Solubilization of the ileal receptor for intrinsic factor–vitamin B-12 complex by digestion with papain

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Brush-border-membrane vesicles isolated from hamster ileum were incubated with either papain or Pronase P and subsequently centrifuged to obtain soluble (supernatant) and insoluble (pellet) fractions. Papain (4 units/ml) solubilized 95–100% of the sucrase and leucine naphthylamide-hydrolysing activities but only 30% of the alkaline phosphatase. Digestion with papain also resulted in the solubilization of more than 75% of the ileal receptor for intrinsic factor–vitamin B-12 complex with a corresponding decrease in receptor activity in the pellet. Essentially 100% of the receptor activity was recovered. In contrast, digestion with Pronase P resulted in a decrease in total receptor activity. Papain-solubilized receptor was not sedimented by centrifugation at 105 000g for 90 min and was eluted in the included volume of Sepharose 6B. Like the binding to more intact preparations, binding of intrinsic factor–vitamin B-12 complex to papain-solubilized receptor was rapid, reaching 50% of maximum in 8 min, and required Ca^{2+} . Although Mg^{2+} could not completely substitute for Ca^{2+} , Mg^{2+} did stimulate Ca^{2+} -dependent binding at low Ca^{2+} concentrations. These results demonstrate that the ileal receptor for intrinsic factor–vitamin B-12 complex can be solubilized with papain, and suggest that papain solubilization may be a useful first step in the isolation and purification of this receptor.

Efficient intestinal absorption of vitamin B-12 (B-12) requires that the vitamin first be complexed with intrinsic factor (IF), a glycoprotein secreted by gastric parietal cells (Wilson & Strauss, 1959; Cooper, 1964). A second requirement for efficient absorption is the binding of IF–B-12 complex to a specific receptor localized in the distal portion of the small intestine, the ileum (Herbert & Castle, 1961; Wilson, 1963; Donaldson *et al.*, 1967). Binding of IF–B-12 complex to its receptor, which is located on the brush-border plasma membrane of ileal enterocytes (Donaldson *et al.*, 1967), requires Ca^{2+} (Mackenzie & Donaldson, 1972). Furthermore, when the availability of Ca^{2+} is limited, Mg^{2+} can enhance Ca^{2+} -dependent binding of IF–B-12 complex to its receptor (Beesley & Bacheller, 1980).

A number of investigators have utilized mechanical shearing forces (Cotter & Rothenberg, 1976; Okuda & Fujii, 1977) and/or detergent solutions

(Katz & Cooper, 1974; Schneider *et al.*, 1974; Marcoullis & Gräsbeck, 1977) to solubilize the ileal IF–B-12-complex receptor from several species. In the present investigation I utilized another technique, digestion with proteolytic enzymes, which has been successfully employed in solubilization of a variety of brush-border enzymes (Dahlqvist, 1963; Auricchio *et al.*, 1963; Maestracci, 1976). As reported below, digestion of ileal brush-border-membrane vesicles with papain resulted in solubilization of more than 75% of the ileal IF–B-12-complex receptor with essentially 100% recovery.

Methods and materials

Brush-border-membrane vesicles were prepared essentially as previously described (Beesley & Faust, 1979). In brief, intact brush borders were suspended in a solution containing 300 mM-Mannitol, 10 mM- MgCl_2 , 2 mM- CaCl_2 and 20 mM-Hepes/NaOH buffer, pH 7.4, and disrupted by freezing at -78°C followed by thawing at 37°C . The disrupted brush borders were then centrifuged at 3000g for 10 min. The pellet was resuspended, again subjected to the freeze–thaw procedure, and centrifuged at 3000g for

Abbreviations used: IF, intrinsic factor; B-12, vitamin B-12; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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10 min. The pellet was discarded, and the two 3000g supernatants were combined, again centrifuged at 3000g for 10 min to remove residual debris and finally centrifuged at 48000g for 60 min to obtain brush-border-membrane vesicles.

Digestion of brush-border-membrane vesicles and binding of IF-B-12 complex were accomplished by using the following procedures. Vesicles (approx. 0.25 mg of protein) were suspended in 0.05 ml of a solution containing 300 mM-mannitol, 2.5 mM-cysteine, 20 mM-Hepes/NaOH buffer, pH 7.4, and various concentrations of papain (EC 3.4.22.2). After 30 min at room temperature 0.45 ml of incubation medium was added to give the following final concentrations: 300 mM-mannitol, 0.25 mM-cysteine, 1 mM-CaCl₂, 2 mM-MgCl₂, 20 mM-Hepes/NaOH buffer, pH 7.4, and 450 pg of [⁵⁷Co]B-12 (as IF-B-12 complex)/ml. After 45 min at room temperature, 0.25 ml of a solution containing 300 mM-mannitol, 1 mM-CaCl₂, 2 mM-MgCl₂ and 20 mM-Hepes/NaOH buffer, pH 7.4, was added, and the samples were immediately centrifuged at 40000g for 1 h. The supernatant was removed, and the pellet was rinsed gently and its radioactivity counted. The supernatant and rinse were combined and filtered through Millipore filters with pore diameters of 0.45 μ m. The filters were washed twice with 2 ml of medium containing 300 mM-mannitol, 1 mM-CaCl₂, 2 mM-MgCl₂ and 20 mM-Hepes/NaOH buffer, pH 7.4, and their radioactivities were counted. Digestion with Pronase P and subsequent binding of B-12 was accomplished as described above with the exception that cysteine was not present.

To measure the time course of IF-B-12-complex binding, brush-border-membrane vesicles were digested as described above and centrifuged at 40000g for 1 h. The resultant supernatant was incubated with IF-B-12 complex as described above. Binding was terminated by filtration through Millipore filters.

Both IF and IF-B-12 complex were prepared as previously described (Beesley & Bacheller, 1980). Sucrase (EC 3.2.1.48), alkaline phosphatase (EC 3.1.3.1) and leucine naphthylamide-hydrolysing activities were measured essentially as described by Dahlqvist (1964), Eichholz (1967) and Goldberg & Rutenberg (1958) respectively. Chromatography was performed on a 1.5 cm $\times$ 40 cm column eluted with medium containing 300 mM-mannitol, 1 mM-CaCl₂, 2 mM-MgCl₂ and 20 mM-Hepes/NaOH buffer, pH 7.4.

Mannitol used in the present study was deionized by passage through the mixed-bed ion-exchange resin AG 501-X8D (Bio-Rad Laboratories, Richmond, CA, U.S.A.). [⁵⁷Co]B-12 (as cyanocobalamin) was purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.) and had a specific radioactivity of 220 μ Ci/ μ g. Papain (type IV;

19 units/mg) and Pronase P (protease type VI; 5 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Digestion of ileal vesicles with papain resulted in the release of several brush-border enzymes from these membranes. The activities of three of the enzymes present in the supernatant, after digestion for 30 min with various concentrations of papain and subsequent centrifugation, are shown in Fig. 1. Sucrase and leucine naphthylamidase were readily solubilized. Thus, after digestion with 200 μ g of papain/ml, essentially 100% of each of these activities was recovered in the supernatant. Similar treatment resulted in release of less than 40% of the alkaline phosphatase. The lower alkaline phosphatase activity in the supernatant was not simply due to inactivation of this enzyme since, when both supernatant and pellet were assayed, 80–90% of the activity could be recovered.

Digestion of ileal vesicles with papain also resulted in release of a substance that bound B-12. The amounts of B-12 bound in the supernatant and pellet after digestion of ileal vesicles with various concentrations of papain are shown in Fig. 2. Even in the absence of papain, incubation of ileal vesicles at room temperature for 30 min resulted in an apparent solubilization of as much as 25% of the

Fig. 1. Activities of several enzymes in the supernatant after digestion of ileal vesicles with papain

For experimental details see the text. Activities are expressed as the percentages of total activity (supernatant plus pellet) measured before digestion. ●, Sucrase; ▲, leucine naphthylamidase; ■, alkaline phosphatase. Values are means $\pm$ s.d. for four determinations.

Fig. 2. B-12 binding to the pellet and supernatant after digestion of ileal vesicles with papain

For experimental details see the text. ●, Pellet; ○, supernatant; ■, total. Values are the means $\pm$ s.d. for four or five determinations.

B-12 binder. The amount of solubilization of the binder in the absence of papain varied in different preparations and may have reflected variable contamination of the preparation with proteolytic enzymes. Nevertheless, digestion with papain resulted in an increase of B-12 binding in the supernatant and a reciprocal decrease of binding in the pellet. At the highest papain concentration tested (200 μ g/ml) approx. 77% of the original binding capacity was in the supernatant, whereas only 21% remained in the pellet. Centrifugation of the supernatant at 105 000 g for 90 min failed to sediment the B-12 binder. Papain apparently did not damage the B-12 binder, since more than 95% of the binding activity could be recovered. Supernatant obtained after papain digestion of jejunal brush-border-membrane vesicles did not bind significant amounts of B-12.

Ileal brush-border-membrane vesicles were also digested with Pronase P. Digestion with Pronase P resulted in a small increase of B-12 binding in the supernatant and a drastic decrease in the amount of B-12 bound by the pellet. At the highest Pronase P concentration used (200 μ g/ml) approx. 29% of the original binding capacity was in the supernatant and only 7% in the pellet. Incubation of IF–B-12 complex with Pronase P before addition of vesicles did not decrease binding. Thus the low total recovery (36%) of B-12-binding capacity indicates that Pronase P inactivated the B-12-binder.

The time course for uptake of B-12 by papain-solubilized B-12 binder is shown in Fig. 3. When IF was present, binding was rapid, reaching 50% of maximum in less than 8 min. Replacement of active

Fig. 3. Time course of B-12 binding to supernatant obtained by digestion of ileal vesicles with papain

For experimental details see the text. B-12 binding in the presence of active IF (●) and IF inactivated by boiling (○) are shown. Values are means $\pm$ s.d. for four determinations (●) or the means of two determinations (○).

Table 1. Effects of Ca^{2+} , Mg^{2+} and heat treatment on binding of B-12 to supernatant obtained by digestion of ileal vesicles with papain

Binding was performed in incubation media that contained 2 mM- MgCl_2 , 1 mM- CaCl_2 or 1 mM- CaCl_2 plus 2 mM- MgCl_2 . The amount of B-12 bound by supernatant that had been heated at 90°C for 10 min (heat-treated) is also given. Values are the means $\pm$ s.d. for three determinations.

Addition(s)	B-12 bound (pg)
MgCl_2	0.8 ± 0.3
CaCl_2	12.0 ± 1.0
$\text{MgCl}_2 + \text{CaCl}_2$	20.6 ± 2.3
$\text{MgCl}_2 + \text{CaCl}_2$ (heat-treated)	0.9 ± 0.8

IF with IF inactivated by boiling essentially abolished binding.

As shown in Table 1, binding of B-12 to papain-solubilized B-12 binder was dependent on Ca^{2+} . Substitution of Mg^{2+} for Ca^{2+} decreased binding to less than 10% of that observed with Ca^{2+} . Although Mg^{2+} itself did not support binding of B-12, it did stimulate Ca^{2+} -dependent binding. Thus binding with 1 mM- Ca^{2+} plus 2 mM- Mg^{2+} was approx. 1.7 times that observed with 1 mM- Ca^{2+} alone. Table 1 also shows that heating the papain-solubilized B-12 binder at 90°C for 10 min completely abolished its activity.

The papain-solubilized B-12 binder was complexed with IF–B-12 and chromatographed on

Sephacryl S200 (exclusion limit mol.wt. 4×10^6) and Sepharose 6B (exclusion limit mol.wt. 2.5×10^5). The B-12 binder was eluted in the excluded volume of Sephacryl S200 and in the included volume of Sepharose 6B a little before thyroglobulin, suggesting a molecular weight of about 1×10^6 . Chromatography in the presence of 0.05% Triton X-100 did not alter the elution volume of the solubilized B-12 binder. The B-12 binder was quantitatively retained by Millipore filters with pore diameters of $0.45 \mu\text{m}$, but was not retained by Uni-Pore or Nucleopore filters with pore diameters of $0.4 \mu\text{m}$ and $0.2 \mu\text{m}$ respectively.

Discussion

Digestion of ileal brush-border-membrane vesicles with papain resulted in the release of a substance that bound B-12. The appearance of a B-12 binder in the 40000g supernatant was not simply due to non-specific disruption of the vesicles into particles too small to be sedimented at the centrifugal forces employed. If the vesicles had simply been disrupted, one would expect that the release patterns of all brush-border enzymes would be similar. To the contrary, digestion with papain released more than 95% of the sucrase and leucine naphthylamidase activities but only 38% of the alkaline phosphatase. Similar differential release of enzymes from jejunal brush borders digested with various proteolytic enzymes has been reported by Maestracci (1976), who also demonstrated that these membrane vesicles remained intact after digestion with papain.

The B-12 binder released by digestion with papain fulfils the major criteria for solubility. Thus the B-12 binder was not sedimented after centrifugation at 105000g for 90min and was eluted in the included volume of Sepharose 6B. The apparent molecular weight of the binder was about 1×10^6 , a value that is somewhat less than that reported by Marcoullis & Gräsbeck (1977) for the most abundant B-12 binder isolated from pig ileum. In spite of the relatively large size of the B-12 binder, it was surprising that this material was quantitatively retained by Millipore filters with pore diameters of $0.45 \mu\text{m}$. Retention of the binder was apparently due to adsorption on these cellulose filters, since the B-12 binder was not retained by polycarbonate Uni-Pore and Nucleopore filters with pore diameters of 0.4 and $0.2 \mu\text{m}$ respectively.

The characteristics of B-12 binding to the solubilized B-12 binder conform to those previously observed by ourselves (Beesley & Bacheller, 1980) and other investigators (Wilson & Strauss, 1959; Herbert & Castle, 1961; Donaldson *et al.*, 1967; Mackenzie & Donaldson, 1972) with more intact preparations. Thus binding of B-12 was dependent on IF, had a time course similar to that observed

with ileal vesicles (Beesley & Bacheller, 1980) and required Ca^{2+} . Furthermore, as was originally demonstrated with ileal brush borders and membrane vesicles (Beesley & Bacheller, 1980), Mg^{2+} stimulated Ca^{2+} -dependent binding when the availability of Ca^{2+} was limited. It therefore seems apparent that this solubilized B-12 binder is the ileal receptor for IF-B-12 complex. In addition, the finding that the solubilized B-12 receptor was inactivated by heating and by digestion with Pronase P suggests that the receptor possesses an essential proteic component.

A variety of procedures, including mechanical shearing (Cotter & Rothenberg, 1971) and alkaline buffer (Okuda & Fujii, 1977), have previously been utilized to solubilize the ileal receptor for IF-B-12 complex in the absence of detergent. Although some of these methods have been successful, data for the percentage of the receptor solubilized (yield) and percentage recovery were either lacking or difficult to interpret. The non-ionic detergent Triton X-100 has also been utilized for solubilization of the ileal IF-B-12-complex receptor (Katz & Cooper, 1974; Marcoullis & Gräsbeck, 1977), and yields as high as 93% have been reported. By using papain digestion of ileal brush-border-membrane vesicles I have achieved yields of at least 75% with essentially 100% recovery. Since this digestion and solubilization does not require detergent, the disadvantages inherent in the use of detergents, such as inhibition of binding (Schneider *et al.*, 1974) and false apparent receptor activity due to binding of B-12 to detergent micelles (Marcoullis & Gräsbeck, 1977), are avoided.

Recently, while this manuscript was in preparation, Kouvonen & Gräsbeck (1981) reported the solubilization of the receptor for IF-B-12 complex by papain digestion of particulates from pig ileal mucosae. When their papain-solubilized receptor was chromatographed on Sephadex G-200 in the presence of 0.05% Triton X-100, the receptor-IF-B-12 complex was eluted in the region of serum transcobalamin I, indicating that the molecular weight of the receptor was less than 1×10^5 . This is in contrast with the receptor investigated in the present study, which has a molecular weight of about 1×10^6 even in the presence of Triton X-100. The reason for this difference in molecular weights is not clear, but may be related to differences in digestion procedures (24 h at 4°C versus 30 min at room temperature) or different sources of starting material (pig ileal particulates versus hamster ileal brush-border-membrane vesicles).

Solubilization of the ileal receptor for IF-B-12 complex by digestion with papain should provide a useful first step in attempts to isolate and purify this receptor. In addition, comparison of papain-solubilized receptor with receptor solubilized by a

non-digestive procedure should give important information on the nature of that portion of the receptor that anchors it to the brush-border membrane.

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