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Reconstitution of purified dipeptidyl peptidase IV. A comparison with aminopeptidase N with respect to morphology and influence of anchoring peptide on function

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The pig small intestinal dipeptidyl peptidase IV was asymmetrically integrated into egg phosphatidylcholine and microvillar lipid vesicles prepared by a β-octylglucoside dialysis method. The enzyme molecules appeared dumbell-shaped ((11.0–11.5) × (5.0–5.5)nm) and were separated from the liposomal membrane by a stain-filled gap of about 2.5 nm, representing the 'junctional segment'. The influence of lipid bilayer and detergents on the kinetic parameters of amphiphilic and hydrophilic forms of aminopeptidase N and dipeptidyl peptidase IV was studied. Since the lipid bilayer and detergents, which interact only with the anchoring root, had no crucial effect on the kinetic parameters of the different forms of the enzymes, it is concluded that the anchoring roots exert little effect on the catalytic domain of the stalked integral membrane proteins.

Introduction

Amphiphilic aminopeptidase N and dipeptidyl peptidase IV have been purified to homogeneity from the detergent-solubilized pig small intestinal microvillar membranes [1,2]. These enzymes are anchored to the membrane by a small hydrophobic segment. Their catalytically active domains are hydrophilic and are exposed on the non-cytoplasmic side of the membrane. These domains constitute the dominating part of the protein mass and contain substantial amounts of carbohydrates.

Aminopeptidase N and dipeptidyl peptidase IV have complementary specificity towards oligopeptides. In a concerted action they are thought to play an important role in the terminal digestion of the oligopeptides generated by the action of pancreatic proteinases in the small intestinal lumen.

Since proteinase solubilization of microvillar hydrolases does not result in loss of enzymatic activity, it is generally believed that the anchoring peptides do not affect the enzymatic activity. In contrast to this view, Ugolev et al. [3] reported that the proteinase and detergent forms of the microvillar enzymes differ in kinetic properties and that the activities of the amphiphilic enzymes could be modulated by effectors. On this basis they proposed that the anchoring peptide segment exerts a regulatory effect on the catalytic function of the hydrophilic domains. Since this hypothesis implies for the hydrophobic segment a regulatory role of possible functional significance, it is of interest further to evaluate and study the effect of anchors on the enzymatic activities of the stalked integral microvillar proteins. In this paper an experimental method for membrane reconstitution of dipeptidyl peptidase IV is reported and its morphology and relation to the membrane are studied. The results are compared to previous similar studies on aminopeptidase N [4].

It also reports on the importance of the anchoring peptide and the milieu around it, on the catalytic function of purified amphiphilic dipeptidyl peptidase IV and aminopeptidase N. To assess the role of anchoring peptides on the catalytic functions, the kinetic parameters of the two amphiphilic enzymes were determined in the following states: after incorporation into artificial membranes, in detergent solution and in detergent-free solution. The results were compared to the kinetic parameters of the hydrophilic forms.

Materials

Chemicals. Proteinase K (research grade) was obtained from Serva (Heidelberg, F.R.G.), [^{3}H]Triton X-100 from the Radiochemical Centre (Amersham U.K.) and bovine trypsin (type III) from Sigma Chemical Co., St. Louis, MO. All other chemicals were as previously described [4].

Enzymes. Pig intestinal amphiphilic aminopeptidase N and dipeptidyl peptidase IV were purified using immunoadsorbent chromatography [1,2]. The enzymes remained in 2 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and aprotinin (2.8 mg $\cdot$ l^{-1}).

Hydrophilic aminopeptidase N was prepared by treatment of the amphiphilic form with trypsin [1]. The enzyme was then isolated from the reaction mixture by chromatography on a column of *Ricinus communis* lectin Sepharose.The enzyme bound to the column was eluted by 30 mM lactose in 25 mM Tris-HCl, pH 7.4. The eluted enzyme solution was then dialyzed against 25 mM Tris-HCl, pH 7.4, in order to remove lactose. The loss of detergent-binding properties was controlled by charge-shift immunoelectrophoresis [5].

The hydrophilic form of dipeptidyl peptidase IV was obtained from the amphiphilic one by proteinase K treatment. 17 mg proteinase K were added to 84 U of dipeptidyl peptidase IV (corresponding to 3.4 mg) in 60 ml of 2 mM Tris-HCl, pH 8.0, 0.1% Triton X-100 and incubated for 60 min. After this treatment the enzyme was completely changed to the hydrophilic form as tested by charge-shift immunoelectrophoresis [5]. The hydrophilic dipeptidyl peptidase IV was then isolated from the reaction mixture by *Ricinus communis* lectin Sepharose chromatography as described above for aminopeptidase N.

Methods

Analytical methods. Aminopeptidase N and dipeptidyl peptidase IV activities were determined spectrophotometrically using 1 mM L-alanine-4-nitroanilide in 50 mM Tris-HCl, pH 7.3, and 1.5 mM glycyl-L-proline-4-nitroanilide in 50 mM Tris-HCl, pH 8.0, as substrates, respectively. One unit of enzyme activity is defined as 1 μmol substrate hydrolysed per min at 37°C [1,2].

^{14}C and ^{3}H radioactivities were determined in a Nuclear Chicago scintillation counter using Aqualuma Plus as scintillant. Sucrose concentration was measured at 25°C by polarimetry using the 577 nm Hg line (Perkin-Elmer polarimeter, model 241, Norwalk, USA) using a dilution series of sucrose in the proper buffer as standard.

Detergent exchange. Triton X-100 in the dipeptidyl peptidase IV preparations was exchanged with β-octylglycoside using gradient centrifugation [6]. 0.5–1.0 ml (250–500 μg) of enzyme solution containing trace amounts of [^{3}H]Triton X-100 was applied on top of a linear sucrose gradient (10 ml, 0.464 M–0.8 M sucrose in 25 mM Tris-HCl, pH 7.4, containing 30 mM β-octylglycoside). The samples were centrifuged (284000 $\times$ g, Beckman SW 40 rotor, 4°C, 35–45 h). Fractions of 800–1000 μl were collected from the bottom and analyzed for enzyme activity, sucrose concentration and radioactivity. Fractions containing more than 0.5 U $\cdot$ ml^{-1} of enzyme activity were pooled and used for reconstitution.

Reconstitution. Reconstitution was carried out essentially according to the method of Helenius et al. [6]. 5 mg lipid in 20 μl chloroform containing trace amounts of [^{14}C]phosphatidylcholine were evaporated by nitrogen to a film in a conical test tube. The dry lipid film was dissolved in 5 ml of 25 mM Tris-HCl, pH 7.4, containing 30 mM β-octylglucoside and 0.8 M sucrose. 5 ml enzyme solution (0.2 mg dipeptidyl peptidase IV) kept in the same buffer were added and the solution was thoroughly mixed. The detergent concentration was then reduced by either dialysis against 5 mM Tris-HCl, pH 7.4 (4°C), for 48 h with a change of buffer after 17 h, or by 10-times dilution using the same buffer followed by overnight dialysis and concentration of the dialysed sample by ultrafiltration using a PM-10 filter (Amicon, Lexington, MA, U.S.A.).

Preparation of detergent-free amphiphilic forms. Aminopeptidase N and dipeptidyl peptidase IV were made detergent-free by the method described for Semliki Forest virus spike proteins [7]. About 200 μg of either aminopeptidase N or dipeptidyl peptidase IV in 2 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 were placed on top of a linear sucrose gradient (0–0.54 M sucrose in 25 mM Tris-HCl, pH 7.5). The sample was then centrifuged for 40 h at 248000 × *g* using a Beckman SW40 rotor. Fractions of about 1 ml were collected from the bottom and analyzed for enzymatic activity.

Sucrose-density-gradient centrifugation. Analysis of the lipid vesicles formed by reconstitution was carried out by gradient centrifugation. 0.25 or 1 ml of the vesicle-containing solution was layered on top of a linear sucrose gradient (0–1.58 M) in 25 mM Tris-HCl, pH 7.4. The sample was then centrifuged either in a Beckman SW40 rotor (248000 × *g*) or in a Beckman 50.1 rotor (300000 × *g*) for 40 or 20 h, respectively (4°C). Fractions of 0.5 ml or 0.3 ml, respectively, were collected from the bottom and analysed for enzymatic activity, radioactivity and sucrose concentration. Selected fractions were analyzed by electron microscopy after dialysis against 25 mM Tris-HCl, pH 7.4.

Treatment with proteolytic enzymes. Dipeptidyl peptidase IV in the lipid vesicles was treated with proteinase K or trypsin. 10 μl of proteolytic enzyme (5 mg/ml water) were added to 200 μl of dipeptidyl peptidase IV-containing vesicle solution (1.2 U, 50 μg, of dipeptidyl peptidase IV per ml). The mixture was incubated for 1 h and then analysed by sucrose-density-gradient centrifugation.

Electron microscopy. Enzyme samples were examined by negative staining using 2% (w/v) sodium silicotungstate, pH 7.2. A droplet of the sample (50–100 μg of protein/ml) was applied to a carbon-coated Formvar film carried on a 400-mesh copper grid, glow-discharged shortly before use. The grid was then flushed by ten drops of stain solution. Excess stain was drained to leave a thin film on the grid which was left to dry at room conditions. The specimens were examined in a Philips 300 or a Jeol 100 CX electron microscope operated at 60 or 80 kV, respectively. The magnification was calibrated by using a Fullam grating replica No. 1002X.

Measurements of molecular dimensions were performed on images printed to total magnifications between 185000 and 310000 using a measuring magnifier (magnification factor 8) with a metric graticule read to 0.1 mm.

Kinetic experiments. The kinetic epxeriments were performed in the Reaction Rate Analyzer 8600 (LKB, Stockholm, Sweden) using L-alanine-4-nitroanilide (aminopeptidase N) or glycyl-L-proline-4-nitroanilide (dipeptidyl peptidase IV) as substrates at different concentrations. The reactions were carried out at 37°C in 25 mM Tris-HCl buffer, pH 7.5. When detergent was present in the reaction mixture it was added 30 min before the start of the reaction by the addition of the substrate. The initial reaction velocities were measured by using the slope of the tangent to zero time. The kinetic parameters were calculated by a desk computer using the statistical method of Wilkinson [8].

Results and Discussion

Reconstitution of amphiphilic dipeptidyl peptidase IV into liposomes made from egg phosphatidylcholine using β-octylglucoside resulted in complete association of the enzyme with the lipid (Fig. 1b). The enzyme-containing liposomes were positioned at a density between 1.02 and 1.08 $g \cdot cm^{-3}$, the density corresponding approximately to that of vesicles formed by phosphatidylcholine alone (Fig. 1a). Reconstitution of dipeptidyl peptidase IV into liposomes made with lipids extracted from microvillar membranes resulted in a rather low degree of incorporation (Fig. 1c). This low incorporation is similar to that of aminopeptidase N reconstitution with phosphatidylcholine and microvillar lipid vesicles using the β-octylglucoside dialysis method [4], and is in contrast to the complete incorporation of dipeptidyl peptidase IV in phosphatidylcholine vesicles. Such differential incorporation during reconstitution using β-octylglucoside has been reported for Semliki Forest virus protein and penicillinase from *Penicillium lichenoformis* [9].

When the sodium cholate method described by Kagawa and Racker [10], which was successfully

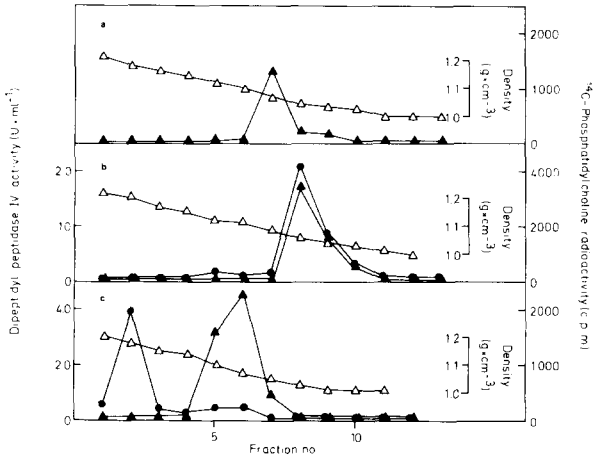

Fig. 1. Sucrose density centrifugation experiments on reconstituted dipeptidyl peptidase IV. (a) egg phosphatidylcholine; (b) dipeptidyl peptidase IV reconstituted with egg phosphatidylcholine; (c) dipeptidyl peptidase IV reconstituted with intestinal microvillus membrane lipids. ▲, ^{14}C radioactivity in phosphatidylcholine; △, density (sucrose concentration); ●, dipeptidyl peptidase IV activity.

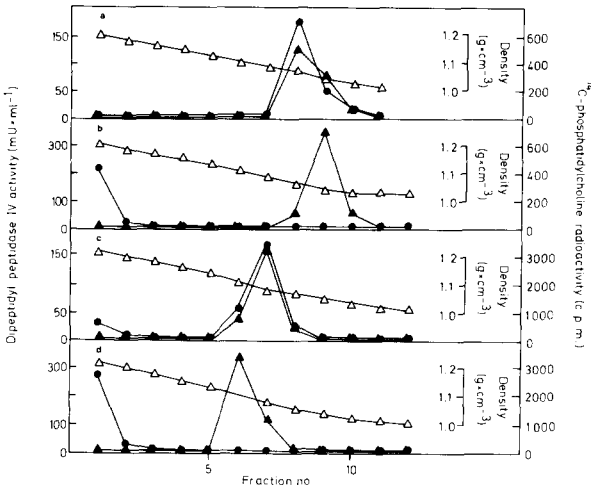

Fig. 2. Sucrose density gradient centrifugation experiments on reconstituted dipeptidyl peptidase IV after proteolytic treatment. Dipeptidyl peptidase IV reconstituted with egg phosphatidylcholine from fractions 8 and 9 of Fig. 1b, incubated 1 h at 37°C (a) and treated with proteinase K (50 μg proteinase K, 1 h at 37°C) (b); dipeptidyl peptidase IV reconstituted with intestinal microvillus membrane lipids (from fraction 5 and 6 of Fig. 1C) incubated 1 h at 37°C (c) and treated with proteinase K (50 μg proteinase K, 1 h at 37°C) (d); symbols as in Fig. 1.

used for aminopeptidase reconstitution [4], was used, no dipeptidyl peptidase IV was incorporated into the vesicles because the enzyme during dialysis at room temperature was converted to the hydrophilic form. This conversion could be prevented by the addition of 1 mM EDTA to the solutions but not by phenylmethylsulphonyl fluoride or iodoacetic acid. However, the addition of EDTA to the buffer solutions during dialysis could not be combined with the addition of magnesium ions, thus making this approach impossible.

Relation to membrane and morphology

Trypsin treatment of dipeptidyl peptidase IV-containing vesicles resulted in about 20% release of the enzyme from the vesicles. Proteinase K treatment of dipeptidyl peptidase IV-containing vesicles, however, resulted in complete release of the enzyme from the lipid vesicles (Fig. 2). Although the proteinase: protein ratio was high, i.e., within the range normally used in this type of experiment [6], the enzymatic activity was not affected. Subsequent incubation of proteinase K-treated vesicles in 1% Triton X-100 did not reveal any additional enzymatic activity. Thus all dipeptidyl peptidase IV molecules were positioned on the external side of the liposomes. The asymmetric orientation of dipeptidyl peptidase IV observed in these liposomes is similar to that of aminopeptidase N present in the liposomes [4].

An electron micrograph of negatively stained reconstituted dipeptidyl peptidase IV (fraction 8 of Fig. 1b) is shown in Fig. 3. Umilamellar vesicles of an average diameter of about 60 nm containing several copies of dumbell-shaped structures ((11.0–11.5) × (5.0–5.5)nm) are seen. These structures have dimensions similar to those of free amphiphilic dipeptidyl peptidase IV, suggesting that they represent the incorporated enzyme molecules. The dumbell-shaped structures are seen at a maximum distance of approx. 2.5 nm from the surface of the vesicles (Fig. 4), i.e., a value close to that reported for a kidney endopeptidase [11]. The broad range of gap-widths measured for dipeptidyl peptidase IV and aminopeptidase N (see later) does not represent a true variation in distance between head groups and membrane. It is due to the varying angle of projection of individual molecules, as delineated in the lower panel of Fig. 4.

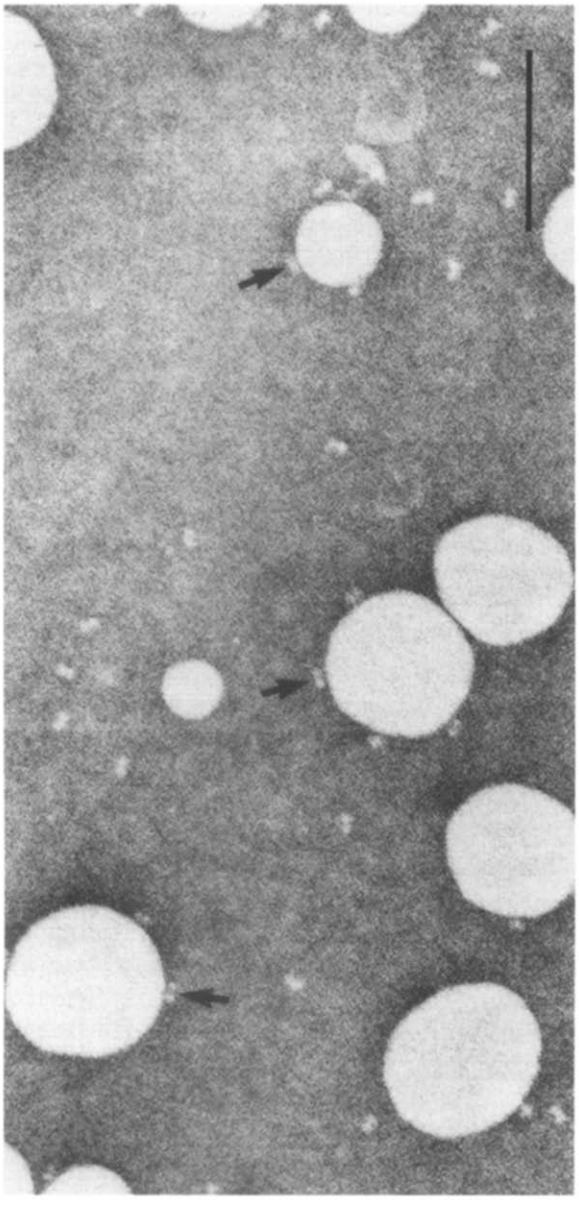

Fig. 3. Electron micrograph of negatively stained reconstituted dipeptidyl peptidase IV (egg phosphatidylcholine). Fraction 8 from Fig. 1b was used after removal of sucrose by dialysis. Scale bar 100 nm. Arrow indicates typical dumbell structures.

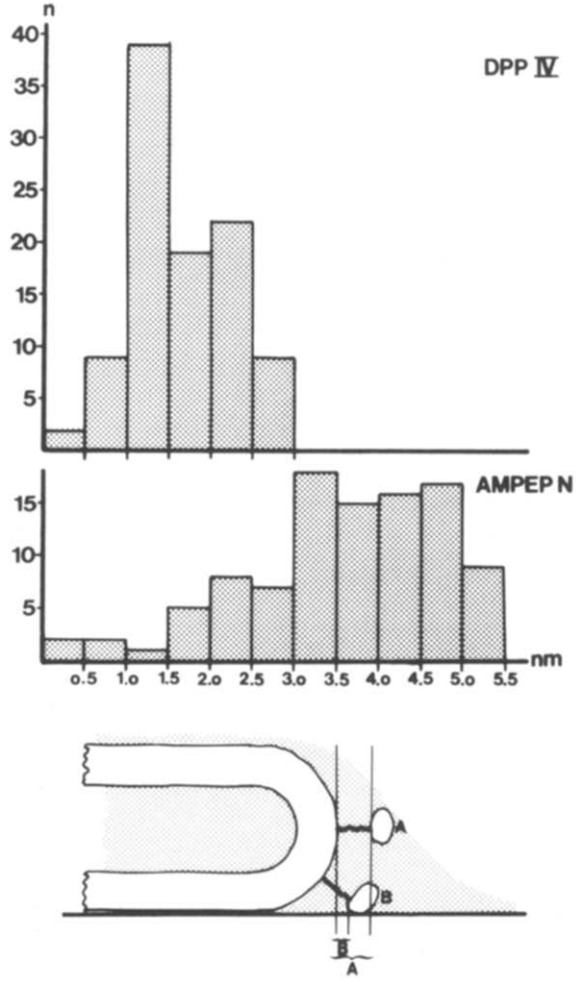

Fig. 4. Graphic presentation of 100 random measurements of gap widths between liposomal membranes and enzyme 'heads' of reconstituted dipeptidyl peptidase IV and of aminopeptidase N (data from Ref. 4). The width of the gaps is influenced by the angle of projection, as exemplified by positions A and B in the lower panel. Only the maximum widths can be taken to represent the full length of the junctional peptides. Details for the preparation of reconstituted aminopeptidase N are given in Ref. 4.

The distance between the hydrophilic part of the enzyme and the membrane is believed to be bridged by the hydrophilic part of the anchoring peptide called the junctional peptide [4]. The molecular weight of this peptide is calculated to be 700–1700 provided the secondary structure is either a fully stretched peptide or an α-helix. The hydrophobic part of the anchoring peptide when just spanning the membrane has a molecular weight of 1600–3500 (corresponding to a fully stretched peptide or an α-helix). The total molecular weight of the anchoring peptide (2300–5200) falls into the range of the molecular weight determined for the anchor of pig kidney dipeptidyl peptidase IV [12].

Calculating the volume, using a cylindrical model of the enzyme, the molecular weight of the amphiphilic dipeptidyl peptidase IV is about 200 000 assuming a partial specific volume of 0.7 $cm^3 \cdot g^{-1}$. This corresponds to an enzyme composed of two subunits each of a molecular weight of about 100 000. This molecular weight is in reasonable agreement with that obtained by SDS-polyacrylamide gel electrophoresis [2].

The membrane relations of dipeptidyl peptidase IV resemble those of aminopeptidase N [4] except for a difference in length of the junctional peptides (Fig. 4). Thus the microvillar peptidases appear to consist of a major hydrophilic domain and a minor hydrophobic root. These domains are connected through a hydrophilic junctional segment. The hydrophilic domain contains the active site of the enzymes, whereas the anchoring root has a clear function of immobilizing the enzymes to the membranes. The junctional segments are the preferred sites for attack by the proteolytic enzymes, during the conversion of the amphiphilic form of the enzyme into the hydrophilic form, and may be important for the in vivo degradation of these enzymes.

Kenny et al. [11] stated that the inability of papain to hydrolyse the microvillar endopeptidase from the microvillar lipid vesicles was due to its inability to reach the junctional segment (2.5 nm) of the endopeptidase. They argued that at least 4 nm space is required for papain, an ellipsoidal molecule with dimensions 5.0 × 3.7 × 3.7 nm [13]. In the present study dipeptidyl peptidase IV was released from the phosphatidylcholine and microvillar liposomal membranes by proteinase K but not very efficiently by trypsin. The molecular dimensions of trypsin and proteinase K are 6.8 × 5.9 × 5.5 [14] and 5.4 × 3.4 × 3.4 nm [15], respectively. Since the proteinase K has the smallest axis of 3.4 nm it needs at least 3.4 nm distance for it to attack between the hydrophilic domain and the anchoring root. The junctional segment of dipeptidyl peptidase IV is in this work shown to be 2.5 nm. The fact that proteinase K can release the enzyme from the liposomal membrane in spite of having a larger minimum dimension might ir
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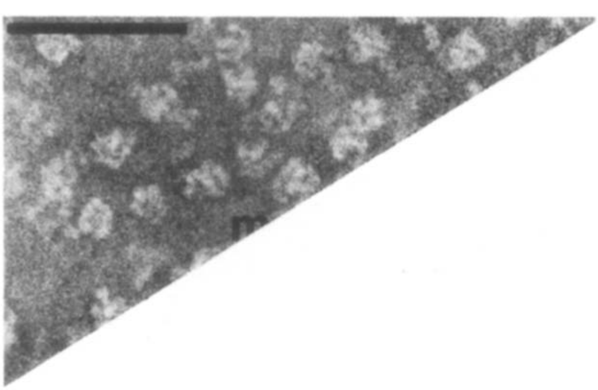

(about 5 nm) between the major hydrophilic domain and the membrane (Fig. 4).

Influence of lipids and detergents on the kinetic parameters

The influence of lipids and detergent on the kinetic parameters of amphiphilic and hydrophilic forms of aminopeptidase N and dipeptidyl peptidase IV was studied to obtain information on the importance of the anchoring peptide for the function of the enzymes. The influence of lipids was studied using reconstituted enzymes. To study the effects of detergents, it was essential to have detergent-free hydrophilic and amphiphilic forms of the enzymes. Detergent-free hydrophilic forms could be obtained by *R. communis* lectin chromatography. Detergent-free amphiphilic forms were obtained by centrifugation of purified enzymes in Triton X-100 into detergent-free sucrose gradients. By this procedure aminopeptidase N molecules became associated into regularly ordered rosettes of approx. 200 nm in diameter (Fig. 5a). In contrast, a majority of dipeptidyl peptidase IV molecules did not form ordered structures by this procedure, although some were seen (Fig. 5b).

Amphiphilic aminopeptidase N and dipeptidyl peptidase IV, either in detergent-free or detergent-containing solution or incorporated into liposomes, displayed Michaelis-Menten kinetics, as the correlation coefficient b_1 [8] was close to unity (range 0.93–1.05). This was also true for the hydrophilic forms. Triton X-100 (0.1%) or β-octylglucoside (30 mM) had no influence on the K_m values of the hydrophilic forms of dipeptidyl peptidase IV or aminopeptidase N. The K_m values determined for the detergent-free amphiphilic forms of the enzymes and for the reconstituted enzymes (in phosphatidylcholine liposomes) were both similar to that of the hydrophilic form. Likewise, no effect was observed on the V_{max} parameter. Thus it seems reasonable to conclude that the anchoring peptides do not have an important role in regulating the catalytic activity of intestinal aminopeptidase N and dipeptidyl peptidase IV.

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