johnson & Scarpa, 1976; Russell, 1984), CPE would be maximally active. Finally, on the secretion of CPE from the cell, the neutral pH of the extracellular fluid would substantially decrease CPE activity. Thus pH values in the physiological range presumably regulate CPE activity *in vivo*.

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REFERENCES

- Anderson, R. G. W. & Pathak, R. K. (1985) *Cell* **40**, 635–643
- Auld, D. S. & Vallee, B. L. (1970) *Biochemistry* **9**, 4352–4359
- Auld, D. S. & Vallee, B. L. (1971) *Biochemistry* **10**, 2892–2897
- Bourdais, J., Devilliers, G., Girard, R., Morel, A., Benedetti, L. & Cohen, P. (1990) *Biochem. Biophys. Res. Commun.* **170**, 1263–1272
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Bradshaw, R. A., Ericsson, L. H., Walsh, K. A. & Neurath, H. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 1389–1394
- Clauser, E., Gardell, S. J., Craik, C. S., MacDonald, R. J. & Rutter, W. J. (1988) *J. Biol. Chem.* **263**, 17837–17845
- Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol.* **45**, 273–388
- Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103–138
- Davidson, H. W. & Hutton, J. C. (1987) *Biochem. J.* **245**, 575–582
- Davies, R. C., Riordan, J. F., Auld, D. S. & Vallee, B. L. (1968) *Biochemistry* **7**, 1090–1099
- Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W. & Drayna, D. (1991) *J. Biol. Chem.* **266**, 21833–21838
- Fersht, A. (1983) in *Enzyme Structure and Mechanism*, pp. 134–155, W. H. Freeman and Co., San Francisco
- Folk, J. E. (1971) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn., pp. 57–79, Academic Press, New York
- Fricker, L. D. (1988) *Annu. Rev. Physiol.* **50**, 309–321
- Fricker, L. D. (1991) in *Peptide Biosynthesis and Processing* (Fricker, L. D., ed.), pp. 199–230, CRC Press, Boca Raton, FL
- Fricker, L. D. & Snyder, S. H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3886–3890
- Fricker, L. D. & Snyder, S. H. (1983) *J. Biol. Chem.* **258**, 10950–10955
- Fricker, L. D., Evans, C. J., Esch, F. S. & Herbert, E. (1986) *Nature (London)* **323**, 461–464
- Fricker, L. D., Adelman, J. P., Douglass, J., Thompson, R. C., von Strandmann, R. P. & Hutton, J. (1989) *Mol. Endocrinol.* **3**, 666–673
- Fricker, L. D., Das, B. & Angeletti, R. H. (1990) *J. Biol. Chem.* **265**, 2476–2482
- Gardell, S. J., Craik, C. S., Clauser, E., Goldsmith, E. J., Stewart, C. B., Graf, M. & Rutter, W. J. (1988) *J. Biol. Chem.* **263**, 17828–17836
- Gebhard, W., Schube, M. & Eulitz, M. (1989) *Eur. J. Biochem.* **178**, 603–607
- Hartsuck, J. A. & Lipscomb, W. N. (1971) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn., pp. 1–56, Academic Press, New York
- Hook, V. Y. H. & Loh, Y. P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2776–2780
- Johnson, R. G. & Scarpa, A. (1976) *J. Biol. Chem.* **251**, 2189–2191
- Jung, Y.-K., Kunczt, C. J., Pearson, R. K., Dixon, J. E. & Fricker, L. D. (1991) *Mol. Endocrinol.* **5**, 1257–1268
- Kaiser, E. T. & Kaiser, B. L. (1972) *Acc. Chem. Res.* **5**, 219–224
- Makinen, M. W., Wells, G. B. & Kang, S. O. (1985) *Adv. Inorg. Biochem.* **6**, 1–69
- Manser, E., Fernandez, D., Loo, L., Goh, P. Y., Monfries, C., Hall, C. & Lim, L. (1990) *Biochem. J.* **267**, 517–525

- Mock, W. L. & Tsay, J. T. (1988) *J. Biol. Chem.* **263**, 8635–8641
- Narahashi, Y. (1990) *J. Biochem. (Tokyo)* **107**, 879–886
- Narahashi, Y. & Yoda, K. (1979) *J. Biochem. (Tokyo)* **86**, 683–694
- Orci, L., Ravazzola, M., Storch, M. J., Anderson, R. G. W., Vassalli, J. D. & Perrelet, A. (1987) *Cell* **49**, 865–868
- Plummer, T. H., Jr. & Erdos, E. G. (1981) *Methods Enzymol.* **80**, 442–449
- Quinto, C., Quiroga, M., Swain, W. F., Nikovits, W. C., Jr., Standring, D. N., Pictet, R. L., Valenzuela, P. & Rutter, W. J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 31–35
- Reynolds, D. S., Stevens, R. L., Gurley, D. S., Lane, W. S., Austen, K. F. & Serafin, W. E. (1989a) *J. Biol. Chem.* **264**, 20094–20099
- Reynolds, D. S., Gurley, D. S., Stevens, R. L., Sugarbaker, D. J., Austen, K. F. & Serafin, W. E. (1989b) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9480–9484
- Rodriguez, C., Brayton, K. A., Brownstein, M. & Dixon, J. E. (1989) *J. Biol. Chem.* **264**, 5988–5995
- Roth, W. W., Mackin, R. B., Spriss, J., Goodman, R. H. & Noe, B. D. (1991) *Mol. Cell. Endocrinol.* **78**, 171–178
- Russell, J. T. (1984) *J. Biol. Chem.* **259**, 9496–9507
- Schmid, M. F. & Herriott, J. R. (1976) *J. Mol. Biol.* **103**, 175–190
- Schnabel, E., Mains, R. E. & Farquhar, M. G. (1989) *Mol. Endocrinol.* **3**, 1223–1235
- Skidgel, R. A., Davis, R. M. & Tan, F. (1989) *J. Biol. Chem.* **264**, 2236–2241
- Smyth, D. G., Maruthinar, K., Darby, N. J. & Fricker, L. D. (1989) *J. Neurochem.* **53**, 489–493
- Tan, F., Chan, S. J., Steiner, D. F., Schilling, J. W. & Skidgel, R. A. (1989) *J. Biol. Chem.* **264**, 13165–13170
- Titani, K., Ericsson, L. H., Walsh, K. A. & Neurath, H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1666–1670
- Titani, K., Ericsson, L. H., Kumar, S., Jakob, F., Neurath, H. & Zwilling, R. (1984) *Biochemistry* **23**, 1245–1250
- Vallee, B. L. & Auld, D. S. (1990) *Biochemistry* **29**, 5647–5659

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