Conformational Change of Rabbit Aminopeptidase N into Enterocyte Plasma Membrane Domains Analyzed by Flow Cytometry Fluorescence Energy Transfer

Jean-Pierre Gorvel, Zohair Mishal,* Fanny Liegey, Alain Rigal, and Suzanne Maroux

Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, BP 71, F 13402 Marseille Cedex 9; and *Centre Technique pour le soutien de la Recherche sur le Cancer, Service de Fluorimétrie, Centre National de la Recherche Scientifique, F 94800 Villejuif, France

Abstract. Membrane vesicle preparations are very appropriate material for studying the topology of glycoproteins integrated into specialized plasma membrane domains of polarized cells. Here we show that the flow cytometric measurement of fluorescence energy transfer used previously to study the relationship between surface components of isolated cells can be applied to membrane vesicles. The fluorescein and rhodamine derivatives of a monoclonal antibody (4H7.1) that recognized one common epitope of the rabbit and pig aminopeptidase N were used for probing the oligomerization and conformational states of the enzyme integrated into the brush border and basolateral membrane vesicles prepared from rabbit and pig enterocytes. The high fluorescent energy transfer observed in the case of pig enzyme integrated into both types of vesicles and in the case of the rabbit enzyme integrated into basolateral membrane vesicles agreed very well with the existence of a dimeric organization, which was directly demonstrated by cross-linking experiments. Although with the latter technique we observed that the rabbit aminopeptidase was also dimerized in the brush border membrane, no energy transfer was detected with the corresponding vesicles. This indicates that the relative positions of two associated monomers differ depending on whether the rabbit aminopeptidase is transiently integrated into the basolateral membrane or permanently integrated into the brush border membrane. Cross-linking of aminopeptidases solubilized by detergent and of their ectodomains liberated by trypsin showed that only interactions between anchor domains maintained the dimeric structure of rabbit enzyme whereas interactions between ectodomains also exist in the pig enzyme. This might explain why the noticeable change in the organization of the two ectodomains observed in the case of rabbit aminopeptidase N does not occur in the case of pig enzyme.

AMINOPEPTIDASE N is an ubiquitous ectoenzyme widely used as a specific marker of the apical domain of the plasma membrane of several epithelial cells and is particularly abundant in the brush border of the kidney and small intestine (22, 29). The entire amphiphilic molecule can be extracted from the membrane by neutral detergents, and the hydrophilic ectodomain can be solubilized by proteases (28). The corresponding d- and p-form from various species have been purified (22). Analysis of all these aminopeptidases by SDS-PAGE has shown that they contain only one type of subunit with a molecular weight of ~130,000, which in the pig can be split into two smaller (95,000- and 50,000-mol wt) subunits by limited proteolysis (3). Determination of the molecular weight of the native p-form of rabbit and pig enzymes by means of the Yphantis method showed that the former was a monomer (125,000 mol wt) (11) whereas the latter was a dimer (247,000 mol wt) (30). The dimeric state of the pig aminopeptidase N integrated into the membrane and after detergent extraction was confirmed by a cross-linking study (41). Electron microscopic studies on the d-form of pig aminopeptidase N reincorporated into liposomes have shown that it has a dimeric symmetrical structure of ~13.5 × 5.5 nm separated by a 5-nm gap from the membrane (19).

Debated for a long time, the presence of low amounts of aminopeptidase N into the basolateral domain of enterocytes was definitively proved by flow fluorometry analysis of basolateral membrane vesicles (32). Recently, we showed that the aminopeptidase N integrated into the basolateral domain was the newly synthesized enzyme en route to the brush border (31). The same transient integration of some apical glycoproteins into the basolateral domain was also observed in the hepatocytes (2). This suggest that the sorting of the basolateral and at least some apical glycoproteins occurred in the basolateral membrane where only basolateral markers stopped. The conformational state and oligomerization of the newly synthesized glycoproteins seem to be crucial for their transport through the various organelles involved in their

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synthesis and sorting (9, 23, 35, 37, 44). It is therefore of particular interest to analyze the subunit organization and conformational state of aminopeptidase both during its passage through the basolateral membrane and after its permanent integration into the brush border membrane.

Fluorescence resonance energy transfer between dyes bound to a single protein or to a protein complex has been a very useful means of obtaining information about intramolecular and intermolecular distance relationships (7, 10, 15, 40, 42, 43, 46). It was shown that flow cytometric measurements have several advantages over spectrofluorimetric measurements to study the topology and association of surface antigens of isolated cells (7, 10, 42, 43, 46). In epithelial cells, however, the presence on the plasma membrane of distinct, functionally specialized domains characterized by their unique protein composition is maintained by tight junctions between cells (38). Disruption of these junctions, when cells are isolated, is followed by a redistribution of proteins from different domains over the whole surface (12). In topological studies on the proteins integrated into the various plasma membrane domains of polarized cells from epithelia, it was necessary to use membrane vesicles obtained during tissue homogenization under conditions that prevented the breakage of tight junctions and the ensuing redistribution of proteins (32). Here we show that the method involving flow cytometric measurement of fluorescence energy transfer can be applied to membrane vesicles.

Fluorochromes coupled to monoclonal antibodies specific to one epitope of each subunit of a complex form suitable probes for determining the relative distance between these subunits (10, 42). Fluorescein and rhodamine dyes constitute an excellent donor–acceptor couple since the energy transfer from the excitated fluorescein to the rhodamine provides a "spectroscopic ruler" in the range of 1–10 nm (40, 46). In the present study, we used fluorescein and rhodamine derivatives of a monoclonal antibody (4H7.1) that recognizes the rabbit and pig aminopeptidases N for probing the proximity between two monomers of these enzymes, either integrated into brush border and basolateral membrane vesicles or solubilized by Triton X-100.

We have also investigated the subunit association of aminopeptidase N by chemical cross-linking using bifunctional reagents of different lengths (1, 6, 8, 23, 24, 27, 41).

Materials and Methods

Materials

Peroxidase-labeled anti-guinea pig immunoglobulins and peroxidase-labeled anti-mouse immunoglobulins were from Cappel Laboratories (Malvern, PA). FITC and TRITC were purchased from Sigma Chemical Co. (St. Louis, MO). Bifunctional reagents and Iodogen were from Pierce Chemical Co. (Rockford, IL). Na ^{125}I (carrier free) was obtained from Amersham Corp. (Arlington Heights, IL; 15 mCi/μg). Composition of PBS (pH 7.4) was 8.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, and 0.137 mM NaCl; that of buffer A (pH 7.4) was 20 mM Tris, 1 mM $MgCl_2$, 10 mM KCl, 0.14 M NaCl.

Membrane Vesicle Preparations

Brush border and basolateral membrane vesicles from rabbit and pig enterocytes were separated as described in the case of the rabbit (32). The final purification of basolateral membrane vesicles by immunoprecipitation was omitted because it was incompatible with fluorometry analysis, which can be applied only to free particle suspension. The flow fluorometry analysis of the rabbit basolateral membrane fraction used in the present study showed, however, that all the vesicles bearing accessible aminopeptidase were basolateral membrane vesicles (32).

Antisera, Immunoglobulins, and Monoclonal Antibodies

Immunoglobulins from goat anti-pig aminopeptidase serum and from guinea pig anti-α subunit of pig aminopeptidase serum were previously used and their specificities determined (3).

The mouse monoclonal antibody Cl 3.3 against human blood group A was a gift from Dr. Bara (IRSC, Villejuif) (18), and the mouse monoclonal antibody B9.12 against HLA class I used as control was a gift from Dr. Mawas (INSERM U119 Marseille).

Hybridomas producing the monoclonal antibodies studied in Table I were obtained by fusion of spleen cells (75×10^6) from two BALB/c mice immunized with pure p-form of rabbit aminopeptidase N (11).

The schedule of immunization of BALB/c mice was as follows (taking the day of fusion as day 0): on days 28 and 14, 100 μg of pure aminopeptidase in 0.2 ml of Freund's complete adjuvant were injected intraperitoneally; on day 3, mice received 100 μg of aminopeptidase injected intravenously.

Fusion was performed as previously described (17). After fusion the cells were distributed among 768 200-μl wells. Within 24 d, 85 hybridomas were tested. 24 hybridomas gave a positive dot blot reaction on 1 μg of pure aminopeptidase. These hybridomas were cloned by limiting dilution as previously described (17). Eight clones secreting the antibodies studied in Table I were selected. These antibodies, which are designated by the same name as the corresponding hybridoma, were purified as previously described (17). They were IgGs. When coupled to Ultrogel AcA 22 (45) they specifically bound aminopeptidase N present in a detergent extract of the brush border membrane (see Fig. 1).

Each antibody was labeled using ^{125}I and Iodogen as described (14).

Solubilization, Immunoprecipitaion of Aminopeptidase, and SDS-PAGE Analysis

Detergent extraction of aminopeptidase before or after cross-linking reaction, trypsin hydrolysis of the detergent extracts, and immunoprecipitation were performed as previously described (3, 13). Pig and rabbit aminopeptidases were immunoprecipitated by goat polyclonal antibodies against pure pig aminopeptidase and monoclonal antibody 1D8.1 coupled to Ultrogel AcA 22, respectively (45). The gel was washed and prepared for gel electrophoresis as previously described in the case of direct immunoprecipitates (13), except that DTT was generally not added.

Separation of cross-linked aminopeptidases was performed in slab gels (0.75 mm thick, 5 cm long) made by a 3.5–10% linear gradient of polyacrylamide with a 3.5% stacking gel as described by Blobel and Dobberstein (4). The immunoblotting procedure has been described elsewhere (13).

Cross-linking Experiments

Freshly prepared membrane vesicles in suspension in PBS buffer or detergent extracts before or after trypsin hydrolysis, containing 6 μg of aminopeptidase/ml (5×10^{-8} M); i.e., 0.2 and 4 mg of total proteins from brush border and basolateral membranes, respectively, were treated at 4°C for 1 h with dimethyl pimelimidate (6), dithiobis (succinimidyl-propionate) (27), or ethylene glyco bis (succinimidyl succinate) (1) at a final concentration of 0.25 mM. These bifunctional reagents were selected because of the differences in their reported bridge span sizes: 0.9, 1.2, and 1.7 nm for dimethyl pimelimidate, dithiobis (succinimidyl-propionate), and ethylene glyco bis (succinimidyl succinate), respectively (8). The excess of cross-linker was then inactivated by adding 10 mM glycine. The vesicles were centrifugated and the aminopeptidase was solubilized by detergent. Reaction mixtures of solubilized aminopeptidase were dialyzed against a large volume of PBS buffer for 6 h before aminopeptidase immunoprecipitation.

Conjugation of Antibodies with Fluorescein and Rhodamine

Monoclonal antibodies were conjugated with FITC or TRITC as described by Le Bouteiller et al. (25, 26). After extensive dialysis against PBS, the molar fluorochrome-to-protein ratios were 2.2, 2.5, 3.7, and 4.8 for FITC/4H7.1, TRITC/4H7.1, FITC/1D8.1, and TRITC/1D8.1, respectively.

Immunofluorescence Staining of Membrane Vesicles

The concentration of each of the conjugated antibodies permitting saturation of surface vesicles was determined by flow fluorometry analysis as previously described (32).

For flow cytometric energy transfer measurements 100 μl of freshly prepared brush border membrane fractions and basolateral membrane fractions containing 100 U of aminopeptidase N were either singly or doubly labeled by incubating them in the presence of 1% FCS with either 30 μg of one fluorescent antibody or with a mixture of 30 μg of one FITC and 30 μg of one TRITC derivative for 30 min at 4°C. The volume was brought up to 2 ml with buffer A containing 1% FCS, and the vesicles were injected into the flow system for analysis.

For spectrofluorometric energy transfer measurements, 250 μl of brush border membrane fractions and basolateral membrane fractions containing 225 U of aminopeptidase N were either simply or doubly labeled by incubating them in the presence of 1% FCS with either 50 μg of fluorescein-labeled (F)-4H7.1 plus 50 μg of unlabeled 4H7.1 or with 50 μg of F-4H7.1 plus 50 μg of rhodamine-labeled (R)-4H7.1 for 30 min at 4°C. The samples were loaded on 15% saccharose cushions (500 μl in Eppendorf tubes) and centrifuged at 15,000 rpm for 30 min at 4°C in a centrifuge (2 MK; Sigma Chemical Co.). The pellets were resuspended in either 1 ml of buffer A and homogenized in a Potter homogenizer or in 1 ml of buffer A containing 2% Triton X-100 and 0.1% SDS. The former vesicle suspensions were immediately used for spectrofluorometric analysis. After an overnight incubation of the latter samples at 4°C, the detergent extracts of membrane vesicles were obtained by centrifugation at 15,000 rpm for 30 min at 4°C in a centrifuge (2 MK; Sigma Chemical Co.).

Fluorescence Energy Transfer Experiments

In the fluorescein–rhodamine couple, the donor is the fluorescein (410 nm < excitation wavelength < 530 nm with maximum at 488 nm and 500 nm < emission wavelength < 600 nm with maximum at 530 nm) and the acceptor is the rhodamine (460 nm < excitation wavelength < 590 nm with maximum at 555 nm and 550 nm < emission wavelength < 650 nm with maximum at 590 nm) (7).

When resonance energy transfer occurred between a donor–acceptor pair, the following phenomena could be observed: quenching of the fluorescence emission of the donor molecule; sensitization of the emission of the acceptor; and a reduction in the anisotropy of the acceptor emission, which was dependent on the ratio of excitation through direct energy transfer from the donor to the acceptor. Because of the energy change correspondence requirement, the latter kind of process is sometimes referred to as a resonance energy transfer. The depolarization is caused by the partial randomization through the emission and reexcitation processes underlying the preferential orientation of excited chromophores initially produced by photoselection with linearly polarized light. The emission anisotropy (*EA*) is given by

$$EA = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}},$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities recorded when fluorescent light passes through polarizers oriented in parallel and perpendicularly, respectively, to the polarized excitation light. The emission anisotropies of singly and doubly labeled vesicles are calculated by computer from the measured $I_{\parallel}$ and $I_{\perp}$.

The three phenomena cited above, resulting from the energy transfer between fluorescein and rhodamine antibodies bound to membrane vesicles, were analyzed using a flow cytometer FACS 440 modified as previously described for measuring the emission intensities (20) and anisotropies (34) in each particle. To determine the fluorescence emission intensities, the labeled vesicles were excited sequentially at 488 and 514 nm, and fluorescence emissions were detected using suitable filter combinations. We detected fluorescence emissions at 530 and >590 nm. Four intensities were collected: the fluorescence at 530 and >590 nm when excited at 488 nm, the forward angle light-scatter signal, and the fluorescence emission >590 nm when excited at 514 nm. For fluorescence anisotropy measurements, the FACS was calibrated by means of half wave retardation plates (Oriel Corp., Stamford, CT). The excitation source was a laser (model 2025; Spectra-Physics, Mountain View, CA), at 514 nm with a power of 800 mW in the TEM_{00} mode. For rhodamine emission, a 590-nm band pass filter and a 580-nm cut off filter were used.

The quenching of the donor (F-4H7.1) fluorescence of doubly labeled vesicles and their detergent extract was measured using a spectrofluorometer (SFM 25; Kontron Analytical, Everett, MA) at 6°C as described by Schreiber et al. (36). The excitation wavelength was 488 nm and the emission wavelength was 530 nm. The slit was 10 nm for excitation and emission. Since under our experimental conditions both light scattering and the optical density at 488 nm varied in a linear relationship with the dilutions of the samples studied, the fluorescence values can be corrected for light scattering using the equation

$$F_{corr} = \frac{F_{obs}}{OD_{obs}} - \frac{F_o}{OD_o},$$

where F_{obs} and OD_{obs} are the fluorescence and optical density measured, respectively, in a given sample, and F_o and OD_o are the fluorescence and the optical density in an unlabeled sample. The fluorescence energy transfer was expressed as percent quenching (Q) of the donor fluorescence at 530 nm with

$$Q(\%) = \left[1 - \frac{F_{corr.FR}}{F_{corr.F}}\right] \times 100,$$

where $F_{corr.FR}$ is the corrected fluorescence value of the sample doubly labeled with a mixture of F-4H7.1 and R-4H7.1, and $F_{corr.F}$ is the value of the sample labeled with a mixture of F-4H7.1 and unlabeled 4H7.1.

Results

Cross-linking Experiments

Chemical cross-linkings with 0.25 mM dithiobis (succinimidyl-propionate), ethylene glyco bis (succinimidyl succinate), or dimethyl pimelimidate were performed on brush border and basolateral membrane vesicles, detergent extracts of the brush border membrane, and trypsin hydrolysates of these detergent extracts. The concentration of the total proteins (0.2 and 4 mg/ml in the case of brush border and basolateral membranes, respectively) was kept low to prevent nonspecific coupling. Free and cross-linked aminopeptidases were immunoprecipitated and analyzed by SDS-PAGE.

When cross-linking was performed with pure hemoglobin at 0.2–1 mg/ml, no more than 25–50% of the material recovered was dimerized, and very low amounts of tri- and tetramer were observed (1, 27). So, considering that aminopeptidase N represents only 6 and 0.15% of the total proteins from brush border and basolateral membranes, respectively, the relatively low amounts of specific cross-linking between two aminopeptidase N monomers observed in Fig. 1 can be considered as proof of the dimeric organization of the enzymes.

In the case of pig aminopeptidase N, membrane bound and both forms of solubilized pig enzyme (Fig. 1 *A*) gave the same pattern of cross-linked molecules. Since the molecular weight of the intact α subunit and that of the two polypeptides, β and γ, resulting from the α subunit proteolysis (3) was 140,000, 95,000, and 50,000, respectively, the molecular weight of the major cross-linked molecules (250,000, 225,000, and 200,000) might correspond to the association α_2, $\alpha\beta$, and β_2, respectively. These results agree very well with the existence of a dimeric structure that is maintained after solubilization by detergents or proteases.

As expected, no cross-linking of the p-form of rabbit aminopeptidase was obtained (Fig. 1 *B*, lane *5*). By contrast, the enzyme integrated into the plasma membrane (Fig. 1 *B*, lanes *2* and *3*) or solubilized by Triton X-100 (Fig. 1 *B*, lane *4*) gave a cross-linked molecule with a molecular weight corresponding to that of a dimer. The possibility that one aminopeptidase subunit might be associated with another glycoprotein can be ruled out since, after the cleavage of the

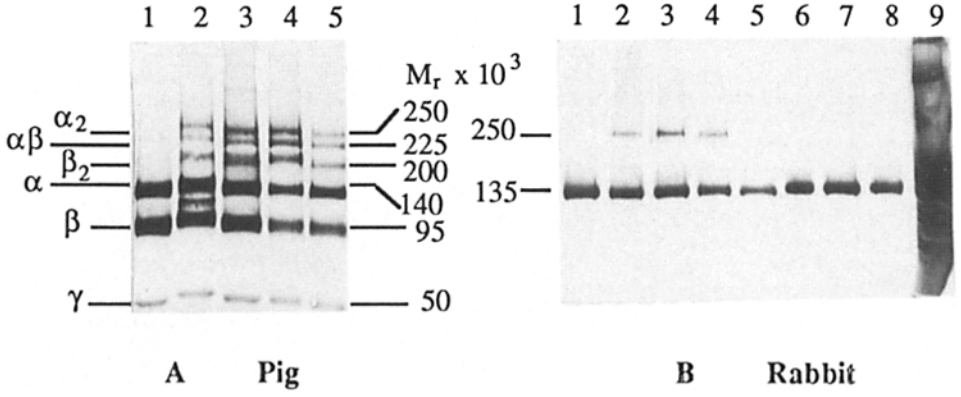

Figure 1. Cross-linking of pig (*A*) and rabbit (*B*) aminopeptidase N with dithiobis (succinimidyl-propionate). The reaction was performed with basolateral membrane vesicles (lanes *2* and *6*); brush border membrane vesicles (lanes *3* and *7*); or detergent extract of brush border membrane vesicles either before (lanes *4* and *8*) or after (lanes *5*) trypsin hydrolysis. Control experiments, where dithiobis (succinimidyl-propionate) was omitted, were performed with brush border membrane vesicles (lanes *1*). After cross-linking, 0.8 μg of aminopeptidase N were specifically immunoprecipitated and prepared for gel electrophoresis analysis (see Materials and Methods) either in the absence (lanes *1–5*) or in the presence (lanes *6–9*) of DTT. In lane *9* all the brush border membrane proteins were analyzed. Stainings were performed after transfer onto nitrocellulose with guinea pig antibodies against the α subunit of the pig aminopeptidase N in *A* and the monoclonal antibody CL3.3 against the human blood group A in *B*.

cross-linker by DTT (*27*) (Fig. 1 *B*, lanes *6–8*), no other glycoprotein was detected using anti-human blood group A antibodies which, as shown in lane *9* and in Gorvel et al. (16), revealed almost all the glycoproteins of the enterocyte plasma membrane.

It was previously shown by flow fluorometry analysis of the rabbit basolateral membrane fraction used in the present study (see Materials and Methods) that all the accessible aminopeptidase was integrated into basolateral membrane vesicles. However, this fraction also contained inaccessible aminopeptidase, located on the intravesicular side of Golgi membrane vesicles, that cannot be cross-linked. This explains why the efficiency of the cross-linking appeared to be lower in the case of the enzyme integrated into the basolateral membrane than in the case of the brush border aminopeptidase (Fig. 1 *A* and *B*, lanes *2* compared with lanes *3*).

Identical results were obtained using ethylene glyco bis (succinimidyl succinate) as a bifunctional reagent, whereas no cross-linked aminopeptidase was obtained with dimethyl pimelimidate.

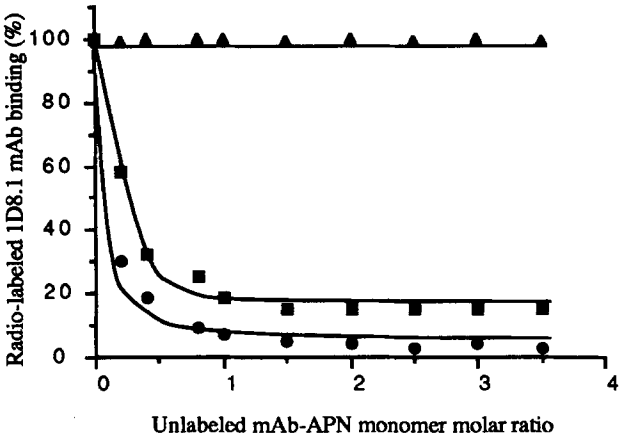

Figure 2. Competition between ^{125}I-1D8.1 monoclonal antibody (*mAb*) and unlabeled antibodies: 1D8.1 (●), 7H1.3 (■), and 4C11.1 (▲) for epitopes of rabbit aminopeptidase N (*APN*) integrated into brush border membrane vesicles.

Immunological Epitopes of Rabbit Aminopeptidase N Recognized by Monoclonal Antibodies

Competition of monoclonal antibodies for immunological epitopes on the surface of the aminopeptidase was studied. The concentration of antibodies required to saturate aminopeptidase integrated into brush border membrane was first determined by incubating 100 μl of vesicle suspension in PBS buffer containing 1 μg of enzyme for 1 h at 4°C with increasing amounts of each ^{125}I-labeled antibody. After centrifugation and washing, the vesicles were counted. Competition between unlabeled and labeled antibodies was then evaluated by determining the binding of ^{125}I-antibodies used at a concentration corresponding to the saturation on vesicles previously incubated with increasing amounts of unlabeled antibodies. Fig. 2 gives an example of the curves obtained. In each case 1 mol of an unlabeled monoclonal antibody/mol of rabbit aminopeptidase monomer was necessary and sufficient to obtain maximal inhibition of ^{125}I-labeled antibody binding. The following equation gives the value of the competition: 100 − 100 × (cpm in the presence of unlabeled antibody/cpm in the absence of unlabeled antibody). The results obtained are given in Table I. They show that the eight

Table I. Competition between the Different Monoclonal Antibodies against Rabbit Aminopeptidase N

	mAb							
^{125}I-mAb	4H7.1	1D8.1	7H1.3	4C11.1	3H1.4	4H11.1	2D12.1	3G4.1
4H7.1	100	0	10	0	5	0	0	0
1D8.1	20	97	85	0	0	5	0	9
7H1.3	30	63	75	0	0	0	0	13
4C11.1	0	7	0	100	70	26	0	0
3H1.4	0	0	0	93	94	0	14	0
4H11.1	20	7	0	6	25	90	74	68
2D12.1	31	34	20	35	9	80	94	89
3G4.1	23	22	43	26	34	80	85	90

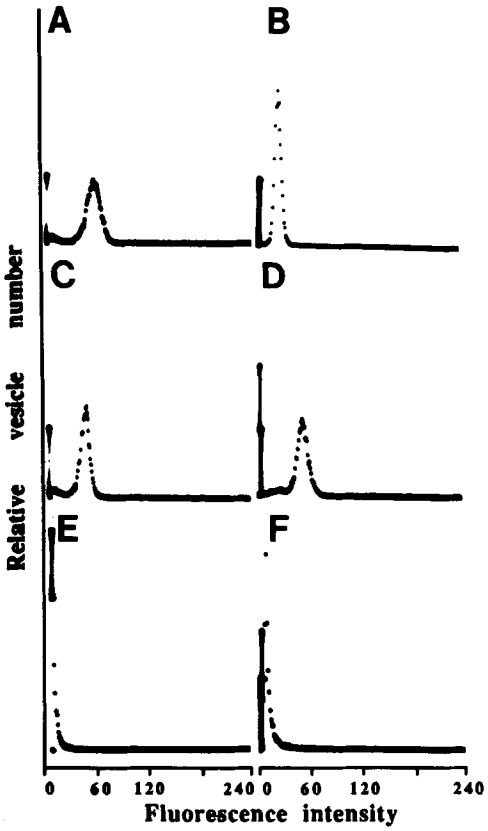

Figure 3. Frequency distribution of emission intensities of rabbit brush border membrane vesicles labeled with fluorescent anti-aminopeptidase monoclonal antibodies: (*A*) labeling with the monoclonal antibody 1D8.1 coupled with fluorescein; (*B*) labeling with the monoclonal antibody 4H7.1 coupled with rhodamine; (*C* and *D*) labeling with equimolar mixture of both antibodies. A control experiment was performed with the monoclonal antibody B9.12 against HLA class I coupled with either fluorescein (*E*) or rhodamine (*F*). After excitation at 488 nm the emission intensities were detected at 530 nm in *A*, *C*, and *E* and >590 nm in *B*, *D*, and *F*. Approximately 5,000 vesicles were analyzed per distribution.

antibodies can be classified in four groups that recognize four different epitopes at the surface of aminopeptidase N. One group contained only 4H7.1 antibody, which was found to cross react with the pig aminopeptidase. One group contained 1D8.1 and 7H1.3, which inhibited the enzymic activity of the aminopeptidase N. The other two groups consisted of 4C11.1 and 3H1.4, and 4H11.1, 2D12.1, and 3G4.1 antibodies.

Flow Cytometric Measurements of Fluorescence Energy Transfer

In a preliminary experiment, the fluorescence energy transfer between fluorescein and rhodamine coupled to two monoclonal antibodies that can bind simultaneously onto two different epitopes of rabbit aminopeptidase monomer was analyzed. Rabbit membrane vesicles were singly labeled with either F-1D8.1 or R-4H7.1 and doubly labeled with both F-1D8.1 and R-4H7.1. After excitation either at 488 nm (maximal excitation of fluorescein) or at 514 nm (low excitation of fluorescein and rhodamine), the fluorescence emission at 530 nm (maximum of fluorescein emission) and/or 590 nm (maximum of rhodamine emission) of the singly and doubly labeled vesicles were analyzed. Patterns of fluorescent vesicles (Fig. 3) and their corresponding maximum emission intensities (Table II) were given by the computer. The differences in patterns distribution of emission intensities between the singly or doubly labeled vesicles which can be observed in Fig. 3 and Table II, respectively, are typical of an energy transfer between the donor–acceptor couple of doubly labeled vesicles: the intensity of the fluorescence emission of the donor (fluorescein) decreased and the intensity of the emission of the acceptor (rhodamine) increased (7).

When the same experiment was performed with pig membrane vesicles (Table II), no energy transfer was observed since only the antibody 4H7.1 recognized pig aminopeptidase N. This constitutes an efficient negative control.

For probing the proximity between two monomers of the aminopeptidase, we doubly labeled pig and rabbit membrane vesicles with the fluorescein and rhodamine derivatives of 4H7.1. The results of the flow cytometric analysis of emission intensities are given in Table III. The decrease in fluorescein emission (I_{530}) when excited at 488 nm and the increase in the rhodamine emission (I_{590}) when excited at either 488 nm or 514 nm showed that an energy transfer took place from fluorescein to rhodamine in the case of doubly labeled pig brush border and basolateral membrane vesicles and rabbit basolateral membrane vesicles. By contrast, no difference was noted between singly and doubly labeled rabbit brush border vesicles. Antibodies specific to three other epitopes of the rabbit aminopeptidase—1D8.1, 2D12.1, and 4C11.1—were used in the same type of experiment with rabbit membrane vesicles. 4C11.1 gave the same results as 4H7.1: energy transfer was obtained with basolateral membrane but not with brush border membrane (data not shown). In the case of 1D8.1 (Table III) and 2D12.1 (data not shown), no energy transfer occurred either in brush border or basolateral membrane vesicles.

Table II. Fluorescence Intensities of Brush Border and Basolateral Membrane Vesicles from Pig and Rabbit Enterocytes Labeled with Fluorescent Monoclonal Antibodies against Rabbit Aminopeptidase N

			488-nm excitation		514-nm excitation
Membrane vesicles		Label	I_{530}*	I_{590}	I_{590}
Rabbit	BB‡	F-1D8.1§	54	11	0
		R-4H7.1	0	22	50
		F-1D81 + R-4H7.1	36	49	69
	BL	F-1D8.1	47	6	0
		R-4H7.1	0	6	31
		F-1D81 + R-4H7.1	20	37	42
Pig	BB	F-1D8.1	0	0	0
		R-4H7.1	0	22	39
		F-1D8.1 + R-4H7.1	0	22	35
	BL	F-1D8.1	0	0	0
		R-4H7.1	0	10	30
		F-1D8.1 + R-4H7.1	0	8	32

* I_{530} and I_{590} are the recorded emission intensities (see Material and Methods).
‡ *BB*, brush border; *BL*, basolateral.
§ F, fluorescein-labeled antibody; R, rhodamine-labeled antibody.

Table III. Fluorescence Intensities of Brush Border and Basolateral Membrane Vesicles from Pig and Rabbit Enterocytes Labeled with Fluorescent Monoclonal Antibodies against Rabbit Aminopeptidase N

Membrane vesicles		Label	488-nm excitation		514-nm excitation
			I_{530}*	I_{590}	I_{590}
Pig	BB‡	F-4H7.1§	30	0	0
		R-4H7.1	0	22	39
		F-4H7.1 + R-4H7.1	20	31	58
	BL	F-4H7.1	14	0	0
		R-4H7.1	0	10	30
		F-4H7.1 + R-4H7.1	10	33	53
Rabbit	BB	F-4H7.1	23	0	0
		R-4H7.1	0	22	50
		F-4H7.1 + R-4H7.1	25	24	52
	BL	F-4H7.1	14	0	0
		R-4H7.1	0	6	31
		F-4H7.1 + R-4H7.1	10	24	41
	BB	F-1D8.1	54	11	0
		R-1D8.1	0	21	58
		F-1D8.1 + R-1D8.1	54	22	57
	BL	F-1D8.1	47	6	0
		R-1D8.1	0	12	40
		F-1D8.1 + R-1D8.1	43	15	41

* I_{530} and I_{590} are the recorded emission intensities (see Material and Methods).
‡ *BB*, brush border; *BL*, basolateral.
§ F, fluorescein-labeled antibody; R, rhodamine-labeled antibody.

In a separate experiment, we doubly labeled pig and rabbit membrane vesicles with the fluorescein and rhodamine derivatives of 4H7.1 and analyzed the emission anisotropy. Fig. 4 shows that a significant shift took place in the fluorescence anisotropy of rhodamine with doubly labeled pig brush border and basolateral membrane vesicles (Fig. 4, *A* and *B*) and rabbit basolateral membrane vesicles (Fig. 4 *D*) whereas no difference was to be seen between singly and doubly labeled rabbit brush border vesicles (Fig. 4 *C*).

Spectrofluorometric Measurements of Fluorescence Energy Transfer

The spectrofluorometric method was used to study the effect of detergent extraction on the dimeric conformation of aminopeptidases. Pig and rabbit membrane vesicles were doubly labeled with fluorescein and rhodamine derivatives of 4H7.1. Quenching of the donor fluorescence (*Q*) (see Materials and Methods) was determined before and after treating vesicles with detergent. Table IV gives the results obtained: after extraction by detergent, fluorescence energy transfer occurred in the case of pig aminopeptidase but not in the case of rabbit aminopeptidase extracted from both types of membranes.

The data obtained with membrane vesicles agreed very well with those obtained with the flow cytofluorometric method. It is worth noting, however, that the quenching values determined in the case of pig and rabbit basolateral membrane vesicles were lower than those of the pig brush border vesicles. This was probably due to the higher amounts of material present in the basolateral membrane vesicle samples (15 times more than in the brush border membrane vesicle samples) that perturbed the measurements by greatly increasing the light scattering (5–6 times). This disturbance was avoided with the flow fluorocytometric method (43) where the efficiency of the fluorescence energy transfer as indicated by the sensitization of the acceptor (Table III) and the reduction in the anisotropy of the acceptor emission (Fig. 4) was very similar with all the three types of vesicles.

Discussion

Aminopeptidases N are ectoenzymes composed of only one type of subunit anchored in the membrane by its NH_2-terminal sequence of ~20 hydrophobic residues. This membrane-spanning hydrophobic domain is followed by a hydrophilic sequence of ~15 hydrophilic residues, emerging from the lipid bilayer and forming the "stalk" on which the hydrophilic globular domain of the molecule is set (29). Proteolysis of a peptide bond at the junction between the stalk and the globular domain or along the stalk generates the hydrophilic p-form of the enzyme and the anchoring domain (3, 11, 22, 28, 29).

The cross-linking experiments described here show that pig and rabbit aminopeptidases are polymerized in the membrane and that their polymeric organization is maintained after detergent extraction. After protease solubilization, as

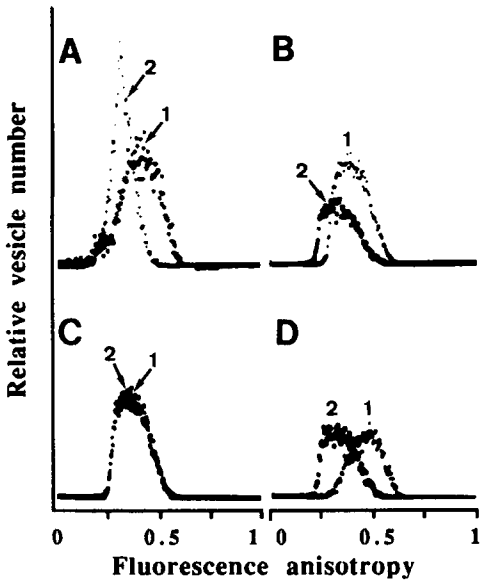

Figure 4. Frequency distribution of emission anisotropies of membrane vesicles labeled with a fluorescent monoclonal antibody (4H7.1) directed against one epitope of a monomer of rabbit and pig aminopeptidase N, measured with the flow cytometer. (*A*) Pig brush border membrane vesicles; (*B*) pig basolateral plasma membranes vesicles; (*C*) rabbit brush border membrane vesicles; and (*D*) rabbit basolateral membrane vesicles were singly labeled with rhodamine-4H7.1 (peaks *1*) or doubly labeled with equimolar mixture of R-4H7.1 and F-4H7.1 (peaks *2*). Vesicles were analyzed as they passed through the polarized beam of the laser by simultaneous detection of the apparent fluorescence intensity (>590 nm) in parallel and perpendicular planes, respectively, to the polarization plane of the excitation light (514 nm). Emission anisotropies were calculated from the equation given in Materials and Methods.

Table IV. Donor Fluorescence Quenching of Brush Border and Basolateral Membrane Vesicles from Pig and Rabbit Enterocytes Doubly Labeled with Fluorescein and Rhodamine Derivatives of the Monoclonal Antibody 4H7.1 before and after Detergent Extraction

		Quenching	
		Membrane vesicles	Detergent extracts
		%	%
Pig	BB*	51.5	56
	BL	13.1	31
Rabbit	BB	−0.7	−0.2
	BL	15	−2

* *BB*, brush border; *BL*, basolateral.

previously established by molecular weight determinations (3, 11, 30), the pig enzyme still behaves like a dimer, whereas the rabbit enzyme behaves like a monomer. These results strongly suggest that in rabbit aminopeptidase the interaction between two monomers occurs only between anchoring domains whereas in the pig enzyme interactions between globular domains also take place.

The cross-linking experiments did not reveal any differences in monomer association in the aminopeptidases depending on whether they were integrated into the brush border or into the basolateral membrane.

However, when the relative position of two monomers was investigated with fluorescence energy transfer by labeling monomers with fluorescein and rhodamine derivatives of a monoclonal antibody (4H7.1) that specifically bound to one epitope of aminopeptidase monomers, a difference in the monomer association of integrated aminopeptidase was observed between brush border and basolateral membranes in the case of the rabbit. In basolateral vesicles, the distance between two (4H7.1) epitopes was short enough (<10 nm) for energy transfer to take place between the two fluorochromes, whereas in brush border vesicles, this distance was too great (>10 nm) for energy transfer to occur. During detergent extraction, this difference disappeared. The enzymes extracted from both types of membranes behave like the enzyme integrated into the brush border membrane. The stalked structure of each aminopeptidase N monomer (19, 29) gives their globular ectodomain a large degree of freedom. In the absence of any strong interactions between the two ectodomains of a dimer, as occurs in the case of the rabbit enzyme (see above), they can easily move so that they remain in contact when the distance between two epitopes changes. Our results show that in the basolateral membrane some constraints must exist that maintain the two ectodomains of a dimer in a transient association so that the distance between the 4H7.1 epitopes is <10 nm. These constraints are lost either during detergent extraction or when the enzyme reaches the brush border membrane. The distance between the 4H7.1 epitopes then becomes >10 nm. No such change occurs, or if it does, it is less noticeable, in the case of pig aminopeptidase, since energy transfer occurs in pig brush border vesicles as well as in the basolateral membrane vesicles and after detergent extraction. The transitional conformation of the rabbit enzyme in basolateral membrane might be similar to the dimeric conformation of the pig enzyme that, in this species, is definitively stabilized by interactions between globular domains. This dimeric organization might be decisive for the intracellular transport and sorting of the aminopeptidase N from the endoplasmic reticulum to the brush border via the basolateral domain (9, 23, 35, 37, 44). It might be initiated and/or stabilized by a transient association with another constituent of the intracellular and basolateral membranes as has been described in other systems such as histocompatibility class II antigens (21), lysosomal enzymes (33), secreted IgA (39), and immunoglobulins (5).

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