Enhanced Co^{2+} activation and inhibitor binding of carboxypeptidase M at low pH

Similarity to carboxypeptidase H (enkephalin convertase)

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Carboxypeptidases H and M differ in their distribution and other properties, but both are activated by Co^{2+} and inhibited by guanidinoethylmercaptosuccinic acid. The higher degree of activation or inhibition of carboxypeptidase H by these agents at acid pH has been employed to identify this enzyme in tissues. We found that the activation or inhibition of both purified and plasma-membrane-bound human carboxypeptidase M depends on the pH of the medium. CoCl_2 activated over 6-fold at pH 5.5, but less than 2-fold at pH 7.5. Guanidinoethylmercaptosuccinic acid inhibited the membrane-bound carboxypeptidase M more effectively than the purified enzyme, and the IC_{50} was about 25–30 times lower at pH 5.5. As purified human plasma carboxypeptidase N and pancreatic carboxypeptidase B were also activated more at pH 5.5, we conclude that the increased activation by CoCl_2 is due to the enhanced dissociation of Zn^{2+} below the pK_a of the ligands that co-ordinate the cofactor in the protein. Thus increased activation or inhibition at acid pH would not differentiate basic carboxypeptidases.

INTRODUCTION

Many prohormones are processed by cleavage between two basic residues followed by removal of the newly revealed C-terminal arginine or lysine by a carboxypeptidase (CP) [1]. This, and the fact that many peptides are inactivated by liberation of C-terminal arginine or lysine [1], have generated a great deal of interest in basic CPs. (We use the term 'basic CP' to denote a CP that specifically removes C-terminal arginine or lysine.) Besides pancreatic CPB [2] and plasma CPN [3], two more recently discovered basic CPs can catalyse these reactions. CPH, an intragranular enzyme, has a pH optimum of 5.6 and Zn^{2+} as cofactor. Some reagents that combine with thiol groups inhibit it [4]. Because CPH cleaves $[\text{Arg}^6]$ - and $[\text{Lys}^6]$ -enkephalins [4–6], it was first named enkephalin convertase, although obviously the enzyme is not specific for enkephalin hexapeptides. Another basic CP, CPM, differs from CPN, CPB or CPH in most properties such as M_r , kinetics or neutral pH optimum [1,7,8], and its recently determined cDNA sequence proves its unique character (F. Tan, S. Chan, D. F. Steiner, J. W. Schilling & R. A. Skidgel, unpublished work). CPM is a constituent of plasma membranes in many types of human and animal cells [8–11].

All of the basic CPs (B, N, H and M) are activated by Co^{2+} , which replaces Zn^{2+} as cofactor [1–4], and inhibited by compounds such as guanidinoethylmercaptosuccinic acid (GEMSA; [1]). It has been reported that CPH is inhibited by lower concentrations of GEMSA and activated more by Co^{2+} than are the other CPs [5,12,13].

Since CPH is active at acid pH, these activation and inhibition reactions, carried out around pH 5.5, have been used to detect CPH activity in crude tissue extracts. Binding of radiolabelled GEMSA at pH 5.6 was also employed in autoradiographic studies to show the distribution of CPH in heart [14], brain [15] and other organs.

We report here the activation and inhibition of purified and membrane-bound CPM at an acid pH. We found that CPM and other basic CPs are more sensitive to Co^{2+} and GEMSA at acid pH than at above neutrality. Thus measurement of ' Co^{2+} -stimulated activity' or high-affinity binding of GEMSA at acid pH [4,5,12–15] will not exclusively identify CPH in tissues that contain other basic CPs.

EXPERIMENTAL

Materials

The substrate dansyl-Ala-Arg was synthesized as previously described [7,16]. GEMSA was provided by Dr. Yehuda Levin of the Weizmann Institute of Science, Rehovot, Israel. All other chemicals used were of reagent grade or better. As sources of human CPM, both the washed placental microvillar membrane fraction (membrane-bound CPM) obtained after differential centrifugation [10] and the solubilized highly purified enzyme (purified CPM) [7] were used. CPN was purified from outdated human plasma [17], and human pancreatic CPB was purified from autopsy samples [17,18].

Abbreviations used: CP, carboxypeptidase; GEMSA, guanidinoethylmercaptosuccinic acid.

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Determination of enzyme activity

Enzymic activity with dansyl-Ala-Arg was measured by using a fluorimetric assay [5,7], except that the reactions were carried out either in 0.1 M-sodium acetate buffer, pH 5.5 (acetate buffer), or in 0.1 M-Mes buffer, pH 7.5. In general, buffer, enzyme and inhibitor (GEMSA) were preincubated at 4 °C for 20 min. The mixtures were warmed to 37 °C and substrate (dansyl-Ala-Arg) was added to give a final concentration of 0.2 mM in a final volume of 250 μ l. After incubation at 37 °C the reactions were stopped by the addition of 150 μ l of 1 M-sodium citrate buffer, pH 3.1, before extraction with 1 ml of chloroform.

The effect of Co^{2+} on enzymic activity at various pH values was assayed by incubating the enzyme in Mes buffer adjusted to the desired pH (5.0 to 7.5) either in the presence or in the absence of 1 mM- CoCl_2 for 2 h at 4 °C, before the addition of substrate at 37 °C.

The GEMSA concentrations that inhibited enzyme activity by 50% (IC_{50}) were determined graphically by plotting the percentage enzyme activity (enzyme without GEMSA = 100%) versus $-\log[\text{GEMSA concentration (M)}]$. All points were measured in duplicate and at least two different determinations were done for each enzyme preparation at each pH value. GEMSA concentrations ranged from 0.01 μ M to 100 μ M. The membrane-bound CPM and purified CPM preparations were diluted to give similar catalytic activities with dansyl-Ala-Arg at pH 7.5.

RESULTS

CPM is maximally active between pH 7.0 and 8.0 and is much less active at acid pH [7]. At pH 5.5 both membrane-bound CPM and purified CPM retain only about 35% of the activity that they have at pH 7.5 with the dansyl-Ala-Arg substrate. Fig. 1 compares the inhibition of both the membrane-bound and purified CPM

Fig. 1. Increased inhibition of CPM at acid pH by GEMSA

□, Purified CPM, pH 7.5; ●, purified CPM, pH 5.5; △, membrane-bound CPM, pH 7.5; ▲, membrane-bound CPM, pH 5.5. Membrane-bound CPM is inhibited by a lower concentration of GEMSA than is the purified enzyme.

Table 1. Activity of purified carboxypeptidases in the presence and in the absence of CoCl_2 (1 mM)

Activity is expressed as percentage of activity at pH 7.5 in the absence of Co^{2+} . These were 440, 325 and 300 nmol of substrate hydrolysed/min per mg of protein for CPM, CPN and CPB respectively.

Enzyme	Activity (%)			
	pH 5.5		pH 7.5	
	– Co^{2+}	+ Co^{2+}	– Co^{2+}	Co^{2+}
CPM	31	150	100	138
CPN	7	156	100	324
CPB	76	212	100	148

by GEMSA at pH 7.5 and pH 5.5. There are significant differences in the inhibition of the membrane-bound and purified enzyme at either pH. Membrane-bound CPM is inhibited at a lower concentration of GEMSA than is the purified enzyme at both pH 7.5 and 5.5. In addition, at pH 5.5 the IC_{50} of GEMSA, with either the membrane-bound or the purified CPM, is 25–30 times lower than at pH 7.5 (53 nM and 210 nM at pH 5.5, and 1.6 μ M and 5.3 μ M at pH 7.5). It should be noted that at pH 5.5 the IC_{50} of GEMSA (53 nM) for the membrane-bound CPM is 100 times lower than the IC_{50} (5.3 μ M) for purified CPM at pH 7.5.

Many basic CPs are activated by Co^{2+} [1–4], but CPH was reported to be activated much more than the other CPs [5]. We found, however, that the activation of both membrane-bound CPM and purified CPM by Co^{2+} very much depended upon the pH of the reaction. At pH 5.5 the activity of either enzyme preparation was accelerated 3–4 times more by CoCl_2 than at pH 7.5 (Table 1).

Fig. 2. Activation of CPM by CoCl_2 decreases with increasing pH

Activity at pH 7.5 without CoCl_2 = 385 nmol of substrate hydrolysed/min per mg of protein. Error bars indicate $\pm$ S.E.M.

Purified human plasma CPM and pancreatic CPB are also more activated by Co^{2+} at low pH. In fact, all the CPs tested were more active at pH 5.5 in the presence of Co^{2+} than at pH 7.5 in the absence of Co^{2+} . Thus the activity of these enzymes, which is normally low at acid pH and moderately enhanced by Co^{2+} at neutral pH, can be raised significantly by added Co^{2+} at low pH.

Fig. 2 shows that the degree of activation of CPM by Co^{2+} (1 mM) decreases with increasing pH. At pH 5.0 activity is increased 5.5-fold, from 85 to 470 nmol of substrate hydrolysed/min per mg of protein, whereas at pH 7.5 it was only increased 1.4-fold by CoCl_2 (from 385 to 550 nmol/min per mg).

DISCUSSION

These studies show that the effect of an inhibitor and of an activator on CPM varies depending on the pH and whether the enzyme is membrane-bound or is in solution as a purified protein. Membrane-bound human CPM is inhibited by a lower concentration of GEMSA than is the purified enzyme; if the membrane-bound enzyme is assayed at pH 5.5 and the purified one at 7.5, the difference in the IC_{50} is 100-fold. Thus the active site of the membrane-bound enzyme is more receptive to GEMSA, and low pH may induce additional changes that enhance the ability of GEMSA to compete with substrate for the active site.

Radiolabelled GEMSA has been used to localize CPM in tissue sections at pH 5.6, on the basis of the high affinity of this inhibitor for CPM at acid pH [4,14,15]. Such determinations, however, may not be specific. CPM is present in many tissues and in plasma membranes of many cell types, including endothelial and epithelial cells and fibroblasts [8,10]. Recently we found that an established cell line from dog kidney contains CPM that is very similar to human CPM [19]. For example, it cross-reacts specifically with antibodies to human CPM. This enzyme is also inhibited more by GEMSA at pH 5.5 (results not shown) than above neutrality. Thus CPM, widely distributed in tissues, could bind radio-labelled GEMSA with high affinity at low pH.

CoCl_2 activated all CPs (M, N and B) much more at an acid pH than above neutrality. Significantly, all the enzymes tested were more active at pH 5.5 in the presence of Co^{2+} than at pH 7.5 without Co^{2+} . Since CPM activity is usually measured as Co^{2+} -stimulated activity at pH 5.5 that is inhibited by GEMSA, other CPs could contribute significantly to the activity and lead to erroneous conclusions about the distribution of CPM.

Metalloproteinases and peptidases ranging from thermolysin, neutral endopeptidase 24.11 [20] and angiotensin I-converting enzyme to carboxypeptidases A and B have similar Zn^{2+} -binding ligands: two histidine residues and a glutamic acid residue [21,22].

Excess added Zn^{2+} preserves the activity of angiotensin I-converting enzyme at low pH, indicating that the dissociation of Zn^{2+} from the enzyme below pH 7.0 is a major reason for loss of activity [23]. Thus the increased activation of CP-type enzymes by Co^{2+} at low pH can be explained by enhanced dissociation of Zn^{2+} due to the pK_a of the Zn^{2+} -binding histidine residues.

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