Intestinal Brush Border Aminooligopeptidases: Cytosol Precursors of the Membrane Enzyme†

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ABSTRACT: Although brush border aminooligopeptidases play a pivotal role in the intestinal surface hydrolysis of the final products of intraluminal protein digestion, their mode of synthesis and assembly is unknown. We have identified aminooligopeptidases in the 100000*g*/h supernatant which cross-react in a sensitive radioimmunoassay for the brush border peptidases. These cytosol peptidases were purified by antibody-affinity chromatography, and their physical and chemical characteristics were compared with those of their brush border counterparts. Analysis by gel filtration chromatography, velocity gradient ultracentrifugation, and polyacrylamide gel electrophoresis at varying percent total acrylamide revealed that the cytosol enzymes exist as three molecular weight isomers, having a common subunit present as either a monomer (70 000), dimer (140 000), or tetramer (280 000). Two of these cytosol species (I and II) are smaller by 40 000 daltons than the comparable brush border aminopeptidases [Gray, G. M., & Santiago, N. A. (1977) *J. Biol. Chem. 252*, 4922]. Binding of the cytosol peptidases to concanavalin A–Sepharose was less complete than that found for the related brush border enzymes. Incorporation of [^{3}H]-leucine in vivo into cytosol aminopeptidases was maximal at 20 min but was not achieved in the brush border membrane aminopeptidase until 1–3 h. These cytosol aminopeptidases have the appropriate characteristics to serve as precursors of the surface membrane peptidases at some stage of the intracellular assembly process.

The intestinal membrane aminooligopeptidases are integral membrane proteins located at the luminal surface of the small intestinal cell. These digestive hydrolases are probably transmembrane proteins (Louvard et al., 1976) whose active site is available at the lumen–cell interface to ensure surface hydrolysis of oligopeptides of four to six amino acid residues which cannot be assimilated intact across the intestinal surface membrane (Smithson & Gray, 1977). Despite the importance of these enzymes for protein digestion, their intracellular site of synthesis, mode of intracellular translocation, and mechanism of membrane insertion are unknown.

Although pancreatic proteases destined for secretion are synthesized on endoplasmic reticulum membranes and sequestered within the cisternal space of these membranes until their discharge from the cell by exocytosis (Jamieson & Palade, 1968, 1967), the synthesis and assembly process for integral membrane proteins in mammalian systems has not been defined. The cytosol of mouse spleen cells appears to contain small amounts of a precursor of the functional membrane glycoprotein, HLA antigen (Wernet, et al., 1973), and a cytosol form of another intestinal membrane protein, sucrase-isomaltase, has recently been described (Cezard et al., 1979).

We have identified aminopeptidases within the intestinal cytosol that appear to be closely related to the brush border membrane peptidases. The findings have implications for the intracellular synthesis and assembly of the final surface membrane aminopeptidase.

Experimental Procedures

Animals. Male Sprague–Dawley rats were allowed Purina rat chow ad libitum. Animals were killed by a blow on the head, and the jejunum was removed and irrigated with 0.9% NaCl at 4 °C. One-half milliliter per gram of tissue of 0.02 M sodium potassium phosphate buffer, pH 7.0 (buffer A)–10 mM mercaptoethanol was added to the mucosal scrapings, and the mixture was homogenized in a Brinkman Polytron (Type PT 10 20 350 D) for 30 s at a setting of 4.0. The homogenate was centrifuged at 105000*g* for 1 h, and the supernatant was recovered. This constituted the cytosol fraction. The pellet was resuspended again in the same buffer and recovered as the 105000*g*/h precipitate.

Enzyme Assays. Leucyl-β-naphthylamide hydrolase activity was assayed as previously described (Wojnarowska & Gray, 1975) with the addition of 0.2 mM *p*-(hydroxymercuri)-benzoate (PHMB) in the reaction mixture to inhibit activity of nonspecific cytosol peptidases. When used in this way, the assay is specific for brush border aminopeptidase and the related cytosol peptidases that react in the brush border radioimmunoassay. Catalase (Boehringer Mannheim) and alcohol dehydrogenase (Sigma Chemical Co.) were assayed as previously described (Conklin et al., 1975).

Solubilization of Brush Border Peptidase. The pellet was resuspended in buffer A–1 mM cysteine and the protein content was determined by the Lowry method (Lowry et al., 1951). Papain (twice crystallized; Sigma) was complexed with (*p*-aminobenzyl)cellulose (Celltex PAB; Bio-Rad) (Eichholz, 1968), and 1 mg of the dry complex was added per mg of brush border protein; the mixture was incubated at 37 °C for 15 min and centrifuged at 105000*g* for 1 h. This released 65–85% of the membrane aminopeptidase activity into the supernatant.

Gel Filtration Chromatography. A 2.5 × 90 cm column of agarose–acrylamide copolymer (Ultrogel ACA 34; LKB Produktor, Bromma, Sweden) was packed according to the manufacturer's instructions (Instruction Manual 1-2204-#01, Dec 1976) and used to determine the Stokes radius of the aminopeptidase (Conklin et al., 1975).

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Density Gradient Ultracentrifugation. The cytosol aminopeptidases (0.1 mL) were applied onto 5.0-mL linear gradients of 4–16% D-mannitol in 0.01 M sodium potassium phosphate, pH 7.8, and then centrifuged at 65 000 rpm in an SW65TI rotor for 6 h. The bottom of the tube was then punctured, and the fluid was collected in 0.1-mL (7 drops) fractions by increasing the pressure at the top of the tube. Sedimentation coefficients were determined (Martin & Ames, 1961); catalase, alcohol dehydrogenase, and bovine albumin were the standard proteins.

Nondenaturing Polyacrylamide Gel Electrophoresis. Acrylamide gel electrophoresis was carried out as described by Rodbard & Chrambach (1971) by use of multiphasic buffer system no. 2515 (Wojnarowska & Gray, 1975). The 10-cm separation gel contained either 7.5, 8.75, or 10% total acrylamide (% T) with constant 2% cross-linking (% C). The 2.5-cm stacking gel consisted of 3.1% T and 21.0% C. The pH values for the upper reservoir buffer, stacking gel buffer, separating gel buffer, and lower reservoir buffer were 9.1, 9.5, 9.4, and 7.5, respectively. The aminopeptidases (0.1 mL), made 1% in mannitol or 25% in glycerol, were electrophoresed at 2 mA/gel for 4 h. The proteins were stained with Coomassie blue, and aminopeptidases were specifically identified by the histochemical assay (Nachlas et al., 1960) with leucyl-β-naphthylamide as the substrate.

Antibody-Affinity Chromatography. A monospecific antiserum to brush border aminooligopeptidase was raised in New Zealand white rabbits by immunizing with 50 μg of pure brush border aminooligopeptidases I and II as previously described (Gray & Santiago, 1977; Conklin et al., 1975). A recall dose was given 1 month later in incomplete Freund's adjuvant. The 0–30% saturated ammonium sulfate fraction was recovered and coupled to Affigel 10 (Bio-Rad) at a ratio of 50 mg of globulin per mL of gel. Eighty percent of the globulin fraction was coupled by this method. Partially purified cytosol or membrane peptidase was applied to the 10-mL affinity column in 0.01 M sodium potassium phosphate–0.9% NaCl, pH 7.8 (buffer B). After 2 column volumes had been eluted, the direction of flow was reversed and the bound peptidase was desorbed with 4 M urea in buffer B. The specific eluate was subjected to vacuum dialysis, and the aminopeptidase isomers were separated as detailed under Results.

Solid-Phase Radioimmunoassay of the Aminopeptidases. Polystyrene plates (Cooke Engineering, Alexandria, VA) were used as the solid matrix for adsorbing the antiaminopeptidase globulin. Two micrograms of the globulin fraction was applied to each well in 200 μL of buffer B and incubated for 4 h at room temperature. The globulin solution was then removed, and the wells were filled and aspirated 3 times with bovine albumin (10 mg/mL)–buffer B to remove any unbound antiaminopeptidase globulin and to ensure that all protein binding sites on the well surface were occupied. The wells were then filled with 300 μL of albumin (10 mg/mL)–buffer B and stored at 4 °C. For the solid-phase assay, the albumin–buffer B solution was aspirated, and 200 μL of the sample containing aminopeptidase in bovine albumin (2 mg/mL)–buffer B was added to the well. Incubation for 4 h at room temperature, followed by 12 h at 4 °C, allowed maximal binding to the immobilized specific antiaminopeptidase globulin. The liquid was then aspirated from each well, and 200 μL (1 mg) of affinity-purified (Cezard et al., 1979) ^{125}I-labeled (17.4 Ci/mg; New England Nuclear) antiaminopeptidase globulin (1×10^6 cpm/mg) was added to the well. After incubation at room temperature for 4 h, the unbound labeled antibody was aspirated, and the well was washed 3 times with buffer B. The top of the plate was cut off, and the individual well radioactivity was determined in a γ counter. Nonspecific binding was determined either by substitution of normal rabbit globulin for the specific antiaminooligopeptidase as the immobilized antibody or by use of bovine albumin instead of the peptide antigen in the second step. This did not exceed 5% of the total bound radioactivity.

Lectin Binding Experiments. A 150-μL column of concanavalin A covalently bound to Sepharose 4B (Pharmacia) was prepared in a glass wool stoppered 3-mL plastic syringe barrel (Pharmaseal Laboratories) and equilibrated with 0.01 M sodium potassium phosphate, pH 7.0, with 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ (buffer C). The purified brush border and cytosol aminopeptidases were applied in 2 mL of buffer C–albumin (2 mg/mL). After the column and sample had equilibrated for 45 min at room temperature, the unbound peptidase was separated from the bound fraction by centrifuging the syringe at $50g \times 2$ min in a 16×100 mm glass tube. The bound aminopeptidase was then specifically eluted with 10 mM α-methyl mannoside in buffer C.

[^{3}H]Leucine Incorporation into Aminopeptidase in Vitro. Rats were anesthetized with sodium pentobarbital (6 mg/100 g of body weight), and a 25-cm loop of jejunum beginning just distal to the ligament of Treitz was isolated and cannulated at the proximal and distal ends (Smithson & Gray, 1977). [^{3}H]Leucine (1 mCi; New England Nuclear; 50 Ci/mmol) in 5 mL of 0.9% NaCl was instilled into the loop and allowed to remain for 5 min. The isotope was then flushed out by a solution of 150 mM NaCl with 1 mM unlabeled leucine; this solution was then perfused at 0.23 mL/min until the end of the experiment. Rats were sacrificed at 2, 5, 10, 15, 20, 30, 40, 60, and 180 min after the [^{3}H]leucine was chased, and the cytosol and brush border fraction was prepared as described above. Radioactive aminopeptidase was specifically immunoprecipitated by the monospecific antibody raised against brush border aminooligopeptidase, the pellet was taken up in aquasol (New England Nuclear), and the radioactivity was determined in a scintillation counter. Nonspecific precipitation was determined by incubation of the sample with normal rabbit globulin. To confirm that the immunoprecipitate contained only aminopeptidase, we dissociated the antigen–antibody complex by treatment with 0.2 M Na_2CO_3, pH 11.4 (Hoag et al., 1975), and then applied the sample to acrylamide gels as described above. Gel slices (2 mm) were incubated overnight at 60 °C in H_2O_2 (Grower & Bransome, 1970) and dissolved in aquasol, and the radioactivity was determined in a scintillation counter.

Sodium Dodecyl Sulfate–Acrylamide Electrophoresis. Gels were prepared and experiments were carried out as described by Weber & Osborn (1969). To determine the molecular weight of the glycoprotein subunits, we made gels at different percent total acrylamide (10.1, 8.5, and 7.0%) with constant cross-linking (1.33% C). The K_r values and derived molecular weights were determined as described under Results and in Table III.

Results

Aminooligopeptidase Activity in Intestinal Cytosol. The high-speed intestinal supernatant contained less than 1% of total mucosal glucose-6-phosphatase, succinic dehydrogenase, and galactosyl transferase activities and hence did not appear to be appreciably contaminated by organelle proteins. Because this cytosol fraction contained 2–4% of intestinal PHMB-resistant aminopeptidase activity, experiments were designed to ascertain whether this soluble activity represented material that had been solubilized from brush border membrane or

Table I: Effect of Preparative Conditions on Aminooligopeptidase in Intestinal Supernatant[a]

condition	% in cytosol	enzyme type
[K+] (mM)		
0	2.3	cytosol[b]
10	2.7	cytosol
50	2.3	cytosol
buffer A (mL)–musosa (g)		
1:1	2.7	cytosol
10:1	2.8	cytosol
homogenization time (s)		
20	3.8	cytosol
40	3.8	cytosol
temp (°C)		
4	2.3	cytosol
23	3.4	cytosol and brush border[c]

[a] Rat small intestine was prepared as described under Experimetnal Procedures, and the effect of varying a particular condition was analyzed. Each condition represents an experiment with a single intestine which serves as its own control. [b] Cytosol-type aminooligopeptidases by analysis on acrylamide gel electrophoresis (cf. Figure 1). [c] Brush border type aminooligopeptidases (Gray & Santiago, 1977).

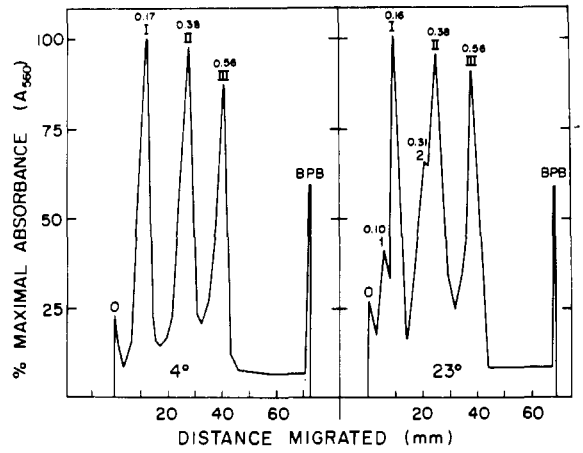

FIGURE 1: Polyacrylamide gel electrophoresis in nondenaturing system no. 2515 (cf. Experimental Procedures) of intestinal cytosol prepared at 4 °C (left) and 23 °C (right). Aminopeptidases were histochemically stained and peaks were localized by a Gilford scanner. Values above enzyme peaks denote R_f vs. the bromphenol blue front. The additional peaks 1 and 2 (R_f 0.10 and 0.31) found at 23 °C migrate identically with brush border enzymes I and II (Gray & Santiago, 1977). In this and subsequent figures, a relative scale is used with 100 arbitrarily set to the peak of highest peptidase activity for cytosol or brush border membrane samples. This permits precise comparative analysis of the samples having markedly different hydrolytic activities.

whether it constituted a distinct intracellular species of the surface membrane aminopeptidase.

The proportion of aminopeptidase recovered in the high-speed supernatant was maintained at the same fraction of total cell activity despite variation in the time of homogenization, the ratio of buffer volume to mucosal weight, or the concentration of KCl in the homogenizing buffer (Table I). These conditions had been shown to have marked effects on the intracellular distribution of pancreatic proteases (Scheele et al., 1978). Only homogenization and incubation at 23 °C yielded an increase in soluble aminopeptidase as compared to that of 4 °C preparations (Table I), and this was found to be due to release of aminooligopeptidases I and II from brush border (Figure 1). All other manipulations of the mucosal homogenate yielded soluble aminopeptidases that migrated distinctly differently from their brush border counterparts in

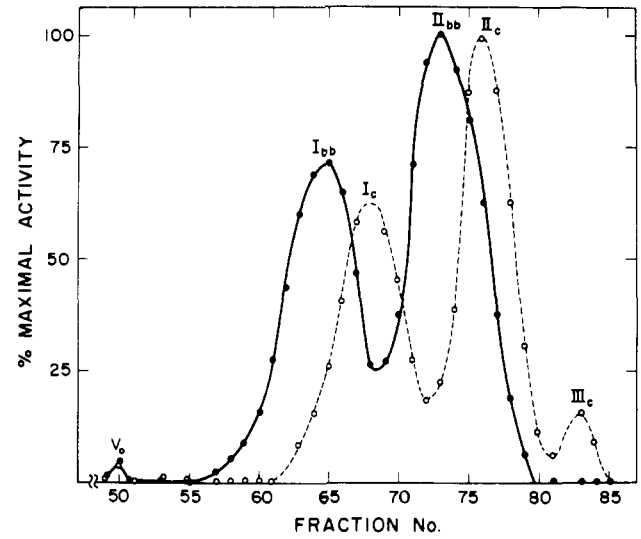

FIGURE 2: Gel filtration chromatographic profiles of brush border (●) and cytosolic (○) PHMB-resistant aminooligopeptidase activities. The cytosol and solubilized brush border fractions were prepared as described under Experimental Procedures, and samples were placed on the 2.5 × 9.0 cm Ultragel ACA 34 column in separate experiments. V_0 = void volume. Roman numerals indicate the designated AOP species where bb = brush border and c = cytosol.

Table II: Comparison of Molecular Parameters of Aminooligopeptidases[a]

enzyme no.	location	s (S)	a (Å)	$K_r \times 10^2$	M_r
I	brush border[b]	9.5	66	12.7	320 000
	cytosol	8.9	58	12.2	280 000
II	brush border[b]	7.8	49	9.4	180 000
	cytosol	7.5	47	9.1	140 000
III[c]	cytosol	4.3	42	7.5	70 000

[a] s = sedimentation coefficient determined from velocity centrifugation; a = Stokes' radius from gel filtration chromatography; K_r = retardation coefficient from acrylamide gel electrophoresis. [b] Data for brush border enzymes were published previously (Gray & Santiago, 1977). Techniques and standard proteins used are given under Experimental Procedures. [c] Enzyme III was not present in brush border membrane.

three different systems that are known to separate the brush border aminopeptidases (Gray & Santiago, 1977). This is considered in detail below (cf. Figures 1–3).

Comparison of the Molecular Size of Cytosol and Brush Border Aminooligopeptidases. (1) Gel Filtration Chromatography. Aminooligopeptidase solubilized from brush border has been shown to exist as two molecular weight isomers (Gray & Santiago, 1977). Figure 2 compares gel filtration profiles of the cytosol aminopeptidases to those for the brush border membrane. Notably, each cytosol species of AOP elutes behind the corresponding brush border enzyme. In addition, a trailing cytosol peak (III) that does not have a brush border counterpart was consistently identified in the cytosol fraction. The derived Stokes radii for cytosol enzymes I and II are distinctly smaller than those for the brush border enzymes (Table II).

(2) *Velocity Gradient Ultracentrifugation.* Velocity gradient experiments revealed that the cytosol species sedimented to a position behind their brush border counterparts (Figure 3). The slight difference in sedimentation rates was verified by adding ^{125}I-labeled brush border aminooligopeptidase to a sample containing the purified cytosol enzymes (data not shown). Comparison of the sedimentation coefficients for the membrane and cytosol peptidases is given in Table II.

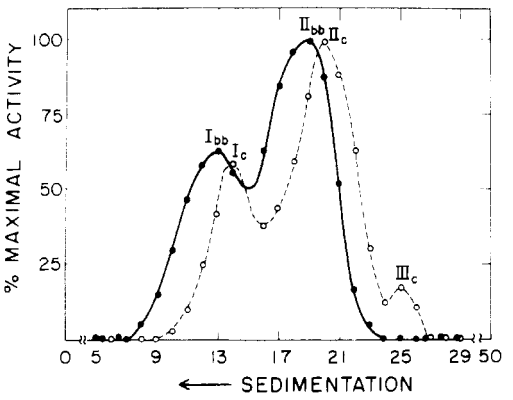

FIGURE 3: Velocity gradient centrifugation of intestinal brush border and cytosol PHMB-resistant aminopeptidase. The 0.1-mL sample was layered on a 5.0-mL linear gradient of 4–16% D-mannitol–buffer B and centrifuged at 65 000 rpm in the SW 65 Ti rotor as described under Experimental Procedures. Each 0.1-mL fraction was analyzed for aminopeptidase activity. Data from adjacent tubes in the same centrifugation experiment are shown. (●) Brush border; (○) cytosol. When added directly to the cytosol sample, the ^{125}I-labeled brush border AOP's sedimented more rapidly than cytosol species to the corresponding fraction found for the native brush border AOP's (data not shown).

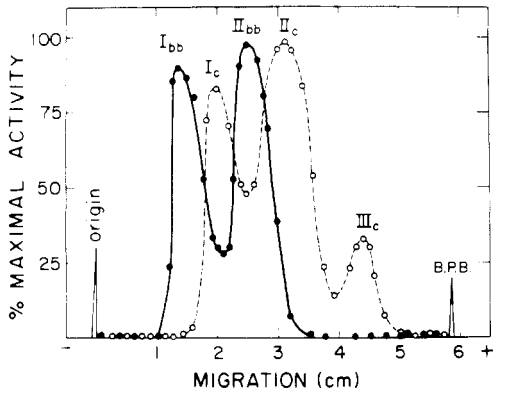

FIGURE 4: Composite scan of acrylamide gels at 7% T comparing brush border and cytosol aminooligopeptidases (cf. Figure 1 for details). Cytosol enzymes I and II migrated behind the corresponding brush border species. There is no brush border counterpart to cytosol III (cf. Figure 5).

(3) Nondenaturing Polyacrylamide Electrophoresis. Acrylamide electrophoresis in nondenaturing gels at 7.5, 8.75, and 10% T revealed that the cytosol aminopeptidases migrated behind their brush border counterparts (Figure 4). This was observed at each gel concentration, and a linear relationship was found when log R_f was plotted against percent total acrylamide (Figure 5). The slope (Δ log R_f/Δ % T) allowed determination of the retardation coefficient and estimation of molecular weights (Rodbard & Chrambach, 1971). Both cytosol peptidases I and II were smaller than their brush border counterparts by ~40 000 daltons. The molecular weight of cytosol III suggested that it may constitute the monomeric form of the larger cytosol species. The K_r values and molecular weights of the cytosol species are compared with those for the brush border in Table II.

Isolation of Cytosol Aminopeptidases. The cytosol PHMB-resistant aminooligopeptidases were purified to homogeneity by gel filtration and affinity chromatography using the monospecific antibody against brush border enzymes (Gray & Santiago, 1977). The cytosol aminopeptidases were purified ~100-fold by the two-step procedure, and no contaminating proteins were identified by Coomassie blue staining of acrylamide gels (not shown). The average specific activity of

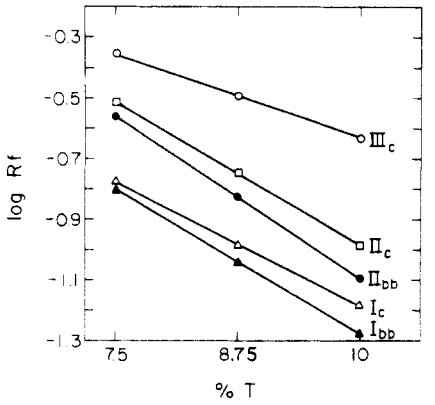

FIGURE 5: Ferguson plot based on acrylamide gel analysis of cytosol and brush border enzymes I, II, and III at three different values for percent total acrylamide. The retardation coefficient (K_r) was determined from the slope and molecular weights estimated by comparison with standards (Rodbard & Chrambach, 1971) (cf. Table II).

Table III: Enzymatic Properties of Cytosol and Brush Border Aminopeptidases

property	brush border	cytosol
K_m (μM)[a]	93 ± 5	93 ± 1
pH optimum[b]	6.9–7.3	6.9–7.3
% inhibn of EDTA[b] (2.5 mM)	53	48

[a] Mean ± SE from linear regression analysis of Eadie plots; six substrate concentrations ranging from $0.1K_m$ to $5K_m$ were used. [b] Analyzed in 0.05 M sodium potassium phosphate buffer (Wojnarowska & Gray, 1975).

870 milliunits/mg of protein was ~5% of that found previously in our laboratory for the brush border aminopeptidases (Gray & Santiago, 1977). This lower specific activity of the cytosol enzymes may reflect a greater susceptibility to denaturation during purification, a lower affinity of the enzyme for the naphthylamide substrate, or the presence of some molecules devoid of an active hydrolytic site. The enzymatic and immunochemical studies detailed below suggest that neither a lowered substrate affinity nor a discrete absence of a hydrolytic site can explain the relatively lower activity of the cytosol aminopeptidases. Instead, it appears that the cytosol enzymes may be more readily denatured than the corresponding membrane peptidases.

Enzymatic Properties of the Cytosol and Brush Border Aminooligopeptidases. The purified cytosol and brush border aminopeptidases were studied for pH optimum, K_m, and EDTA inhibition. As shown in Table III, the cytosol and brush border enzymes had an identical K_m and pH optimum and were inhibited equally by 2.5 mM EDTA. Hence, the active site of the cytosol peptidases appears to function no differently than that of its larger membrane counterparts.

Subunits of Cytosol Aminopeptidases. Because of the small quantities of cytosol aminopeptidase that could be isolated, it was not possible to compare the size of subunits with those of the brush border enzymes by conventional NaDodSO$_4$–acrylamide electrophoresis. Since the aminopeptidases could be readily radiolabeled and subsequently purified by antibody-affinity chromatography, we studied the iodinated forms of these enzymes.

The purified cytosol peptidases (cf. Table II) and brush border aminooligopeptidase (Gray & Santiago, 1977) were radiolabeled with ^{125}I (17.4 Ci/mg; Amersham/Searle) by the Chloramine T method (Hunter & Greenwood, 1962) and separated from any peptide fragments by antibody-affinity chromatography as detailed under Experimental Procedures.

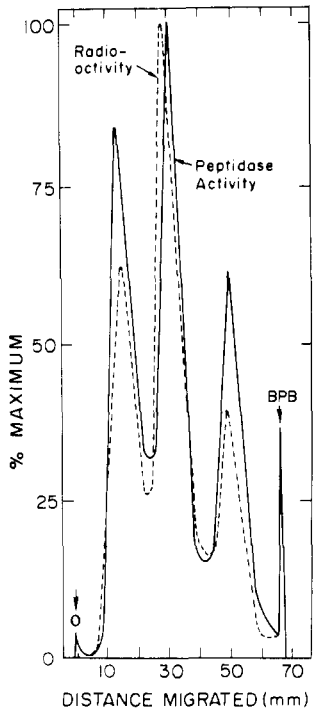

FIGURE 6: Nondenaturing acrylamide electrophoresis of ^{125}I-labeled and affinity-purified cytosol aminopeptidases. Details are given under Experimental Procedures. Radioactivity migrated coincident with enzymatic activity, and no inactive peptide fragments were identified. The individual isomers were cut from gels, and the subunits were studied by $NaDodSO_4$–acrylamide electrophoresis (cf. Figure 7).

Table IV: Molecular Parameters for Subunits of Cytosol Aminooligopeptidases[a]

enzyme no.	$K_r \times 10^2$	M_r
I	5.4 ± 0.7	72 000 ± 10 000
II	5.6 ± 0.9	75 000 ± 14 000
III	5.4 ± 0.1	70 000 ± 3000

[a] The ^{125}I-labeled aminopeptidases were analyzed by sodium dodecyl sulfate-acrylamide gels (Weber & Osborn, 1969) at 10.1, 8.5, and 7.0% T with constant cross-binding (1.33% C). Relative mobility expressed as a ratio to the bromphenol blue font was plotted vs. % T, and the K_r was determined from the slope. Plots of K_r vs. molecular weight of standard proteins (cf. Figure 7) produced linear relationships. Values ± SE were derived from analysis by linear regression.

Examination of the urea-eluted material by nondenaturing acrylamide electrophoresis revealed comigration of radioactivity and peptidase activity at the expected positions of cytosol enzymes I, II, and III (Figure 6). The individual ^{125}I-labeled cytosol peptidases were eluted from the appropriate gel slices and subjected to sodium dodecyl sulfate–acrylamide gel electrophoresis (Weber & Osborn, 1969). As shown in Figure 7, cytosol enzymes I and II appear to consist of a single subunit that migrates identically with the smaller subunit of brush border enzyme I. Cytosol III migrated slightly behind the subunit of cytosols I and II, but experiments at three different values for percent acrylamide revealed a molecular weight (~70 000) that was not significantly different for all three cytosol enzymes (Table IV). These experiments suggest that the cytosol aminooligopeptidases consist of a single subunit containing the active enzyme site and that it exists in monomeric, dimeric, and tetrameric forms.

Comparison of Cytosol and Brush Border Aminopeptidases by Radioimmunoassay. On the basis of their binding to immobilized antibody against the brush border aminopeptidases and their enzymatic and structural similarities to the membrane enzymes (Tables II–IV), the cytosol aminopeptidases appeared to be related to those in the brush border. To define the structural relationship of the cytosol and membrane enzymes more precisely, we compared the capacity of cytosol species to react in a specific radioimmunoassay designed for analysis of the brush border aminopeptidases. On the basis of their enzyme activity, cytosol aminopeptidases showed ap-

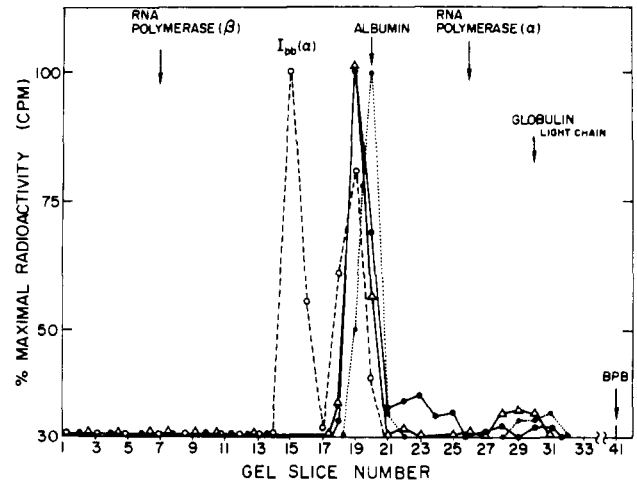

FIGURE 7: Sodium dodecyl sulfate–acrylamide gel electrophoresis (7.0% T; 1.33% C) of ^{125}I-labeled aminooligopeptidases that had been purified by affinity chromatography as described under Experimental Procedures. Position of standard proteins is indicated at the top. The expected 90 000-dalton and 70 000-dalton subunits of brush border aminopeptidase I (O---O) are identified (Gray & Santiago, 1977). The cytosol enzymes I (●—●), II (Δ---Δ), and III (●---●) have only a single 70 000–75 000-dalton subunit. Although the cytosol III subunit migrates slightly ahead of the I and II isomers, analysis at three values of % T did not reveal a significant difference in the subunit size compared to the other isomers (cf. Table III).

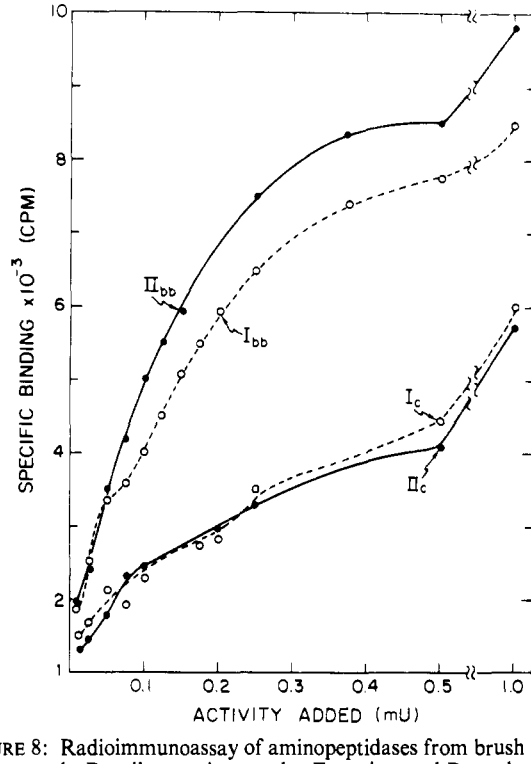

FIGURE 8: Radioimmunoassay of aminopeptidases from brush border and cytosol. Details are given under Experimental Procedures (cf. Figure 2 for symbols). Each assay was reproducible to within 5% of the values plotted. The cytosol enzymes display less affinity throughout the working range of the assay and a lower plateau of maximal binding as compared to the brush border peptidases.

preciably less reactivity in the solid-phase radioimmunoassay throughout the range of the assay (Figure 8). In addition, the maximal binding of the radioactive antibody was appreciably lower for the cytosol enzymes, indicating that the cytosol species probably do not have the full complement of antigen determinants present on the mature brush border aminopeptidases.

Binding of Peptidases to Concanavalin A. The brush border aminopeptidases are known to be glycoproteins consisting of ~20% carbohydrate (Kim & Brophy, 1976), and the smaller size and decreased immunoreactivity of the cytosol peptidases could be accounted for by incomplete or absent carbohydrate chains (Schiffman et al., 1964). Hence, comparative lectin binding studies of the brush border and cytosol enzymes were carried out. Binding to immobilized concanavalin A was linear when 5–30 milliunits was added for both brush border and cytosol enzymes but was significantly lower for the cytosol enzymes (mean ± SE: brush border, 85 ± 2%; cytosol, 51 ± 4%). Reapplication of the unbound aminopeptidase to a fresh concanavalin A–Sepharose column showed the same fractional binding as originally observed, indicating that the individual peptidases are not heterogeneous in regard to mannose or glucose content. All bound peptidase was immediately released and eluted from the Con A column when 10 mM α-methyl mannoside was added to the buffer. Fucose, a monosaccharide that does not interact with Con A, did not elute any peptidase even when present at high (100 mM) concentration.

[^{3}H]Leucine Incorporation into Cytosol Aminopeptidases. The cytosol aminopeptidases appear to be distinct enzyme species that are related to those in the brush border, but they could constitute either a degradative fragment or a precursor of the surface membrane enzyme. To further determine the relationship of these two intestinal aminopeptidase species, we performed in vivo [^{3}H]leucine pulse-labeling experiments and isolated the radiolabeled aminopeptidases from cytosol and brush border by specific immunoprecipitation. Acrylamide electrophoresis of the immunoprecipitate contained >90% radioactivity that comigrated with authentic aminopeptidases I and II (data not shown). The pattern of [^{3}H]leucine incorporation into the cytosol aminopeptidases was distinctly different from that for the brush border species (Figure 9). Whereas labeling of brush border peptidase did not approach a maximum for 1–3 h, the cytosol aminopeptidases were maximally labeled in only 20 min. The early labeling of the brush border fraction at 10 min probably represents incorporation into microsomes that inevitably contaminate such brush border membrane preparations. Thus, the sequential labeling patterns in cytosol and surface membrane suggest that the small cytosol pool constitutes a precursor of the membrane enzyme at an early stage in the synthesis and assembly process.

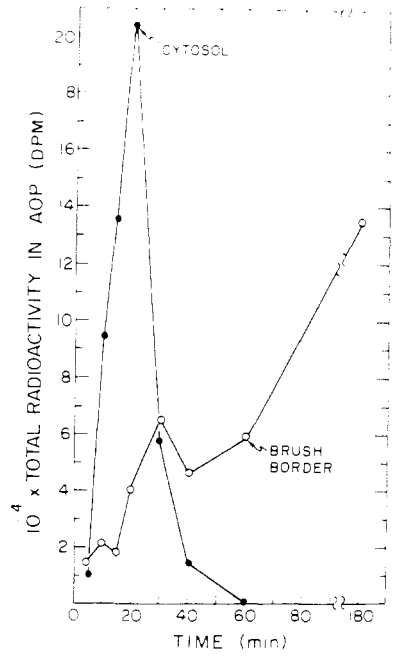

FIGURE 9: Incorporation of [^{3}H]leucine into cytosol and brush border aminooligopeptidase. The 25-cm jejunal loop was exposed to 1 mCi of [^{3}H]leucine for 5 min and then perfused with 1 mM unlabeled leucine for the times indicated on the abscissa. The brush border and cytosol enzymes were prepared and immunoprecipitated, and radioactivity was determined as detailed under Experimental Procedures. The total radioactivity in the brush border (○) and cytosol (●) is given for the jejunum of a single animal at each time period. Results were reproducible within ±10% of the values shown. Maximal incorporation into the cytosol at 20 min exceeded that attained in brush border at 3 h.

Discussion

Aminooligopeptidase, an integral glycoprotein of the specialized intestinal brush border membrane, has its active site available at the luminal membrane where it can perform its essential role in digestion of nutrient protein. This hydrolase is generally believed to be confined to the surface membrane, and the small fraction identified in the 100000g/h supernatant has been considered to represent contamination released from brush border during cell fractionation. In other studies in our laboratory, however, distinct cytosol forms of another membrane hydrolase, sucrase-isomaltase, have recently been identified (Cezard et al., 1979).

Regardless of the conditions of preparation (Table I), rat small intestinal cytosol appears to contain similar amounts of three distinct aminopeptidases that have indistinguishable enzyme properties (Table III) and are structurally and immunochemically (Figure 8) related to the brush border membrane aminooligopeptidase. Although cell supernatant harvested at 23 °C contained more aminopeptidase as compared to cytosol prepared at 4 °C, the increase was due to autolysis of the larger aminopeptidases from brush border membranes (Figure 1). This finding of a cytosol enzyme at constant ratio to its membrane counterpart is in contrast to the marked variation in amounts of pancreatic enzymes recovered from the cytosol depending upon preparative conditions (Scheele et al., 1978). Furthermore, in a previous study, treatment of the brush border membrane fraction with proteases such as papain did not yield the smaller cytosol-type enzymes (Gray & Santiago, 1977), suggesting that the cytosol aminooligopeptidases are not derived from breakdown of the surface membrane. The in vivo [^{3}H]leucine pulse-labeling experiments support this contention (Figure 9).

Unlike the ubiquitous cytosol aminopeptidases identified in many tissues (Kim et al., 1972), these cytosol aminooligopeptidases cross-react with antiserum to the brush border aminooligopeptidases and are not inhibited by PHMB. Although closely related to brush border aminooligopeptidases I and II the corresponding cytosol enzymes are distinctly smaller (by 40000 daltons) when examined by three different separation techniques (Figures 2–5 and Table II). AOP_c III, the smallest of the cytosol species, binds specifically to brush border aminooligopeptidase–antibody columns and yet does not have a homologue of similar size in the brush border. It appears to be the monomeric member of the aminooligopeptidase family (Table II).

As shown in Table IV, comparative analysis of the cytosol and brush border aminooligopeptidase subunits reveals the presence of only a single 70000-dalton monomer for the cytosol

enzymes. The brush border aminooligopeptidases had previously been shown to consist of a 90000-dalton subunit that may carry the catalytic site, and brush border aminooligopeptidase I also contains a second smaller (70000-dalton) subunit (Gray & Santiago, 1977). Thus, the 40000-dalton difference between the cytosol and brush border membranes cannot be explained by the presence of a small insertion subunit in the membrane enzyme. If it is presumed that the 90000-dalton peptide is a true monomeric unit of the brush border enzymes, the cytosol enzymes appear to have a smaller and possibly incomplete subunit. Since the brush border aminooligopeptidases contain about 20% carbohydrate, the smaller cytosol aminooligopeptidases could be missing a considerable portion of the carbohydrate chain present in the membrane enzymes. This is supported by the concanavalin A experiments where fractional binding was less complete for the cytosol enzymes. Unfortunately, the amounts of the cytosol aminopeptidases that could be recovered were insufficient for detailed carbohydrate analysis.

Analysis of the cytosol enzymes by the specific radioimmunoassay designed for the brush border enzymes verifies that the cytosol forms are related to those in brush border (Figure 8), but the maximal antibody binding is appreciably lower, indicating that some antigenic sites of the membrane enzymes are not present in the related cytosol aminopeptidases. This is consistent with their smaller size and reduced affinity for concanavalin A.

Although the exact relationship of the cytosol aminooligopeptidases to the surface membrane enzyme is uncertain, the in vivo pulse labeling experiments demonstrate maximal incorporation into the cytosol aminooligopeptidases at 20 min; in contrast, a maximum was not reached until much later for the surface membrane enzymes (Figure 9). In studies not detailed here, the maximal incorporation into the membrane aminopeptidase occurred at 3–6 h and degradation was very slow (half-life 12–24 h). Hence, the incorporation found in the membrane at ~3 h is representative of maximal radioactivity in the membrane peptidase. In Figure 9, the ordinate is plotted as total radioactivity in the intestinal preparation. Notably, the total incorporation into the cytosol enzymes at 20 min can account for that subsequently found in brush border at the point of maximal incorporation at 3 h. This suggests that the smaller and incomplete cytosol species of the aminooligopeptidase are precursors of the mature surface membrane enzymes. Exactly at what point in the assembly process the peptidases become released into the cytosol is uncertain, but since their molecular weights approach those in the brush border and because all isomers including the monomeric enzyme III have acquired enzymatic activity as well as the capacity to bind to specific antibody and concanavalin A, the cytosol aminooligopeptidases appear to be nearly structurally complete. Despite this, their subsequent movement and assembly into the membrane require a period of hours. This apparent delay in movement of a precursor to the intestinal surface is consistent with previous studies with radioactive monosaccharide precursors by electron microscopic radiography. Bennett & Leblond (1970) noted incorporation of fucose into the golgi region by 2 min, but the labeled sugar did not localize over the brush border membrane for 4 h. The radioautographic grains appeared to accumulate for extended periods of time both in the subapical cytoplasm and in vesicles just beneath the surface membranes prior to their localization in the brush border.

Studies of membrane glycoprotein synthesis in HeLa cells (Bosmann et al., 1969) and in rat liver (Autuori et al., 1975) have revealed labeled glycoproteins in cytosol prior to maximal membrane localization. A well-characterized viral membrane protein, coliphage M_3 coat protein, has recently been shown to be synthesized within the cytoplasm of infected cells prior to its membrane insertion (Ito et al., 1979). Also, recent studies in our laboratory have identified a rapidly synthesized cytosol form of intestinal membrane sucrase that is about 15% smaller than its brush border counterpart (Cezard et al., 1979). However, classical hypotheses for intracellular synthesis and assembly of secretory (Palade, 1975) and plasma-membrane (Blobel & Dobberstein, 1975) proteins require continuous association of the peptide with intracellular membranes. The rapidly labeling aminopeptidase, rather than being a true cytosol component, could actually have been dislodged from an intracellular membrane site during preparation. But if this were the case, some variation in the amount of supernatant aminopeptidase might have been expected with different preparative conditions (Table I).

Although loose attachment of the rapidly labeling precursor to intracellular membrane remains a possibility, our experiments with a specific intestinal membrane glycoprotein are compatible with an initial rapid synthesis of the nascent enzyme, followed by transient release probably from the golgi into cytosol. Subsequent intracellular assembly including addition of a 40000-dalton unit is likely to occur in association with endoplasmic reticulum. Finally, the process culminates with insertion into the brush border surface membrane several hours later. Although further experiments will be required to define the role of the intracellular membrane-associated assembly of intestinal brush border glycoproteins, it appears that there is a distinct cytosol phase early in the assembly process. Whether this phenomenon is peculiar to the specialized intestinal enterocyte or is more ubiquitous will have to await appropriate experiments with individual glycoproteins in other mammalian tissues.

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Nitro Analogues of Citrate and Isocitrate as Transition-State Analogues for Aconitase†

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ABSTRACT: Nitro analogues of citrate (2-hydroxy-3-nitro-1,2-propanedicarboxylic acid) and isocitrate (1-hydroxy-2-nitro-1,3-propanedicarboxylic acid) are carbon acids with pK values of 10.80 and 9.46, respectively. Both compounds are competitive inhibitors of aconitase with their affinity for the enzyme dependent on the ionization state of the carbon acid. When fully ionized, the citrate and isocitrate analogues have K_i values of 59 nM and 72 nM, respectively, which increase to 1.7 mM and 58 μM upon protonation of the carbon acid. In addition to the pH-dependent change in its affinity for aconitase, the isocitrate analogue (but not the citrate analogue) exhibits a slow time-dependent increase in its affinity for enzyme, with an equilibrium constant for the change of 105 and a final K_i of 680 pM for the ionized form and an equilibrium constant of 83 and a final K_i of 680 nM for the protonated form. The rate constant for formation of the tight complex is 6.6 min^{-1}, and that for the reverse isomerization is 0.063 min^{-1}. The Michaelis constants for citrate (160 μM) and isocitrate (49 μM) are 2700 and 72 000 times greater than the final K_i values for the ionized nitro analogues of citrate and isocitrate, respectively. We believe the tight binding of the nitro compounds is due to their similarity in structure to carbanion intermediates in the reaction (bound as aci-acids) which are tetrahedral at the carbon bearing a hydroxyl and trigonal at the carbon lacking a proton.

A renewed interest in the mechanism of action of aconitase (EC 4.2.1.3) has ensued with the realization that the enzyme isolated from beef heart mitochondria is a high-potential iron–sulfur protein (Ruzicka & Beinert, 1978; Kurtz et al., 1979). As reductants other than Fe^{2+} can serve to activate the enzyme (Ruzicka & Beinert, 1978; Schloss, 1979), the long-held role of Fe^{2+} in the activation of aconitase has come into question, and the proposed ferrous wheel mechanism is suspect [for a review of this and other proposed mechanisms, see Glusker (1971)]. Additional support for the absence of a role for Fe^{2+} other than reduction of the iron–sulfur cluster comes from the observation that aconitase can be isolated anaerobically from pig liver cytosol to yield a fully active enzyme (Eanes & Kun, 1974).

While mechanistic details of aconitase action are scanty, it appears that the enzyme plays a role as base, abstracting a proton from citrate or isocitrate (Rose & O'Connell, 1967). The general conclusion that the enzyme proceeds by a carbanion mechanism is not universally accepted, however, as Thomson et al. (1966) proposed a carbonium ion mechanism on the basis of the relatively small primary isotope effects observed with deuterated substrates. In the course of further mechanistic studies of aconitase, we have found that the fully ionized forms of nitro analogues of citrate and isocitrate (which can be considered analogues of carbanion reaction intermediates) are potent inhibitors of the enzyme, and the present report deals with the properties of this inhibition.

Experimental Procedures

Materials. *threo*-DL-Isocitrate, *cis*-aconitate, Mes,[1] Hepes, Tris, Caps, *cis*-oxalacetic acid, and glyoxylic acid were from Sigma Chemical Co. *N*-Ethylmorpholine, ethanolamine, and 3-nitropropionic acid were from Aldrich. The concentration of *threo*-D$_S$-isocitrate in stock solutions of *threo*-DL-isocitrate was determined with isocitrate dehydrogenase by the method of Rose & O'Connell (1967); all references to isocitrate refer to *threo*-D$_S$-isocitrate. *threo*-L$_S$-Isocitrate is a poor inhibitor of aconitase (K_i > 4 mM; Villafranca, 1972; Thomson et al.,

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Caps, 3-(cyclohexylamino)-propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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