Anti-Peptide Antibodies and Proteases as Structural Probes for the Lactose/H^+ Transporter of *Escherichia coli*: A Loop around Amino Acid Residue 130 Faces the Cytoplasmic Side of the Membrane

Robert Seckler,* Tarik Möröy,‡ J. Keith Wright, and Peter Overath
Max-Planck-Institut für Biologie, 7400 Tübingen, FRG
Received July 2, 1985; Revised Manuscript Received January 7, 1986

ABSTRACT: From the amino acid sequence of the *Escherichia coli* lactose/H^+ transporter, 7 hydrophilic segments were selected, 8–13 amino acids in length, and chemically synthesized, and anti-peptide antibodies were raised in rabbits. Apart from the antiserum to the synthetic COOH terminus ($P_{408-417}$), which reacted strongly with the lactose/H^+ transporter and has previously been used to localize the COOH terminus on the cytoplasmic face of the membrane, only those antibodies directed against the peptide corresponding to amino acid residues 125–135 ($P_{125-135}$) exhibited a marked reaction with the transporter, while antibodies to the five other peptides reacted very weakly or not at all, suggesting that most of the hydrophilic segments are conformationally restricted or buried in the interior of the protein. Thermolysin treatment destroys the epitope on the transporter which is recognized by anti-$P_{125-135}$ antibodies. Comparison of the kinetics and the extent of proteolysis of the transporter in right-side-out or inside-out cytoplasmic membrane vesicles or in reconstituted proteoliposomes suggests that the hydrophilic sequence from amino acid 125 to amino acid 135 is accessible to thermolysin only from one side, corresponding to the cytoplasmic face of the membrane. Furthermore, the experiments demonstrate that the transporter is inserted bimodally in a nonpreferential fashion into the proteoliposomes, confirming earlier results using antibodies to the synthetic COOH terminus of the transporter in conjunction with carboxypeptidase A treatment.

The lactose/H^+ transporter of *Escherichia coli* (lactose permease, product of the *lacY* gene) catalyzes the active transport of various galactosides across the cytoplasmic membrane by proton/galactoside symport [for recent reviews, see Overath & Wright (1983), Kaback (1983), and Wright et al. (1985)]. *E. coli* strains carrying a *lacY*-containing plasmid overproduce the transporter more than 10-fold (Teather et al., 1978, 1980). From cytoplasmic membranes of such overproducing strains, the transporter has been solubilized in detergent micelles, purified, and functionally reconstituted into proteoliposomes (Newman et al., 1981; Wright et al., 1983; Foster et al., 1982; Wright & Overath, 1984).

The amino acid sequence of the transporter protein is known (Büchel et al., 1980; Ehring et al., 1980), and circular dichroism and also Raman spectroscopy indicate a high α-helix content of the purified protein (Foster et al., 1983; Wright et al., 1985; Vogel et al., 1985). On the basis of these data and in analogy to the structure of bacteriorhodopsin (Henderson & Unwin, 1975), models have been proposed for the folding of the transporter polypeptide chain in which large parts are organized in membrane-spanning α-helices connected by short hydrophilic looplike peptide segments (Büchel et al., 1980; Foster et al., 1983; Overath & Wright, 1983; Bieseler et al., 1985; Wright et al., 1985; Vogel et al., 1985). To test the validity of such models, the reaction of high molecular weight probes, such as antibodies or proteases, can be used to determine the accessibility of segments of the membrane-embedded polypeptide chain.

Monoclonal antibodies to the transporter have been obtained by Carrasco et al. (1982), but attempts to identify the epitopes recognized by these antibodies have so far been unsuccessful (Herzlinger et al., 1984). Alternatively, antibodies specific for defined segments of a protein can be obtained by using synthetic peptides as antigens (Sutcliffe et al., 1983; Walter & Doolittle, 1983). Antibodies to the synthetic COOH terminus of the lactose/H^+ transporter have been employed to localize this segment of the transporter to the cytoplasmic face of the *E. coli* plasma membrane (Seckler et al., 1983; Carrasco et al., 1984), to determine the orientation of the transporter after reconstitution into proteoliposomes (Seckler & Wright, 1984), and to quantify the amount of wild-type as well as mutant transporter in membranes or proteoliposomes (Wright & Seckler, 1985).

Cleavage of the membrane-embedded transporter by various proteases inactivates transport but does not alter substrate binding. Proteolysis produces a fragment of apparent molecular weight determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (M_{SDS})[1] 20 000 which carries radioactivity introduced by photoaffinity labeling with the substrate *p*-nitro[2-^{3}H]phenyl α-D-galactopyranoside. This fragment was suggested to correspond to the NH_2-terminal region of the protein. Finally, because fragments of indistinguishable M_{SDS} are produced by proteolysis of the carrier in vesicles of native or inverted orientation, cleavages were proposed to occur at different sites on the cytoplasmic or periplasmic end of the same putative membrane-spanning α-helix (Goldkorn et al., 1983). In contrast, Bieseler et al. (1985) reported that the transporter is subject to multiple cleavage by thermolysin in the region from position 134 to 143 of the polypeptide chain from either face of the membrane at the same sites, yielding a COOH-terminal fragment with an

‡Present address: Institute Pasteur, 75724 Paris Cedex 15, France.

[1] Abbreviations: *t*-Boc, *tert*-butyloxycarbonyl; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; P_{n-m}, synthetic peptide comprising amino acid residues *n*–*m* of the lactose/H^+ transporter; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; RSO, right side out; ISO, inside out; M_{SDS}, apparent molecular weight determined by SDS–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-2403$01.50/0

apparent M_{SDS} of 18 000. Therefore, these authors suggested that this highly charged segment traverses the interior of the protein.

This paper describes the production and specificity of antibodies raised against synthetic peptides which correspond to hydrophilic segments of the lactose transporter. From a total of seven antibodies, only those against the COOH-terminal peptide and a peptide corresponding to amino acid residues 125–135 react with the intact polypeptide chain. The latter segment appears to be part of a loop between two putative membrane-spanning α-helices which is directed toward the cytoplasmic face of the *E. coli* cytoplasmic membrane.

Experimental Procedures

Peptide Synthesis. Peptides were synthesized by the solid-phase method (Barany & Merrifield, 1980) using *t*-Boc as the temporary protecting group. The side chains of Arg, Lys, and His were protected by nitro, benzyloxycarbonyl, and dinitrophenyl groups, respectively. Asp, Glu, Ser, and Thr were used as benzyl derivatives. Norleucine was substituted for Met at the NH_2 terminus of P_{1-9}. The first amino acid residue was coupled to the chloromethylpolystyrene resin as described by Gisin (1973). Since P_{1-9} and $P_{130-140}$ were synthesized on an alanyl resin, these peptides carry an additional Ala residue at their COOH terminus.

A double coupling protocol was used for subsequent coupling steps with dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole as coupling reagents. A single tritiated Leu, Val, or Ile residue (925 MBq/mol) was incorporated in each of the peptides. To facilitate coupling to KLH, an additional Tyr residue was added at the N terminus of $P_{130-140}$.

After cleavage from the support by HBr in trifluoroacetic acid, the peptides were further deprotected by catalytic transfer hydrogenation (Anwer & Spatola, 1980) or thiophenol treatment (Lin et al., 1972) if they contained nitro or dinitrophenyl groups. Low molecular weight material was removed by chromatography on Sephadex G-15 and the composition of the peptides confirmed by quantitative amino acid analysis.

Preparation of Immunogens. $P_{130-140}$ was coupled to KLH by bis(diazobenzidine). Benzidine was diazotized as described (Likhite & Sehon, 1967). To a solution of peptide (1 μmol) and KLH (5 mg) in 2.75 mL of borate saline buffer (100 mM boric acid, 25 mM sodium tetraborate, and 75 mM NaCl, pH 8.5) was added 200 μL of neutralized bis(diazobenzidine) (1 volume of diazotized benzidine + 1 volume of 0.5 M sodium borate, pH 8.5); the mixture was stirred for 3 h at 0–4 °C and dialyzed exhaustively against PBS.

P_{34-45}, $P_{125-135}$, $P_{132-144}$, $P_{284-291}$, and $P_{408-417}$ were coupled to KLH by using glutaraldehyde (Reichlin, 1980). Because P_{1-9} was insoluble at neutral pH, coupling by both methods was performed at pH $\sim$11.

Rabbits were injected intradermally or subcutaneously and intramuscularly with 0.4–1.0 mg of the conjugates in complete Freund's adjuvant. A single booster injection of the conjugates in incomplete adjuvant was given 2–4 weeks later, and sera were drawn from day 7 thereafter.

Isolation of Peptide-Specific Antibodies. Soluble peptides were immobilized on CH-Sepharose (Pharmacia, Uppsala, Sweden), and P_{1-9} was coupled to diazonium SP 500 (Serva, Heidelberg, FRG) following the suggestions of the manufacturers. The substitution ratios were 100 nmol/mL of resin for P_{1-9} and 1.5–2 μmol/mL of wet gel for the other peptides.

γ-Globulins from 2–3 mL of anti-peptide serum, obtained by ammonium sulfate precipitation and taken up in 1–2 mL of PBS or borate saline buffer, were shaken overnight with the immobilized peptide (200-μL bed volume). The affinity resin was transferred to a small column (0.5 cm in diameter) and washed extensively with borate saline buffer containing 0.05% Triton X-100, with 0.1 M Tris-HCl and 0.5 M NaCl, and finally with 10 mM sodium phosphate, pH 6.0. Bound antibodies were eluted with 0.8–1.0 mL of 0.2 M glycine hydrochloride, pH 2.5 (containing 0.05% Tween 20 for the elution of anti-P_{1-9}), and neutralized immediately by the addition of 0.5 M sodium borate, pH 8.5.

ELISA. Antisera were tested for anti-peptide reactivity, and the purification of peptide-specific antibodies was followed by ELISA using the free peptides as antigens. The peptides (100 pmol/well) were dried onto poly(vinyl chloride) microtiter plates at 37–42 °C. Blocking of remaining binding sites, incubations, and washes were as in Seckler et al. (1983).

Preparation and Protease Treatment of Vesicles. E. coli strain T206 (Teather et al., 1980) was induced for only 90 min (0.75 generation) with 0.1 mM isopropyl β-D-thiogalactoside to avoid filamentation of the cells. The cells were cooled on ice, harvested, and washed in 50 mM Tris-HCl, pH 8.0 at 4 °C. Right-side-out (RSO) vesicles were prepared as described (Kaback, 1971). Inside-out (ISO) vesicles were prepared by brief sonication of RSO vesicles [cf. Seckler et al. (1983)]. Cytoplasmic membranes from strain T206 were dissolved in dodecyl maltoside and reconstituted with *E. coli* phospholipids (Wright et al., 1983). Both ISO and RSO vesicles were washed in 60 mM Tris-HCl/0.5 mM $MgSO_4$, pH 8.0. RSO vesicles were used immediately, as freezing and thawing of T206 RSO vesicles increases the number of ISO vesicles. RSO and ISO vesicles contained 0.7–0.9 nmol of carrier/mg of membrane protein as determined by substrate binding (Wright et al., 1983).

For proteolysis experiments, vesicles and proteoliposomes were diluted to 0.5 mg of membrane protein/mL in 60 mM Tris-HCl, pH 8.0. Reconstituted proteoliposomes contained 5.7 mg of *E. coli* phospholipid/mg of membrane protein, and the lactose/H^+ transporter was specifically labeled at Cys_{148} with *N*-[^{14}C]ethylmaleimide (120 000 cpm/mg of membrane protein). Digestion at 37 °C was initiated by adding thermolysin (EC 3.4.24.4; Calbiochem, Giessen, FRG; 0.3 mg/mL in 0.1 M Tris-HCl/2 mM $CaCl_2$, pH 8.0) or carboxypeptidase A (EC 3.4.17.1, PMSF-treated; Serva, Heidelberg, FRG; 0.2 mg/mL in 30 mM Tris-HCl, 0.1 M NaCl, and 0.2 mM $CoCl_2$, pH 7.8) to final concentrations of 2 μg of protease/mL. After the indicated times (0–180 min), digestion was halted by adding 5 μL of 0.25 M NaEDTA or NaEGTA saturated with *o*-phenanthroline to a 100-μL sample. The samples were maintained at least 10 min at room temperature, and 35 μL of 0.17 M Tris, 160 mg/mL lithium dodecyl sulfate, 200 mg/mL glycerol, and 0.1 mg/mL bromophenol blue, pH 6.5, was added at room temperature to dissolve the membranes for electrophoresis.

Miscellaneous Procedures. The procedures for the preparation of cytoplasmic membrane vesicles, labeling of the transporter by *N*-ethylmaleimide, radioiodination of peptide-specific antibodies, electrophoresis, and immunoblotting have been described (Wright et al., 1983; Wright & Overath, 1984; Seckler & Wright, 1984; Wright & Seckler, 1985). Fluorography was conducted by using Amplify (Amersham Buchler, Braunschweig, FRG). Polyacrylamide gels were treated with Protosol (New England Nuclear, Dreieich, FRG) before liquid scintillation counting.

Results

Selection of Peptides. From the amino acid sequence of the lactose transporter, three internal peptide segments and

Table I: Antibodies to Synthetic Segments of the Lactose/H^+ Transporter

peptide segment	sequence	average hydropathy[a]	anti-peptide react.		anti-carrier react. of purified antibodies
			serum	purified antibodies	
P_{1-9}	MYYLKNTNF	−0.6	+++	++	−
P_{34-45}	LHDINHISKSDT	−0.9	+++	++	−
$P_{125-135}$	VAEFIEKVSRR	−0.3	+++	+++	++
$P_{130-140}$	EKVSRRSNFEF	−1.4	++++	++	(+)
$P_{132-144}$	VSRRSNFEFGRAR	−1.2	++	+	−
$P_{284-291}$	NRIGGKNA	−1.2	+++	++	−
$P_{408-417}$	LLRRQVNEVA	−0.2	++++	++++	++++

[a] Average of hydropathy values of the individual amino acids according to the scale of Kyte and Doolittle (1982); the average value for the lactose/H^+ transporter is +0.9.

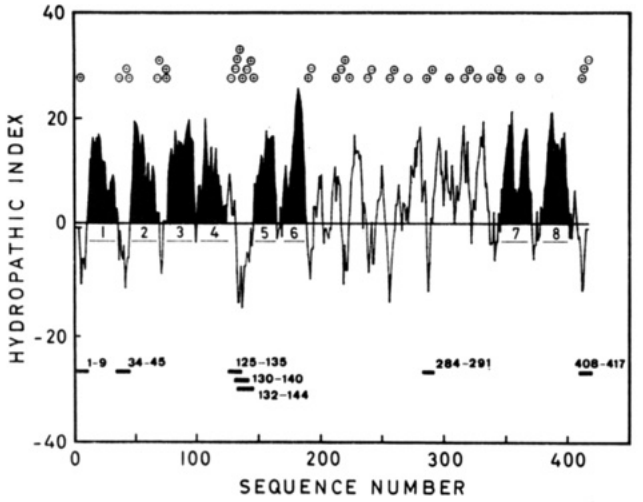

FIGURE 1: Hydropathy profile of the amino acid sequence of the lactose/H^+ transporter. Hydropathy values (Kyte & Doolittle, 1982), summed over seven consecutive amino acid residues, are plotted against the position in the sequence. Putative membrane-spanning hydrophobic α-helices (numbered 1–8), the position of charged amino acid residues, and the location of Cys_{148}, which can be labeled specifically by *N*-ethylmaleimide, are indicated. Horizontal bars represent peptide segments selected for synthesis.

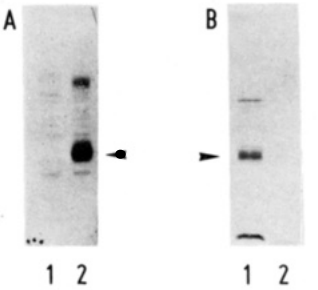

FIGURE 2: (A) Reactivity of anti-$P_{125-135}$ antibodies toward the lactose/H^+ transporter. Cytoplasmic membrane proteins (50 μg) of the carrier-overproducing strain T206 (lane 2) and of strain T184, which lacks the transporter (lane 1), were separated by gel electrophoresis and transferred to a nitrocellulose filter. The filter was bathed in purified anti-$P_{125-135}$ IgG (3.5 μg/mL), then in alkaline phosphatase labeled goat anti-rabbit IgG, and finally in a solution of a chromogenic substrate [compare Wright & Seckler (1985)]. The position of the lactose transporter is indicated by the arrow. (B) Thermolysin treatment abolishes reactivity of the transporter with anti-$P_{125-135}$ IgG. To a suspension of inside-out membrane vesicles [IV in Seckler & Wright (1984)], prepared by brief sonication of RSO vesicles from *E. coli* strain T206 (2 mg of membrane protein/mL in 20 mM Tris-HCl/120 μM $CaCl_2$, pH 8.0), thermolysin was added (20 μg/mL). The reaction was halted immediately (lane 1) or after 90 min at 37 °C (lane 2) by the addition of EGTA (final concentration 2 mM), and aliquots of both samples (65 μg of membrane protein) were subjected to electrophoresis and immunoblotting with purified anti-$P_{125-135}$ IgG as in part A.

the NH_2 terminus were selected for chemical synthesis and the production of antibodies (cf. Figure 1 and Table I). The NH_2 terminus of the polypeptide chain might form a flexible protrusion from the membrane into the aqueous phase. Segments 34–45 and 125–144 represent hydrophilic stretches flanked by hydrophobic, possibly membrane-spanning domains and thus may form looplike structures at the membrane surface. Segment 284–291 represents a hydrophilicity peak in less hydrophobic surroundings. For the synthesis, segment 125–144 was divided into three overlapping peptides of 11–13 amino acid residues.

Reactivity of Anti-Peptide Antibodies. Anti-peptide antibodies were raised in rabbits. The resulting antisera were tested for anti-peptide reactivity by ELISA (Table I): In all cases, antibodies reactive with the synthetic peptide were obtained. The anti-peptide antibodies were purified by affinity chromatography on the immobilized peptides and tested for their cross-reactivity with the lactose transporter. Taking the previously described antibodies to the synthetic COOH-terminal decapaptide ($P_{408-417}$) as a positive control, only those antibodies directed toward $P_{125-135}$ reacted markedly with the transporter on immunoblots. Among the proteins of cytoplasmic membrane vesicles from the carrier-overproducer *E. coli* strain T206, anti-$P_{125-135}$ specifically visualized the broad band of M_{SDS} 33 000, typical for the lactose transporter, and a minor fraction of aggregated transporter at $M_{SDS} \simeq 70\,000$ (Figure 2A, lane 2). Membrane proteins from the transporter-deficient strain T184 (Figure 2A, lane 1) did not show this reaction. Antibodies to $P_{130-140}$ exhibited a much weaker cross-reaction with the transporter, barely distinguishable from the unspecific binding of antibodies to the nitrocellulose filter [(+) in Table I], while the remaining anti-peptide antibodies did not show any reaction with the transporter.

The polyvalent antibodies specific for $P_{125-135}$ were radioiodinated and purified by affinity chromatography. More than 85% of the radioactivity eluted from the affinity resin of $P_{125-135}$, specifically bound to the immobilized peptide subsequently. However, quantitative evaluation of immunoblots demonstrates that only ~0.1% of the radiolabeled anti-$P_{125-135}$ bound to the transporter immobilized on nitrocellulose, while more than 25% of anti-$P_{408-417}$ bound under the same conditions. Presumably due to the low fraction of anti-$P_{125-135}$ antibodies recognizing the transporter, attempts to demonstrate specific binding of ^{125}I-anti-$P_{125-135}$ to carrier-containing membrane vesicles of either ISO or RSO orientation failed. Alternatively, the epitope recognized by this antibody may not be accessible in the intact, membrane-embedded carrier.

Thermolytic Cleavage of the Epitope. ISO *E. coli* membrane vesicles of the carrier-overproducing strain T206, obtained by brief sonication of RSO vesicles (Teather et al., 1977; Seckler et al., 1983), were treated with thermolysin and subjected to immunoblotting with anti-$P_{125-135}$. Thermolysin

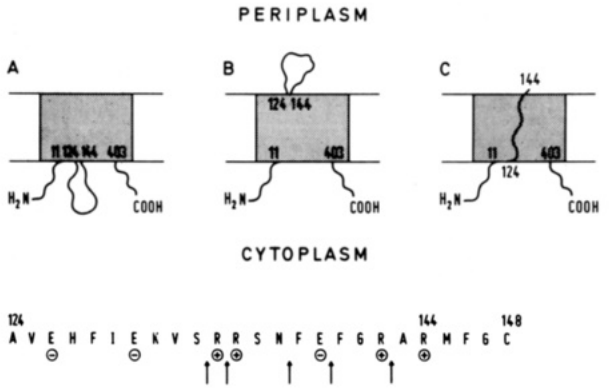

FIGURE 3: Possible locations of amino acids 124–144. Both the NH_2 and COOH termini face the cytoplasm. The sequence from amino acid residues 124–144 may be directed toward the cytoplasm (A) or toward the periplasm (B) or may traverse the membrane (C). The sequence is shown below. Arrows indicate thermolytic cleavage sites according to Bieseler (1985).

treatment completely abolished the binding of anti-$P_{125-135}$ to the transporter, without producing a detectable low molecular weight fragment reactive with the antibodies (Figure 2B, lane 2).

Localization of the Thermolysin Cleavage Site. Because the polypeptide segment around amino acid residue 130 is accessible to antibodies at least in an immunoblot, this segment is probably exposed on the surface of the transporter. The segment may form a loop either on the cytoplasmic face of the membrane (Figure 3A) or on the periplasmic face of the membrane (Figure 3B). A third possibility considered by Bieseler et al. (1985) is that the segment spans that membrane (Figure 3C). The sites of thermolytic cleavage identified by Bieseler et al. (1985) are also indicated. Hydrolysis occurs after residues Ser_{133}, Arg_{134} (the penultimate residue in $P_{125-135}$), Asn_{137}, Glu_{139}, and Arg_{142}. Because there are multiple thermolytic cleavage sites in this region, neither NH_2- nor COOH-terminal fragments would be expected to react with anti-$P_{125-135}$. Proteolysis of the transporter could distinguish among the possibilities in Figure 3 in the following way. Cleavage on the periplasmic face around residue 135 in sealed vesicles should yield a fragment containing a COOH terminus inaccessible to proteases in (B) and (C); no cleavage would be expected in (A). Proteolysis on the cytoplasmic face of the transporter should result in the cleavage of the region around residue 135 in (A) and (C) but not in (B). In each case, the large COOH-terminal fragment should contain Cys_{148} which can be radioactively labeled.

The kinetics of cleavage of the transporter by thermolysin was investigated in right-side-out (RSO) and inside-out membrane vesicles (ISO, Figure 4) initially using the antibody against the COOH terminus (anti-$P_{408-417}$) as a probe. As shown before (Seckler & Wright, 1984), treatment with carboxypeptidase degraded the COOH terminus in ISO vesicles (compare lanes 9 and 10 in Figure 4B) but not in RSO vesicles (lanes 9 and 10 in Figure 4A). This control demonstrates that the COOH terminus is not accessible to this protease in RSO vesicles; i.e., the COOH terminus is directed toward the inside of these vesicles while exposed on the surface of ISO vesicles. Thermolysin treatment of either RSO or ISO vesicles produced a fragment which migrated on a 10.8% polyacrylamide gel with an apparent molecular weight of M_{SDS} 23 000, while the intact transporter exhibited an apparent molecular weight of M_{SDS} 33 000. When the actual molecular weights for two integral membrane proteins, the lactose transporter (M_r 46 500) and bacteriorhodopsin (M_r 26 500,

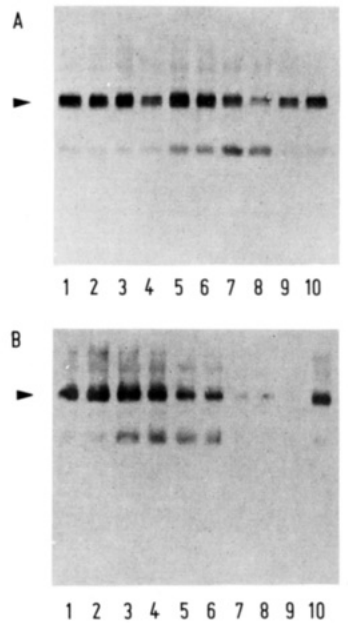

FIGURE 4: Thermolytic cleavage of the lactose transporter in RSO (A) and ISO (B) membrane vesicles. Vesicles (0.5 mg of membrane protein/mL) were untreated (lane 1), were treated with 2 μg/mL thermolysin for 0, 2, 5, 10, 20, 40, or 60 min at 37 °C (lanes 2–8), were treated for 60 min with 2 μg/mL carboxypeptidase A (lane 9), or were maintained at 37 °C without protease (lane 10). After inhibition of the protease, samples (30 μg of protein, 24 pmol of transporter) were subjected to electrophoresis and immunoblotting with anti-$P_{408-417}$ serum. The position of the intact transporter is indicated by the arrows.

M_{SDS} 15 000), were used to calibrate the gel system, the molecular weight of the thermolytic fragment was M_r 35 000 (data not shown).

Although cleavage was observed in both RSO and ISO vesicles, the kinetics differed. In RSO vesicles, the amount of fragment containing the COOH terminus increased until 40 min (Figure 4A, lane 7) and then decreased. After 60 min, a sizable fraction of transporter remained (lane 8). In contrast, in ISO vesicles the amount of fragment containing the COOH terminus peaked at about 5 min while at 40 min hardly any COOH termini could be detected at positions corresponding either to the fragment or to the intact protein. Therefore, thermolysin not only cleaves the protein between residues 133 and 143 but also degrades the COOH terminus. Cleavage at these internal sites is faster than at the COOH terminus. Because the fragment appeared considerably faster in ISO than in RSO vesicles, the cleavage site appears to be more readily accessible in ISO vesicles. Therefore, one possible interpretation of this experiment is that the region around residue 140 is exposed on the surface of ISO vesicles, corresponding to the cytoplasmic face of the membrane (Figure 3A). Although directed to the inside in RSO vesicles, the protease gains access to this region by making the membrane permeable.

Because the experiments with RSO and ISO vesicles did not yield a conclusive answer, the kinetics of thermolysin cleavage in reconstituted proteoliposomes was investigated. Because of the higher lipid to protein ratio, proteoliposomes may be more stable during the proteolytic treatment. In such preparations, about half of the transporter molecules have the same orientation as in cells (RSO orientation) while the other half are inverted (ISO orientation).

First, cross-reactivity of anti-$P_{125-135}$ with the thermolytic fragment in reconstituted proteoliposomes was examined (Figure 5). Samples not exposed to thermolysin (lane 1) or

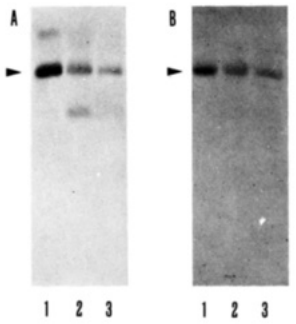

FIGURE 5: Cross-reactivity of anti-$P_{125-135}$ and anti-$P_{408-417}$ sera with thermolytically cleaved purified, reconstituted lactose transporter. Reconstituted transporter (6 μM) was treated for 0, 2, or 10 min (lanes 1–3, respectively) with 2 μg/mL thermolysin in 60 mM Tris-HCl, pH 8.0 at 37 °C. After inhibition of thermolysin, samples (360 pmol of transporter) were subjected to electrophoresis on a 15% polyacrylamide gel and to immunoblotting with a 1:1000 dilution of anti-$P_{408-417}$ serum (A) or with 2 μg/mL purified anti-$P_{125-135}$ (B). The arrow marks the position of the intact transporter.

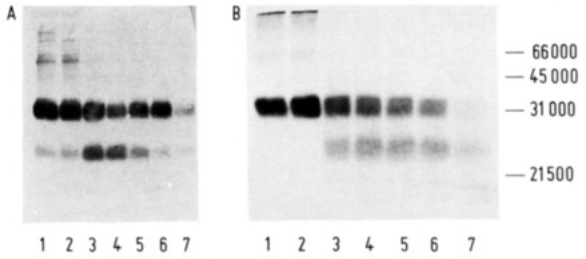

FIGURE 6: Cleavage of the reconstituted lactose/H$^+$ transporter by thermolysin, followed by immunoblotting (A) and autoradiography (B). The transporter in cytoplasmic membrane vesicles from strain T206 was labeled specifically at Cys_{148} by *N*-[^{14}C]ethylmaleimide; membrane proteins were solubilized by dodecyl β-D-maltoside and reconstituted into *E. coli* phospholipids by hydrophobic chromatography. The reconstituted proteoliposomes (0.5 mg of membrane protein/mL) were treated with 2 μg/mL thermolysin at 37 °C as described under Experimental Procedures. The reaction was terminated after 5, 10, 20, and 60 min (lanes 3–6). Lane 1, no thermolysin; lane 2, stopped at $t = 0$; lane 7, sonicated for 5 min in the presence of thermolysin and then incubated 60 min at 37 °C. (A) Samples (13 μg of membrane protein) were subjected to electrophoresis and immunoblotting with anti-$P_{408-417}$ serum. (B) Samples (39 μg of membrane protein) were subjected to electrophoresis and visualized by fluorography.

digested for 2 (lane 2) or 10 min (lane 3) were tested by immunoblotting against anti-$P_{408-417}$ (A) or anti-$P_{125-135}$ (B) after electrophoresis on a 15% polyacrylamide gel. The amount of both antibodies bound at the position of the intact carrier (arrow) decreased due to the digestion of transporters in the inside-out orientation. The fragment transiently retained the COOH terminus (Figure 5A, lane 2) did not cross-react with anti-$P_{125-135}$ (Figure 5B, lane 2). No cross-reactivity of anti-$P_{125-135}$ with any material of lower molecular weight could be detected. Hence, either thermolysin completely degraded the epitope recognized by anti-$P_{125-135}$ or low molecular weight fragments containing this epitope were not efficiently transferred to the nitrocellulose filter.

Second, the kinetics of thermolytic cleavage in reconstituted proteoliposomes was investigated. The transporter was first labeled at Cys_{148} with *N*-[^{14}C]ethylmaleimide (Fox & Kennedy, 1965; Beyreuther et al., 1981). The membrane proteins were then solubilized in dodecyl maltoside and reconstituted into proteoliposomes. The kinetics of thermolysin cleavage of the transporter in these proteoliposomes is shown in Figure 6. Within 5 min, thermolysin produced a fragment of the transporter that reacted with anti-$P_{408-417}$ on immunoblots (Figure 6A, lane 3) and contained the radioactive label at

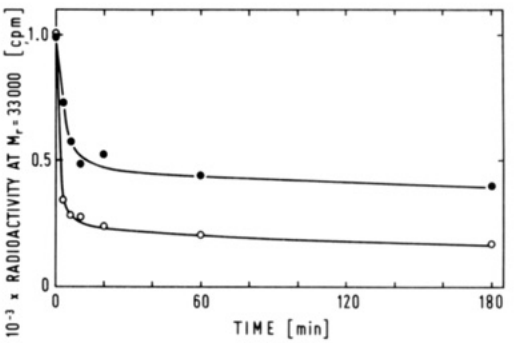

FIGURE 7: Kinetics of cleavage of the transporter by thermolysin. Cytoplasmic membrane vesicles (○) and reconstituted proteoliposomes (●) containing the *N*-[^{14}C]ethylmaleimide-labeled lactose transporter were treated with thermolysin as described previously (cf. Figure 4). The reaction was terminated at the times indicated by the addition of EGTA, and proteins were separated by gel electrophoresis. Radioactive bands at M_{SDS} 33 000 corresponding to the intact transporter were visualized by fluorography and cut out of the dried gel, and the radioactivity was quantified by liquid scintillation counting.

Cys_{148} (Figure 6B, lane 3). While the cross-reactivity of the fragment with anti-$P_{408-417}$ decreased with time, the electrophoretic mobility of the fragment and its fluorographic intensity (*N*-ethylmaleimide at Cys_{148}) remained constant between 10 and 60 min (cf. lanes 4–6 in panels A and B). Thus, the loss of cross-reactivity with anti-$P_{408-417}$ is associated with the loss of only a few COOH-terminal residues in the fragment as its mobility did not change (Figure 6B, lanes 3–6). A fraction of the cotransporters in proteoliposomes evinced changes neither in electrophoretic mobility nor in reactivity with anti-$P_{408-417}$. The membrane of the proteoliposomes protects the primary site of thermolytic cleavage and the COOH terminus of such transporters, implying that the primary cleavage site is on the same side of the membrane as the COOH terminus. Sonication of the proteoliposomes for 5 min in the presence of thermolysin and incubation for 60 min resulted in the thermolytic cleavage of nearly all transporters, as judged by the loss of cross-reactivity with anti-$P_{408-417}$ (Figure 6A, lane 7) and fluorographic intensity (Figure 6B, lane 7). Apparently, sonication in the presence of thermolysin caused additional sites to become susceptible to cleavage as the fluorographic intensity in the region of the polyacrylamide gel where the fragment appears is low.

These results demonstrate that the reconstituted sample contains two populations of transporter molecules, corresponding to the two possible orientations of the transporter. In one population, segment 125–135 *and* the COOH terminus of the polypeptide chain are susceptible to the action of thermolysin (ISO orientation); in the other population, both segments are resistant (RSO orientation). As anticipated by this interpretation, essentially all transporter molecules were cleaved, when the proteoliposomes were sonicated in the presence of thermolysin, thus allowing protease to have access to the inner compartment of the vesicles (Figure 6A,B, compare lanes 6 and 7).

A quantitative evaluation of the kinetics of thermolysin cleavage of the transporter is shown in Figure 7. Inverted cytoplasmic membrane vesicles [cf. V in Seckler & Wright (1984)] and reconstituted proteoliposomes containing labeled transporter were treated with thermolysin and subjected to gel electrophoresis in the presence of SDS. The radioactivity remaining in the transporter band at M_{SDS} 33 000 was quantified by scintillation counting. In the reconstituted sample [Figure 7, (●)], a rapid decrease of the radioactivity to about 50% of the initial value within the first 10 min of thermolysin treatment was followed by a slow further decrease during the

next 3 h. A similar biphasic cleavage was observed with the cytoplasmic membrane vesicles [Figure 7 (○)], which have been shown to retain a preferential inside-out orientation even after repeated sonication during the labeling procedure (Seckler & Wright, 1984). In this case, 75% of the transporter was degraded within 10 min.

Discussion

Antibodies to seven synthetic peptides representing five hydrophilic segments of the lactose/H^+ transporter have been purified and tested for their ability to bind to the intact transporter. Antibodies to the synthetic COOH-terminal decapeptide ($P_{408-417}$) cross-react very strongly with the transporter, and by a competition ELISA, the majority of the peptide-reactive antibodies are also demonstrated to bind to the lactose/H^+ transporter embedded in the *E. coli* cytoplasmic membrane (Seckler et al., 1983). As previously noted, the COOH terminus of the transporter represents an immunodominant epitope when the purified transporter in detergent micelles is used for immunization (Seckler et al., 1983; Seckler & Wright, 1984). Rabbit antibodies to other epitopes on the transporter have only been obtained after repeated immunizations with carboxypeptidease-treated transporter, and more than 50% of a series of mice immunized with the intact transporter produced antibodies to the transporter COOH terminus exclusively (T. Möröy, R. Seckler, and J. K. Wright, unpublished data).

Among the other anti-peptide antibodies, only those directed toward $P_{125-135}$ react specifically with the protein after electrophoresis in the presence of sodium dodecyl sulfate and transfer to nitrocellulose. Anti-$P_{130-140}$ reacts poorly, and no binding of antibodies against P_{1-9}, P_{34-45}, $P_{132-144}$, and $P_{284-291}$ can be detected. Cross-reactivity of an anti-peptide antibody with the intact protein will only be observed if the conformation of the peptide segment in the intact protein is identical with the conformation recognized by the antibody, or if the protein segment is flexible enough to adopt the conformation recognized by the anti-peptide antibody (Walter & Doolittle, 1983). A correlation between segmental flexibility and antigenicity has recently been demonstrated (Westhof et al., 1984; Tainer et al., 1984).

Because most of the antibodies directed against peptides corresponding to internal segments of the transporter fail to cross-react with the complete polypeptide, it appears reasonable to assume that these segments are fixed in a conformation that is not adopted by the corresponding synthetic peptide during antigen presentation or that these segments are buried in the interior of the protein. The structure of the transporter as presented in an immunoblot is likely to be not entirely different from that of the native protein because in a sodium dodecyl sulfate micelle, the secondary structure is very similar to that of the membrane-embedded protein (Vogel et al., 1985). The failure of anti-P_{1-9} antibodies to react with the transporter may have trivial reasons because anti-peptide antibodies were only obtained after coupling to KLH via the NH_2 terminus while a conjugate of P_{1-9} to polylysine via the COOH terminus did not elicit peptide-specific antibody production.

We propose that the segment from amino acid residues 123–145 forms a loop on the cytoplasmic face of the transporter which connects two putative membrane-spanning α-helices [cf. Figure 3A; also see helices 4 and 5 in Figure 1 and Vogel et al. (1985)]. First, anti-$P_{125-135}$ antibodies react with the transporter polypeptide chain in an immunoblot, and this epitope is destroyed by treatment of the native, membrane-embedded protein by thermolysin (Figures 2 and 5). Therefore, this segment is accessible to proteolytic attack, and the cleavage is expected to yield at least two polypeptides, an NH_2-terminal fragment ($M_r \approx 15\,000$), thus far undetected, and a COOH-terminal fragment ($M_r \approx 30\,000$). Together with the observations of Bieseler et al. (1985) that there are multiple cleavage sites between residues 133 and 143 (cf. Figure 3), the data suggest that this region of the polypeptide chain is exposed on the surface of the membrane rather than membrane-spanning as depicted in Figure 3C. Second, thermolysin treatment of reconstituted proteoliposomes in which the transporter molecules have been shown before to be equally distributed in RSO and ISO orientations rapidly produces a fragment from about 50% of all transporter molecules. This fragment initially retains the COOH terminus and the radioactively labeled Cys_{148}. Also, its corrected molecular weight of 35 000 is close to that expected for a fragment extending from a site of cleavage shortly before Cys_{148} to the COOH-terminal Ala_{417}. Upon prolonged treatment, thermolysin destroys the immunological epitope at the COOH terminus without significant alteration of the size of the fragment (Figures 6 and 7). The other half of the molecules having an inverse orientation serves as a control. Their cleavage at both the internal site and the COOH terminus occurs much more slowly. Thus, depending on the orientation of the transporter molecules, both cleavage sites are either sensitive or resistant to thermolysin, demonstrating that they are both exposed on the same side. The experiments exclude models B and C in Figure 3. Model B would predict the rapid appearance of a fragment with a highly resistant COOH terminus in 50% of the carrier molecules while the other half would not be split internally but would lose the COOH terminus. According to model C, all molecules would be fragmented at internal sites, but only 50% of the fragments would rapidly loose the COOH terminus. Because the COOH terminus has been assigned to the cytoplasmic face of the *E. coli* plasma membrane, the segment around amino acid residue 130 is also on the cytoplasmic face of the membrane. This result tallies with the view that this segment is preceded by four membrane-spanning α-helices (cf. helices 1–4 in Figure 1) and consequently assigns the NH_2 terminus to the cytoplasmic face of the membrane (Bieseler et al., 1985).

Our experience with proteolysis experiments with RSO or ISO membrane vesicles is similar to that of Goldkorn et al. (1983) and Bieseler et al. (1985). However, the interpretation is different. As shown in Figure 4, the transporter is susceptible to cleavage by prolonged treatment with thermolysin in vesicles of both orientations albeit at different rates. The same behavior was observed by Goldkorn et al. (1983): Treatment of RSO vesicles with chymotrypsin rapidly produced a fragment of an apparent molecular weight similar to that observed here from about one-third of the transporter molecules while the remaining molecules were essentially resistant to cleavage; in contrast, cleavage of the transporter in ISO vesicles was rapid and nearly quantitative [compare parts a and b of Figure 3 in Goldkorn et al. (1983)]. Finally, Bieseler et al. (1985) observed that multiple cleavages by thermolysin in the region from position 134 to position 143 occurred in ISO vesicles and in a qualitatively similar way in RSO vesicles. All these results can be readily explained if a fraction of the RSO vesicles are leaky or become leaky during protease treatment. Recent experiments have led to the same conclusion (Bieseler, 1985).

Proteolytic enzymes are useful reagents for probing the structure of membrane proteins. Experience with the lactose/H^+ transporter suggests that three points should be considered for an unambiguous interpretation of the results.

The kinetics of the digestion should be adequately resolved so that possible multiphasic behavior is evident, for example, in preparations of RSO and ISO vesicles. Also, proteases should be employed which can be simply and rapidly inhibited. Metalloproteases like thermolysin or carboxypeptidase A, which are inhibited by chelators, and cysteine proteases like papain, which are sensitive to iodoacetate and mercurials, are good choices. In our hands, serine proteases like trypsin and chymotrypsin are difficult to inhibit rapidly and completely. Lastly, high concentrations of proteases should be avoided. The kinetics of digestion could be too rapid, and complete inactivation is more difficult.

ACKNOWLEDGMENTS

We thank Dr. F. Jähnig for critical reading of the manuscript and S. Meessen, S. Grünwald, and B. Erker for expert technical assistance.

Registry No. P_{1-9}, 101226-57-7; P_{34-45}, 101226-58-8; $P_{125-135}$, 101226-59-9; $P_{130-140}$, 101248-09-3; $P_{132-144}$, 101248-10-6; $P_{284-291}$, 101226-60-2; $P_{408-417}$, 101226-61-3; lactose permease, 9068-45-5; thermolysin, 9073-78-3.

REFERENCES

Anwer, M. K., & Spatola, A. F. (1980) *Synthesis*, 929–932.

Barany, G., & Merrifield, R. B. (1980) in *The Peptides* (Gross, E., & Meienhofer, J., Eds.) Vol 2, pp 1–284, Academic Press, New York.

Beyreuther, K., Bieseler, B., Ehring, R., & Müller-Hill, B. (1981) in *Methods in Protein Sequence Analysis* (Elzija, M., Ed.) pp 139–148, Humana Press, Clifton, NJ.

Bieseler, B. (1985) Doctoral Thesis, Universität zu Köln.

Bieseler, B., Prinz, H., & Beyreuther, K. (1985) *Ann. N.Y. Acad. Sci. 456*, 309–325.

Büchel, D. E., Gronenborn, B., & Müller-Hill, B. (1980) *Nature (London) 283*, 541–545.

Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6894–6898.

Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672–4676.

Ehring, R., Beyreuther, K., Wright, J. K., & Overath, P. (1980) *Nature (London) 283*, 537–540.

Foster, D. L., Garcia, M. L., Newman, M. J., Patel, L., & Kaback, H. R. (1982) *Biochemistry 21*, 5634–5638.

Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31–34.

Fox, C. F., & Kennedy, E. P. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 891–899.

Gisin, B. F. (1973) *Helv. Chim. Acta 56*, 1476.

Goldkorn, T., Rimon, G., & Kaback, H. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3322–3326.

Henderson, R., & Unwin, P. N. T. (1975) *Nature (London) 257*, 28–32.

Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688–3693.

Kaback, H. R. (1971) *Methods Enzymol. 22*, 99–120.

Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95–112.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Likhite, V., & Sehon, A. (1967) in *Methods in Immunology and Immunochemistry* (Williams, C. A., & Chase, M. W., Eds.) Vol. 1, pp 164–165, Academic Press, New York.

Lin, M. C., Gutte, B., Caldi, D. G., Moore, S., & Merrifield, R. B. (1972) *J. Biol. Chem. 257*, 4768–4774.

Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804–11808.

Overath, P., & Wright, J. K. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 404–408.

Reichlin, M. (1980) *Methods Enzymol. 70*, 159–165.

Seckler, R., & Wright, J. K. (1984) *Eur. J. Biochem. 142*, 269–279.

Seckler, R., Wright, J. K., & Overath, P. (1983) *J. Biol. Chem. 258*, 10817–10820.

Sutcliffe, J. G., Shinnick, T. M., Green, N., & Lerner, R. A. (1983) *Science (Washington, D.C.) 219*, 660–666.

Tainer, J. A., Getzoff, E. D., Alexander, H., Houghten, R. A., Olson, A. J., Lerner, R. A., & Hendrickson, W. A. (1984) *Nature (London) 312*, 127–134.

Teather, R. M., Hamelin, D., Schwarz, H., & Overath, P. (1977) *Biochim. Biophys. Acta 467*, 386–395.

Teather, R. M., Müller-Hill, B., Abrutsch, U., Aichele, G., & Overath, P. (1978) *Mol. Gen. Genet. 159*, 239–248.

Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Fürst, M., Aichele, G., Wilhelm, U., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223–231.

Vogel, H., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3625–3631.

Walter, G., & Doolittle, R. F. (1983) in *Genetic Engineering* (Setlow, J. K., & Hollaender, A., Eds.) Vol. 5, pp 61–91, Plenum Press, New York.

Westhof, E., Altschuh, D., Moras, D., Bloomer, A. C., Mondragon, A., Klug, A., & Van Regenmortel, M. H. V. (1984) *Nature (London) 311*, 123–126.

Wright, J. K., & Overath, P. (1984) *Eur. J. Biochem. 138*, 497–508.

Wright, J. K., & Seckler, R. (1985) *Biochem. J. 227*, 287–297.

Wright, J. K., Teather, R. M., & Overath, P. (1983) *Methods Enzymol. 97*, 158–175.

Wright, J. K., Dornmair, K., Mitaku, S., Möröy, T., Neuhaus, J.-M., Seckler, R., Vogel, H., Weigel, U., Jähnig, F., & Overath, P. (1985) *Ann. N.Y. Acad. Sci. 456*, 326–341.