Vogel, F., & Lumper, L. (1986) *Biochem. J. 236*, 871-878.
Williams, C. H., Jr., & Kamin, H. (1962) *J. Biol. Chem. 237*, 587-595.
Yabusaki, Y., Murakami, H., & Ohkawa, H. (1988) *J. Biochem. 103*, 1004-1010.
Yamano, S., Aoyama, T., McBride, O. W., Hardwick, J. P., Gelboin, H. V., & Gonzalez, F. J. (1989) *Mol. Pharmacol. 36*, 83-88.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337-5344.
Yasukochi, Y., Peterson, J. A., & Masters, B. S. S. (1979) *J. Biol. Chem. 254*, 7097-7104.
Yoshida, T., Noguchi, M., & Kikuchi, G. (1980) *J. Biol. Chem. 255*, 4418-4420.

Albumins Activate Peptide Hydrolysis by the Bifunctional Enzyme LTA_4 Hydrolase/Aminopeptidase†

Lars Orning* and F. A. Fitzpatrick

Department of Pharmacology, C236, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, Colorado 80262

Received August 23, 1991; Revised Manuscript Received February 3, 1992

ABSTRACT: Albumins from several species activated the bifunctional, Zn^{2+} metalloenzyme aminopeptidase/leukotriene A_4 hydrolase (EC 3.3.2.6). Bovine serum albumin, 1 mg/mL, increased hydrolysis of L-proline-*p*-nitroanilide and leucine-enkephalin by 12-fold and 7-fold, respectively. The apparent K_m for L-proline-*p*-nitroanilide was inversely proportional to the albumin concentration from 0 to 1 mg/mL, declining from 9.4 to 0.7 mM without an appreciable change in apparent V_{max}. These data imply a random activation process in which the enzyme–activator complex is catalytically dominant. Hill plots indicated a 1:1 stoichiometric relationship between albumin and enzyme. Secondary plots of slope versus the reciprocal of albumin concentration indicated that it binds to the enzyme with an affinity constant of 0.9 μM. The pH optimum of the nonactivated enzyme occurred at pH 8; the albumin-activated enzyme had an optimum near pH 7. Neither ultrafiltration nor dialysis of albumin altered its activating effect, but boiling abolished it. Albumin did not affect other cytosolic or microsomal leucine aminopeptidases, or γ-glutamyltransferase. Albumin functions as a nonessential activator, since enzymatic activity was always detectable in its absence. Chloride ions, which activate other Zn^{2+} metalloenzymes, also activated leukotriene A_4 hydrolase/aminopeptidase with an EC_{50} = 50 mM, increasing its initial velocity 2.2-fold in the absence of albumin. Zn^{2+} activated the enzyme, increasing its apparent V_{max} but not its apparent K_m, suggesting it replaced Zn^{2+} lost from the active site, especially at acidic pH. At concentrations greater than 30–50 μM, Zn^{2+} was inhibitory. Albumin mitigated the effect of chloride, but not the effect of Zn^{2+} or that of the competitive inhibitor, captopril. Several other proteins including gelatin, bovine or human γ-globulins, human α_2-macroglobulin, and bovine α-fetoprotein did not stimulate the aminopeptidase activity of LTA_4 hydrolase. Our results suggest that an interaction between LTA_4 hydrolase/aminopeptidase and albumin causes a conformational change in the enzyme, leading to an increased affinity for peptides.

Leukotriene (LT)[1] A_4 hydrolase (EC 3.3.2.6) is a bifunctional Zn^{2+} metalloenzyme (Malfroy et al., 1989; Vallee & Auld, 1990; Toh et al., 1990; Haeggstrom et al., 1990a) which catalyzes the formation of LTB_4 (Radmark et al., 1984; Evans et al., 1985; McGee & Fitzpatrick, 1985) and the hydrolysis of amide derivatives of *p*-nitroaniline or naphthylamine (Minami et al., 1990; Haeggstrom et al., 1990b; Orning et al., 1991a,b). The K_m values for its amide substrates are 50–100 times larger than the K_m for LTA_4. However, the V_{max} values for both aminopeptidase and LTA_4 hydrolase catalysis are comparable; consequently, the k_{cat}/K_m ratio for amide hydrolysis is <2% that for LTB_4 formation (Minami et al., 1990; Orning et al., 1991a,b). Kinetic data reported by different groups contain discrepancies which are relevant to any proposed physiological roles for the aminopeptidase activity. Using homogeneously purified leukocyte enzyme at pH 8.0, 37 °C, with L-leucine-*p*-nitroanilide as a substrate, one group has reported a specific activity of 130 nmol of *p*-nitroaniline/(min·mg) (Haeggstrom et al., 1990b), a value substantially lower than the 500 nmol/(min·mg) we and others obtained using recombinant human leukocyte enzyme and various amide substrates under similar conditions (Minami et al., 1990; Orning et al., 1991a,b). Initially, we attributed this difference to the enzyme preparations, rather than to a minor item, inclusion of bovine serum albumin, 1 mg/mL, in our enzyme assay buffer. Albumin augments formation of LTB_4, an effect ordinarily attributed to sequestration and protection of LTA_4 from spontaneous, nonenzymatic hydration (Fitzpatrick et al., 1982; Maycock et al., 1982). We routinely included BSA in the assay buffer for LTA_4 hydrolase activity and, for consistency, also in the assay for the aminopeptidase

† This work was supported by USPHS Grant HL34303 awarded to F.A.F.
* Corresponding author.

[1] Abbreviations: LT, leukotriene; BSA, bovine serum albumin; PG, prostaglandin; fMLF, formyl-methionyl-leucyl-phenylalanine.

0006-2960/92/0431-4218$03.00/0

activity. Surprisingly, we found that omission of BSA reduced the aminopeptidase activity to levels agreeable with those reported by Haeggstrom et al. (1990b). We now report a detailed kinetic investigation of the activating effect of albumin which increases the specificity (k_{cat}/K_m) of aminopeptidase activity up to 21-fold and facilitates the hydrolysis of physiologically relevant peptides typified by leucine-enkephalin.

Experimental Procedures

Materials. Protease-free, fatty acid-free, and globulin-free bovine serum albumin (BSA), albumins from other species, gelatin, bovine and human γ-globulin, human α_2-macroglobulin, bovine α-fetoprotein, L-proline-*p*-nitroanilide, L-alanine-*p*-nitroanilide, poly(L-lysine), poly(L-glutamate), poly(L-proline), cytosolic and microsomal leucine aminopeptidase from porcine kidney, γ-glutamyltransferase, *o*-phthaldialdehyde reagent, leucine-enkephalin, methionine-enkephalin, formyl-methionyl-leucyl-phenylalanine (fMLF) (Sigma, St. Louis, MO), and dynorphin A 1–6 (Bachem Inc., Torrance, CA) were used. Recombinant LTA_4 hydrolase/aminopeptidase from leukocytes was purified to apparent homogeneity as described (Funk et al., 1987; Radmark et al., 1984). Protein concentrations were determined spectrophotometrically using reagents and methods from Pierce (Rockford, IL).

Enzyme Assays. Aminopeptidase/LTA_4 hydrolase (3–6 μg/mL) in 0.1 M Tris-HCl, pH 7, containing 0.2 M NaCl and 10 μM $ZnSO_4$, was incubated with 0–2 mg/mL BSA and 0.05-5 mM L-proline-*p*-nitroanilide at 22 °C. Formation of *p*-nitroaniline was monitored at 405 nm, $\epsilon = 10800\ M^{-1}\ cm^{-1}$. When leucine-enkephalin or other physiological peptides were used, reactions were performed at 37 °C. At intervals, aliquots were removed, quenched with 4% trichloroacetic acid, and derivatized with *o*-phthaldialdehyde reagent. Formation of the appropriate amino acid derivatives was monitored by RP-HPLC, using columns of Adsorbosphere OPA HR (Altech) with spectrophotometric detection at 340 nm and quantitation by comparison with derivatized standards of known concentrations of amino acid as described (Jones, 1981).

Kinetic Analyses. Kinetic constants, half-lives, and reaction orders were estimated from nonlinear regression analysis using the program GraphPad. The type of activation was determined from Lineweaver–Burk plots, and constants were calculated from secondary plots of the slope of Lineweaver–Burk plots and from reciprocal plots of the difference between the slopes with and without BSA versus reciprocal activator concentration (Segel, 1975).

The pH dependence of activation between pH 6.0 and 9.4 was determined using 0.2 M Tris buffer, containing 0.2 M NaCl. All reagents added (substrate, enzyme, BSA, $ZnSO_4$) were dissolved in the same buffer, and pH was monitored before and after the reaction. Phosphate buffer could not be used due to its inhibitory effect on aminopeptidase/LTA_4 hydrolase (Orning et al., 1991b). Routinely, 100 μM $ZnSO_4$ was added to solutions with a pH < 7.0, 10 μM to solutions between pH 7.0 and 8.0, and 1 μM to solutions with pH > 8.0. This is a conventional approach for examining kinetics of Zn^{2+} metallohydrolases (Bunning & Riordan; 1983). The substrate L-proline-*p*-nitroanilide was used at 50 μM. This concentration is sufficiently low compared to apparent K_m values (<10%) to make initial velocity (v) reflect k_{cat}/K_m conditions (Segel, 1975). Under these conditions, the pH profile will yield values for pK of free enzyme and free substrate. Since L-proline-*p*-nitroanilide does not change its ionization in this pH range, the data reflect the ionization of groups in the free enzyme which influence enzymatic activity.

Table I: Effect of BSA on the Hydrolysis of Peptides by Aminopeptidase/LTA_4 Hydrolase[a]

peptide	aminopeptidase sp act. [nmol of product/(min·mg)] −BSA	aminopeptidase sp act. [nmol of product/(min·mg)] +BSA	stimulation (multiple of control)
fMLP	0.2	0.8	4
dynorphin A (1–6)	0.3	0.8	3
poly(L-proline)	0.1	2.4	24
Met-enkephalin	3.0	13	4
Leu-enkephalin	8	57	7
L-proline-*p*-nitroanilide	34	418	12
L-alanine-*p*-nitroanilide	58	505	9

[a] Each substrate (1 mM) was incubated with 44 μg/mL enzyme in the presence or the absence of 1 mg/mL BSA at 37 °C, in 0.1 M Tris, pH 7.0, containing 0.2 M NaCl and 10 μM $ZnSO_4$. Reaction velocity was determined by measuring the amounts of amino acid released.

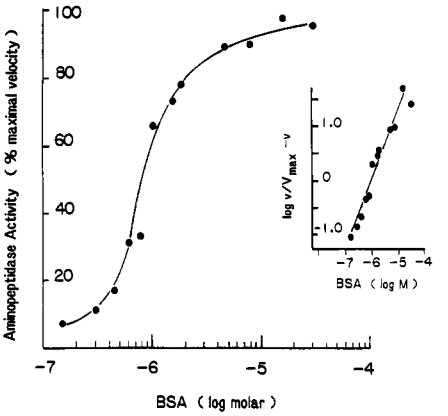

FIGURE 1: Activation of aminopeptidase EC 3.3.2.6 (LTA_4 hydrolase/aminopeptidase) as a function of BSA concentration. Inset: Hill plot. Reaction order = 1.4 for data shown. For $n = 5$ experiments, the reaction order was 1.1 ± 0.2 (mean ± sd) Conditions: 0.1 M Tris, pH 7.0, 0.2 M NaCl, 10 μM $ZnSO_4$, and 1 mM L-proline-*p*-nitroanilide, 22 °C.

Chloride ions can activate other physiologically important Zn^{2+} metallohydrolases (Shapiro et al., 1983; Shapiro & Riordan, 1984a,b); consequently, we investigated their effect on aminopeptidase/LTA_4 hydrolase with and without 1 mg/mL BSA in 0.1 M Tris buffer, pH 7.0, containing 10 μM $ZnSO_4$. NaCl concentrations were varied between 0 and 0.5 M. Effects of Zn^{2+} were studied with or without 1 mg/mL BSA, in 0.1 M Tris buffer, containing 0.2 M NaCl.

Results

Activation of Aminopeptidase/LTA_4 Hydrolase (EC 3.3.2.6) by Albumins. Bovine serum albumin (1 mg/mL) activated the bifunctional enzyme, aminopeptidase/LTA_4 hydrolase (EC 3.3.2.6), enhancing its hydrolysis of several amide substrates (Table I). For instance, at pH 7 the N-terminal hydrolysis of leucine-enkephalin increased 7-fold and the hydrolysis of L-proline-*p*-nitroanilide increased 12-fold. We investigated this process in detail using the latter substrate. Activation was immediate and concentration-dependent, with EC_{50} = 0.6 μM BSA (Figure 1). The slope from Hill plots, 1.1 ± 0.2 ($n = 5$), indicated that one molecule of albumin interacted with one molecule of enzyme (Figure 1, inset). Activation was abolished by boiling solutions of BSA, but not by dialysis against EDTA or ultrafiltration through a membrane with a molecular weight cutoff at 10000 (data not shown). Albumin had no equivalent effect on the enzymatic activity of cytosolic or microsomal leucine aminopeptidase, or γ-glutamyltransferase. Albumins from several other species,

Table II: Effect of Albumin and Other Proteins on the Activation of Aminopeptidase Activity

protein	aminopeptidase activity (% control)	protein	aminopeptidase activity (% control)
none (control)	100[a]	none (control)	100[b]
albumin chicken	115	albumin, cow (1 mg/mL)	744
guinea-pig	244	albumin, cow (0.015 mg/mL)	291
dog	312	fibrinogen, human	331
human	332	γ-globulin, cow	166
rat	346	γ-globulin, human	168
cow	362	α_2-macroglobulin, human	152
goat	362	α-fetoprotein	142
horse	362		
pig	368		
poly(L-lysine)	111		
poly(L-glutamic acid)	131		
poly(L-proline)	134		
gelatin	98		

[a] Enzyme (5 μg/mL) and L-proline-*p*-nitroanilide (1 mM) were incubated with or without serum albumin (1 mg/mL) in 0.1 M Tris, pH 7.0 and 22 °C. The control activity was 0.82 nmol of *p*-nitroaniline/(min·mL). [b] A different batch of enzyme (2 μg/mL) and L-alanine-*p*-nitroanilide (1 mM) were used and the buffer was 0.2 M Tris, pH 7, 0.2 M NaCl and 30 μM $ZnSO_4$. The control activity was 0.86 nmol/(min·mL). Protein concentrations were 1 mg/mL unless noted differently.

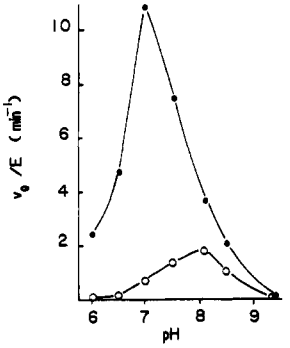

FIGURE 2: pH dependence of aminopeptidase activity: BSA-activated enzyme (●) and control enzyme minus BSA (○).

including human, activated aminopeptidase/LTA_4 hydrolase to a similar degree (Table II). There was no difference between protease-free, fatty acid-free, or γ-globulin-free serum albumin. Furthermore, addition of fatty acids or DL-tryptophan which interact with the fatty acid binding site and the indole binding site of albumin, respectively, did not modulate the stimulation of LTA_4 hydrolase/aminopeptidase by albumin. In contrast to albumin, other proteins including gelatin, bovine γ-globulin or α-fetoprotein, human γ-globulin or α_2-macroglobulin, poly(L-lysine), poly(L-proline), or poly(L-glutamic acid) at concentrations of 1 mg/mL did not stimulate hydrolysis of L-proline-*p*-nitroanilide or leucine-enkephalin significantly (<1.5-fold). Human fibrinogen (95% pure) had a modest effect which was consistent with its contamination by traces of albumin.

pH Dependence of Activation. The pH dependence of aminopeptidase activity was examined in the absence and the presence of 1 mg/mL BSA, using 50 mM L-proline-*p*-nitroanilide in the reaction so that v_0 reflects k_{cat}/K_m (Figure 2). Without BSA, the pH versus rate profile for aminopeptidase activity had an optimum centered at pH 8, with rates decreasing below pH 7.5 and above pH 8.5. To ascertain that the decrease in activity at acidic pH was not a consequence of Zn^{2+} loss, 100 μM $ZnSO_4$ was added at pH < 7.0 and 10 μM $ZnSO_4$ was added at 7.0 < pH < 8.0. In the presence of albumin, the optimum shifted to pH 7.0; both the alkaline and the acidic limbs were shifted toward lower pH. Under k_{cat}/K_m conditions, the pH rate curve yields p*K* values for free substrate and enzyme. Since proline-*p*-nitroanilide is not changing its charge within the pH range used, the p*K* values estimated for the pH curve are those of free enzyme. From

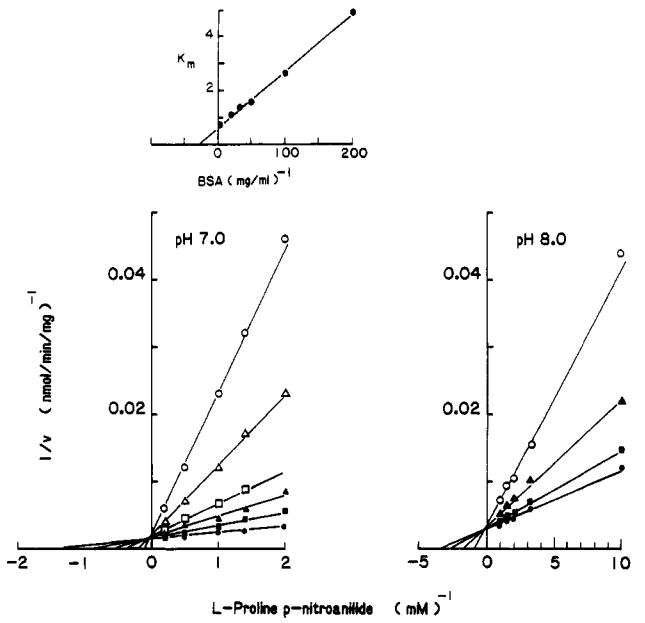

FIGURE 3: Lineweaver–Burk plots for aminopeptidase/LTA_4 hydrolase catalyzed hydrolysis of L-proline-*p*-nitroanilide as a function of BSA concentration, at pH 7.0 (lower left) and pH 8.0 (lower right). BSA concentrations were 0 (○), 0.005 (△), 0.01 (□), 0.02 (▲), 0.05 (■), and 1.0 (●) mg/mL. The upper panel shows determination of K_A according to $K_{m\,apparent} = K_m(1 + K_A/[A])$.

Scheme I: Activation of Aminopeptidase/LTA_4 Hydrolase (EC 3.3.2.6)

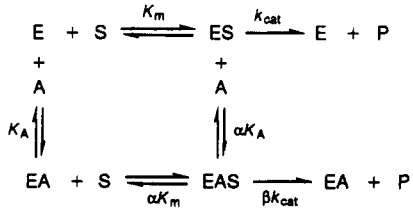

Figure 2, p*K* vaues were 7.3 and 8.3 in the absence of albumin and 6.9 and 7.9 in its presence.

Mechanism of Activation. At either pH 7 or pH 8, the apparent K_m was inversely proportional to the BSA concentration (0–1 mg/mL) but the apparent V_{max} was not appreciably different from the control (Figure 3, lower panels). At pH 7, K_m decreased 13-fold, from 9.4 mM to 0.7 mM L-proline-*p*-nitroanilide, while V_{max} values increased by 1.6-fold

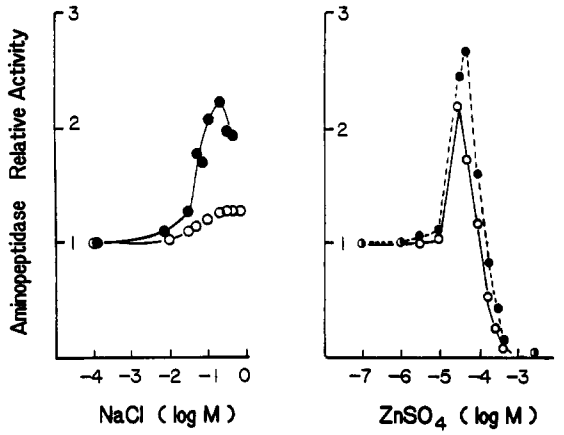

FIGURE 4: Effects of NaCl and $ZnSO_4$ on aminopeptidase/LTA_4 hydrolase in the presence or the absence of BSA. Left panel: Enzyme (5 μg/mL) in 0.1 M Tris, pH 7.0, containing 10 μM $ZnSO_4$, and 1 mM L-proline-*p*-nitroanilide were incubated with 0–500 mM NaCl either minus BSA (●) or plus BSA (○). Right panel: $ZnSO_4$, enzyme (5 μg/mL), 0.1 M Tris, pH 7.0, plus 0.2 M NaCl.

from 471 to 736 nmol/(min·mg). The net effect was a 21-fold increase in selectivity reflected by k_{cat}/K_m. Aminopeptidase activity is always detectable, even in the absence of albumin, suggesting a nonessential, mixed-type mechanism in which activator and substrate bind randomly to enzyme (Scheme I). The kinetic parameters in Scheme I were estimated from secondary plots of the slope from Lineweaver–Burk plots and from reciprocal plots of the difference between the slopes with and without albumin versus the reciprocal of albumin concentration. The values calculated, K_A = 0.9 μM (59 μg/mL), α = 0.083, β = 1.6, indicate that the predominant effect of BSA is to facilitate the binding of the peptides to LTA_4 hydrolase/aminopeptidase and that the major reaction pathway is through the enzyme–BSA complex, since $\alpha K_m \ll K_m$.

Effects of Chloride, Zinc, and Captopril. Chloride ions, from 0 to 200 mM, activated aminopeptidase by 2.2-fold with an EC_{50} = 50 mM; however, at concentrations greater than 200 mM, chloride inhibited the enzyme (Figure 4, left). BSA reduced the magnitude of these effects. Activity was stimulated by only 1.3-fold with an EC_{50} = 37 mM chloride; there was no inhibition at higher chloride concentrations. Zn^{2+} stimulated enzymatic activity at concentrations below 30–50 μM and inhibited activity at higher concentrations (Figure 4, right). The Zn^{2+} activating effect was independent of BSA; EC_{50} was 18 μM and IC_{50} was 106 μM in the presence of BSA; EC_{50} was 17 μM and IC_{50} was 132 μM in the absence of BSA. Activation was more pronounced at pH < 7 (2–6-fold) than at pH > 7 (1.1–1.5-fold). The increased aminopeptidase activity was due to an increase in V_{max} and not to a decrease in K_m (data not shown), indicating replacement of Zn^{2+} lost from the enzyme at acidic pH. BSA had no marked effect on the interaction between the enzyme and captopril, an inhibitor of aminopeptidase/LTA_4 hydrolase (Orning et al., 1991b). A slight, but negligible, decrease in K_i from 0.3 to 0.2 μM was evident in the presence of BSA.

Effects of Albumin on LTB_4 Formation. We reexamined the effect of albumin on LTB_4 formation by LTA_4 hydrolase. Enzyme (7 μg/mL) was incubated with LTA_4 (50 μM) and varying concentrations of BSA (0–1 mg/mL) for 10 s at 25 °C. At concentrations less than 0.2 mg/mL, BSA did not increase LTB_4 formation. At 1 mg/mL, LTB_4 formation increased by 75%. BSA did not alter the pH optimum, pH 7.5–8.0, for LTB_4 formation. The $t_{1/2}$ for LTA_4 in the absence of BSA was about 8 s and in the presence of 1.0 mg BSA/mL it was 11 s. The initial half-life of LTA_4 is not measurably altered by BSA at the low concentrations which can activate aminopeptidase catalysis by EC 3.3.2.6.

Discussion

LTA_4 hydrolase (EC 3.3.2.6) was recently shown to contain a Zn^{2+}-binding domain common for certain metallohydrolases (Malfroy et al., 1989; Vallee & Auld, 1990). Subsequently, Haeggstrom et al. (1990a) have established a stoichiometry of 1:1 for the Zn^{2+}–enzyme complex, and three groups have described an intrinsic aminopeptidase activity associated with this enzyme, indicating that it is catalytically bifunctional (Haeggstrom et al., 1990b; Minami et al., 1990; Orning et al., 1991a). Bestatin, an aminopeptidase inhibitor; captopril, an angiotensin-converting enzyme inhibitor; and thiorphan, a neutral endopeptidase inhibitor, are also inhibitors of aminopeptidase/LTA_4 hydrolase (Orning et al., 1991a,b). We now report that this enzyme exhibits other traits typical for metallohydrolase enzymes as well as certain unique traits. For instance, aminopeptidase/LTA_4 hydrolase is stimulated by low concentrations of chloride and Zn^{2+}, and it is inhibited by higher concentrations of these ions. Similar findings have been reported for the angiotensin-converting enzyme (Dorer et al., 1976; Bunning & Riordan, 1983), carboxypeptidase A (Larsen & Auld, 1989), and neutral endopeptidase (Kerr & Kenny, 1974). Zn^{2+} affected k_{cat} only, suggesting that it replenished cations released from the active site. At lower pH, Zn^{2+} supplementation had a greater effect, supporting this notion. Concentrations of chloride >200 mM and of $ZnSO_4$ > 50 μM decreased enzyme activity. Zn^{2+}, at high concentration, eliminated activity. Like other primary amines, the tris(hydroxymethyl)aminomethane we used as a buffer can chelate Zn^{2+}; consequently, the EC_{50} and IC_{50} values for its modulation of enzymatic activity are probably overestimates of the true values. The Zn^{2+} inhibition reported for carboxypeptidase A depends upon a bridging of Glu-270 in the active site and the catalytic zinc ion by a $ZnOH^+$ complex (Larsen & Auld, 1991). A similar reaction might occur with LTA_4 hydrolase/aminopeptidase.

A feature which seems to distinguish LTA_4 hydrolase/aminopeptidase from other Zn^{2+} metallohydrolases is its activation by albumin. Albumin, with a K_A = 59 μg/mL (0.9 μM), stimulated the hydrolysis of several representative amide substrates. Aminopeptidase/LTA_4 hydrolase interacted 1:1 with albumin itself, not with a low molecular weight contaminant of the albumin preparations. Neither ultrafiltration (cutoff 10000) nor extensive dialysis against EDTA decreased the potency of BSA. In fact, dialysis slightly increased the potency. BSA did not modulate the effects of Zn^{2+} on the enzyme: the EC_{50} and IC_{50} for Zn^{2+} were similar in the presence or the absence of BSA. In contrast, BSA reduced the chloride effect. It is notable that the activation caused by BSA, at pH 7.0, was 10-fold and the activation caused by chloride was 2-fold; thus, the BSA effect is not explained by any chloride it contains. Any conformational changes involved may be similar.

Albumins from different species activated aminopeptidase/LTA_4 hydrolase to a similar extent and there was no difference between protease-free, fatty acid-free, and globulin-free BSA, suggesting that the effects were attributable to albumin. Examination of several other representative proteins confirmed this: gelatin, γ-globulins, α_2-macroglobulin, or α-fetoprotein had no stimulatory effects. Human fibrinogen was able to stimulate the aminopeptidase; however, its effect was substantially lower than that achieved with a corresponding concentration of albumin and it can probably be explained by albumin contamination of the fibrinogen prep-

aration which was 90–95% pure.

LTA_4 hydrolase displays its aminopeptidase activity in the absence of BSA. Therefore, its mechanism is designated as a nonessential one, as shown in Scheme I. The effect of BSA is predominantly on $K_{m\ apparent}$ which is lowered 13-fold with 1 mg/mL BSA, pH 7.0, whereas V_{max} is increased only 1.6-fold. The calculated constants for Scheme I, $\beta = 1.6$ and $\alpha = 0.08$, show that the reaction predominantly proceeds via the enzyme–BSA complex which has a considerably lower binding constant for the substrate, 0.7 mM compared to 9.4 mM. From this, it follows that BSA induces a conformational change which increases the enzyme affinity for amide substrates. The effects of BSA on the pH dependence of aminopeptidase activity support this notion. In the presence of BSA, the optimum is shifted from pH 8 to 7, the alkaline pK shifts from 8.3 to 7.9, and the acidic pK shifts from 7.3 to 6.9. In the presence of BSA, the amino acid residues important for activity seem more accessible for ionization.

In some, but not all, enzyme preparations the activation by BSA surprisingly led to loss of enzymatic activity, which was clearly evident at 37 °C but not at 25 °C. This inactivation was inhibited by captopril and stimulated by Zn^{2+}, indicating involvement of the active site of aminopeptidase/LTA_4 hydrolase. However, we found no direct evidence for autopeptidase activity: there was no detectable L-proline formation and there were no proteolytic fragments detected by SDS–PAGE after deactivation. The reason for the variability of this effect among enzyme preparations is under investigation. It is possible that a conformational change induced by BSA increases the sensitivity of the enzyme to temperature-dependent denaturation at 37 °C. Since supplementation of Zn^{2+} in the buffer increased the loss of enzymatic activity, it cannot be explained by a BSA-induced loss of Zn^{2+}.

Concentrations of BSA which activate aminopeptidase catalysis did not readily activate LTA_4 hydrolase activity. The formation of LTB_4 increased slightly with 1 mg/mL BSA, but as previously shown this is attributable to the stabilizing effect of BSA on the hydrolytically unstable substrate, LTA_4 (Fitzpatrick et al., 1982; Maycock et al., 1982). This differential effect of BSA argues for the two activities to occupy different, but overlapping sites. The 100-fold difference in the K_i value for the aminopeptidase and LTA_4 hydrolase activity determined for captopril also supports this. At the same time, the similar K_i values expressed by bestatin and the mutual exclusion by these two inhibitors (Orning et al., 1991a,b), suggest that the sites are overlapping and not fully different.

Albumin modulates eicosanoid activity in three discrete ways. First, it binds unstable eicosanoids, such as thromboxane A_2 or PGI_2 and spares them from hydrolytic degradation, prolonging their duration of action (Smith et al., 1976; Folco et al., 1977; Wynalda & Fitzpatrick, 1980; Chen et al., 1981). Albumin also binds eicosanoids with β-hydroxycarbonyl functional groups, such as PGE_2 or PGD_2; however, in this case, it catalyzes their dehydration, thereby reducing their duration of action (Fitzpatrick & Wynalda, 1981; 1983). A model involving sequestration and interaction of eicosanoids with an alkaline microenvironment on albumin adequately accounts for these observations (Fitzpatrick et al., 1984). Second, albumin influences the qualitative or quantitative features of eicosanoid biosynthesis. For example, it alters the cell-free metabolism of PGH_2 to favor its conversion into PGD_2 (Hamberg & Fredholm, 1976) and it enhances the formation of the chemotactic agent, LTB_4, by protecting its transient substrate, LTA_4, from spontaneous, nonenzymatic hydrolysis. Data presented in this report indicate that albumin acts in a third way. Using substrates much more chemically stable than LTA_4, we have established that activation by albumin substantially increases the k_{cat}/K_m ratios of aminopeptidase catalysis by the bifunctional Zn^{2+} metallohydrolase EC 3.3.2.6. Thus, release or leakage of LTA_4 hydrolase/aminopeptidase from leukocytes (Evans et al., 1985), erythrocytes (McGee & Fitzpatrick, 1985), or mast cells (Dahinden et al., 1985) during inflammatory processes would accelerate the hydrolysis of naturally occurring peptide substrates. These include enkephalin neuropeptides. The physiological relevance of this is unclear but warrants additional investigation in view of our demonstration that LTA_4 hydrolase/aminopeptidase hydrolyzes endogenous substrates and responds to endogenous activators. Our results stress that the precise nature of in vitro experimental conditions may be vital for detecting hydrolysis of physiologically relevant peptides and for estimating kinetic constants of this enzyme. A similar situation prevails for the angiotensin-converting enzyme (Shapiro & Riordan, 1984; MacFadyen et al., 1991).

Acknowledgments

We thank Dr. Gwen Krivi and Jim Gierse from Monsanto Corporation for a sample of recombinant leukotriene A_4 hydrolase.

Registry No. Zn^{2+}, 7440-66-6; Cl^-, 16887-00-6; LTA_4 hydrolase, 90119-07-6; aminopeptidase, 9031-94-1; L-proline-*p*-nitroanilide, 7369-91-7; captopril, 62571-86-2.

References

Bunning, P., & Riordan, J. F. (1983) *Biochemistry 22*, 110–116.

Bunning, P., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 103–109.

Chen, Y.-C., Hall, E., McLeod, B., & Wu, K. (1983) *Lancet* (August 8), 267–269.

Dahinden, C., Clancy, R., Gross, M., Chiller, J., & Hugli, T. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6632–6636.

Dorer, F., Kahn, J., Lentz, K., Levine, M., & Skeggs, L. (1976) *Biochim. Biophys. Acta. 429*, 220–228.

Evans, J., Nathaniel, D., Zamboni, R., & Ford-Hutchinson, A. W. (1985) *J. Biol. Chem. 260*, 10966–10970.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, Freeman, New York.

Fitzpatrick, F. A., Morton, D. R., & Wynalda, M. A. (1982) *J. Biol. Chem. 257*, 4680–4683.

Fitzpatrick, F. A., & Wynalda, M. A. (1981) *Biochemistry 20*, 6129–6134.

Fitzpatrick, F. A., & Wynalda, M. A. (1983) *J. Biol. Chem. 258*, 11713–11718.

Fitzpatrick, F. A., Liggett, W., & Wynalda, M. A. (1984) *J. Biol. Chem. 259*, 2722–2727.

Folco, G., Granstrom, E., & Kindahl, H. (1977) *FEBS Lett. 82*, 321–324.

Funk, C., Radmark, O., Fu, J., Matsumoto, T., Jornvall, H., Shimizu, T., & Samuelsson, B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6677–6681.

Haeggstrom, J., Wetterholm, A., Shapiro, R., Vallee, B., & Samuelsson, B. (1990a) *Biochem. Biophys. Res. Commun. 172*, 965–970.

Haeggstrom, J., Wetterholm, A., Vallee, B., & Samuelsson, B. (1990b) *Biochem. Biophys. Res. Commun. 173*, 431–437.

Hamberg, M., & Fredholm, B. B. (1976) *Biochem. Biophys. Acta 431*, 189–193.

Jones, B. N. (1983) *J. Chromatogr. 266*, 471–482.

Kerr, M., & Kenny A. (1974) *Biochem. J. 137*, 489–495.

Larsen, K., & Auld, D. (1989) *Biochemistry 28*, 9620–9625.

Larsen, K., & Auld, D. (1991) *Biochemistry 30*, 2613-2618.
Mac Fadyen, R., Lees, K., & Reid, J. L. (1991) *Br. J. Clin. Pharmacol. 31*, 1-13.
Malfroy, B., Kado-Fong, H., Gros, C., Giros, B., Schwartz, J., & Helmiss, R. (1989) *Biochem. Biophys. Res. Commun. 161*, 236-241.
Maycock, A., Anderson, M., DeSousa, D., & Kuehl, F. (1982) *J. Biol. Chem. 257*, 13911-13914.
McGee, J., & Fitzpatrick, F. A. (1985) *J. Biol. Chem. 260*, 12832-12837.
Minami, H., Ohishi, N., Mutoh, H., Izumi, T., Bito, H., Wada, H., Seyama, Y., Toh, H., & Shimizu, T. (1990) *Biochem. Biophys. Res. Commun. 173*, 620-626.
Orning, L., Krivi, G., & Fitzpatrick, F. A. (1991a) *J. Biol. Chem. 266*, 1375-1378.
Orning, L., Krivi, G., Bild, G., Gierse, J., Aykent, S., & Fitzpatrick, F. A. (1991b) *J. Biol. Chem. 266*, 16507-16511.
Radmark, O., Shimizu, T., Jornvall, H., & Samuelsson, B. (1984) *J. Biol. Chem. 259*, 12339-12345.
Segel, I. H. (1975) in *Enzyme Kinetics*, Wiley, New York.
Shapiro, R., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 3850-3857.
Shapiro, R., & Riordan, J. F. (1984a) *Biochemistry 23*, 5225-5233.
Shapiro, R., & Riordan, J. F. (1984b) *Biochemistry 23*, 5234-5240.
Smith, J. B., Ingerman, C., & Silver M. (1976) *J. Clin. Invest. 58*, 1119-1122.
Toh, H., Minami, M., & Shimizu, T. (1990) *Biochem. Biophys. Res. Commun. 171*, 216-221.
Vallee, B., & Auld, D. (1990) *Biochemistry 29*, 5647-5659.
Wynalda, M., & Fitzpatrick, F. A. (1980) *Prostaglandins 20*, 853-861.

Purification and Characterization of a Cathepsin D Protease from Bovine Chromaffin Granules†

Timothy J. Krieger and Vivian Y. H. Hook*
Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814
Received December 28, 1991

ABSTRACT: Purification and potential tachykinin and enkephalin precursor cleaving enzymes from bovine chromaffin granules was undertaken using as substrates the model precursors ^{35}S-(Met)-β-preprotachykinin [^{35}S-(Met)-β-PPT] and ^{35}S-(Met)-preproenkephalin [^{35}S-(Met)-PPE]. Purification by concanavalin A-Sepharose, Sephacryl S200, and chromatofocusing resulted in a chromaffin granule aspartyl protease (CGAP) that preferred the tachykinin over the enkephalin precursor. CGAP was composed of 47-, 30-, and 16.5-kDa polypeptides migrating as a single band in a nondenaturing electrophoretic gel system, and coeluting with an apparent molecular mass of 45–55 kDa by size-exclusion chromatography. These results suggest that two forms exist: a single 47-kDa polypeptide and a complex of 30+16.5-kDa-associated subunits. CGAP was optimally active at pH 5.0–5.5, indicating that it would be active within the acidic intragranular environment. Cleavage at basic residues was suggested by HPLC and HVE identification of ^{35}S-(Met)-NKA-Gly-Lys as the major acid-soluble product generated from ^{35}S-(Met)-β-PPT. Neuropeptide K was cleaved at a Lys-Arg basic residue site, as determined by identification of proteolytic products by microsequencing and amino acid composition analyses. Structural studies showed that the three CGAP polypeptides were similar to bovine cathepsin D in NH_2-terminal sequences and amino acid compositions, indicating that CGAP appears to be a cathepsin D-related protease or cathepsin D itself. The 47- and 16.5-kDa polypeptides of CGAP possessed identical NH_2-terminal sequences, suggesting that the 16.5-kDa polypeptide may be derived from the 47-kDa form by proteolysis. CGAP resembled cathepsin D by cleaving at hydrophobic residues, as shown by CGAP cleavage of neuropeptide K between Leu-Tyr and Phe-Val residues. Processing of proendothelin to endothelin, present in chromaffin granules, requires processing at both hydrophobic and paired basic residues, which would be compatible with CGAP's cleavage site specificity. In addition, CGAP's cathepsin D-like cleavage specificity for hydrophobic residues suggests that it may also be involved in degrading precursor segments that are not part of the active peptide sequences. In summary, CGAP shows substrate selectivity, and cleaves at paired basic residues and at hydrophobic residues. These properties may be compatible with possible participation of CGAP in cleaving some peptide precursors.

Peptide hormones and neurotransmitters are synthesized as protein precursors that require proteolytic processing to form the smaller active neuropeptides (Docherty & Steiner, 1982). Most precursors require endoproteolytic processing at paired basic residue sites (Lys-Arg, Arg-Lys, Lys-Lys, or Arg-Arg), and some also require processing at monobasic arginine sites. Several putative processing enzymes cleaving at such dibasic residues have been identified. These include the yeast Kex2 gene product required for processing the yeast pro-α-mating factor (Julius et al., 1984; Fuller et al., 1989), homologous Kex2-related human and mouse furin genes (Bresnahan et al., 1990; Hatsuzawa et al., 1990), and PC1, PC2, and PC3

† This work was supported by grants from NINCDS (NIH) and NMRDC.
* Address correspondence to this author.

0006-2960/92/0431-4223$03.00/0