Biochemistry

 Volume 34, Number 4 January 31, 1995

Accelerated Publications

Design of a Membrane Protein for Site-Specific Proteolysis: Properties of Engineered Factor Xa Protease Sites in the Lactose Permease of *Escherichia coli*

Miklós Sahin-Tóth,‡ Rhonda L. Dunten,§ and H. Ronald Kaback*

Howard Hughes Medical Institute, Departments of Physiology and Microbiology & Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, California 90024-1570

Received October 19, 1994; Revised Manuscript Received December 5, 1994⊗

ABSTRACT: Lactose permease is a polytopic membrane transport protein with 12 hydrophobic transmembrane domains connected by hydrophilic loops on the cytoplasmic and periplasmic sides of the membrane. By the use of an active permease mutant devoid of Cys residues (C-less permease), single recognition sites (Ile-Glu-Gly-Arg) for the protease factor Xa (fXa) were engineered into hydrophilic loops 7, 8, and 10 in the C-terminal half of the protein. Mutants carrying single sites inserted at position 255, 259 (loop 7), 283, 286 (loop 8), or 341 (loop 10) exhibit significant lactose accumulation (30−70% of C-less permease) and normal levels of expression in the membrane. However, despite solubilization in dodecyl β-D-maltoside, none of the mutant permeases is proteolyzed by fXa to a significant extent. Insertion of two recognition sites in tandem at position 255 results in partial cleavage, and remarkably, introduction of three sites in tandem leads to complete proteolysis by fXa. Importantly, mutants with two or three fXa sites at position 255 accumulate lactose to high levels (70% of C-less) and are present in the membrane in amounts comparable to that of C-less permease. The results indicate that hydrophilic loops 7, 8, and 10 are buried in the tertiary structure of the permease where they are inaccessible to protease. Insertion of tandem sites probably facilitates proteolysis by causing loops to become more accessible to the aqueous phase and by increasing the local concentration of protease recognition sites. The approach should be applicable to other polytopic membrane proteins.

Lactose (lac)[1] permease of *Escherichia coli* is a hydrophobic, polytopic, cytoplasmic membrane protein that catalyzes the coupled translocation of β-galactosides and H^+ with a 1:1 stoichiometry (i.e., symport or cotransport). Encoded by the *lacY* gene, the permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport [reviewed in Kaback (1989, 1992), Kaback et al. (1993), and Poolman and Konings (1993)]. Furthermore, evidence has been presented (Dornmair et al., 1985; Costello et al., 1987; Sahin-Tóth et al., 1994b) indicating that the permease is functional as a monomer. On the basis of circular dichroic studies and hydropathy analysis of the primary amino acid sequence (Foster et al., 1983), a secondary structure was proposed in which the permease has a short hydrophilic N terminus, 12 hydrophobic domains in an α-helical configuration that traverse the membrane in a zig-zag fashion

* Corresponding author. Mailing address: HHMI/UCLA 5-748 MacDonald Research Labs, 10833 Le Conte Ave., Los Angeles, CA 90024-1662. Telephone: (310) 206-5053. Telefax: (310) 206-8623.

‡ Present address: Department of Cell Biology, The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037.

§ Present address: NIMH/DIRP/LGCB, Bldg. 36, Room 3D06, 36 Convent Dr., MSC 4094, Bethesda, MD 20892-4094.

⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1995.

[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; $NaDodSO_4$, sodium dodecyl sulfate; fXa, factor Xa protease; DM, dodecyl β-D-maltoside.

0006-2960/95/0434-1107$09.00/0

connected by hydrophilic loops, and a 17-residue hydrophilic C-terminal tail. Evidence favoring the general features of the model and demonstrating that both the N and the C termini are on the cytoplasmic face of the membrane has been obtained from laser Raman spectroscopy (Vogel et al., 1985), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986; Page & Rosenbusch, 1988), immunological studies with monoclonal (Carrasco et al., 1982, 1984a; Herzlinger et al., 1984, 1985) and site-directed polyclonal antibodies (Seckler et al., 1983, 1986; Seckler & Wright, 1986; Carrasco et al., 1984b), and chemical modification (Page & Rosenbusch, 1988). Finally, unequivocal support for the 12-helix motif has been obtained from analyses of an extensive series of *lac* permease−alkaline phosphatase (*lacY−phoA*) fusions (Calamia & Manoil, 1990).

Due to difficulties inherent in crystallizing hydrophobic membrane proteins, a high-resolution 3-D structure of lac permease is not available. Therefore, in order to obtain structural information, alternative approaches must be developed. By the use of a systematic scanning mutagenesis strategy, single Cys residues have been introduced into a functional lactose permease mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991). The results demonstrate that the great majority of the amino acid residues in the permease can be replaced with Cys or other residues without dramatically altering transport activity or expression [Sahin-Tóth & Kaback, 1993a; Dunten et al., 1993b; Sahin-Tóth et al., 1994a,c; Frillingos et al., 1994; Jung et al., 1995]. Residues where replacement causes inactivation are predominantly intramembrane charged residues located in the C-terminal half of the protein (Sahin-Tóth et al., 1992; Kaback et al., 1994). On the basis of the properties of mutants, it has been shown that Asp237 interacts functionally with Lys358, and Asp240 with Lys319, indicating that these residues are located close to each other in the tertiary structure (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b). Furthermore, purified, reconstituted double-Cys mutants R302C/E325C, E325C/H322C, and E269C/H322C labeled with pyrene exhibit excimer fluorescence, indicating that these pairs of residues are also in close proximity (Jung et al., 1993). Despite strong functional evidence that Asp237 and Lys358, as well as Asp240 and Lys319, form salt bridges, excimer formation is not observed between either set of residues when they are replaced with Cys and labeled with pyrene (H. Jung and H. R. Kaback, unpublished information), and other direct evidence is lacking. Thus, efforts are underway to demonstrate proximity by disulfide bond formation or chemical cross-linking between appropriate pairs of double-Cys replacement mutants. Controlled proteolysis and analysis of fragments appears to be the most direct way to detect such covalent interactions. In the experiments reported here, factor Xa protease (fXa) sites were engineered into hydrophilic loops between helix VII (Asp237 and Asp240) and helices X (Lys319) and XI (Lys358), and the effect of the insertions on transport activity, expression, and fXa cleavage was examined. The results demonstrate that single fXa sites incorporated into hydrophilic loops are inaccessible to the protease and that insertion of three recognition sites in tandem into loop 7 is necessary to achieve complete proteolysis.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose was purchased from Amersham (Arlington Heights, IL). Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984b) was prepared by BabCo (Richmond, CA). Factor Xa protease was purchased from Boehringer Mannheim. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. E. coli HB101 [*hsdS20* (r^-_B,m^-_B), *recA13, ara-14, proA2, lacY1, galK2, rpsL20*(Sm^r), *xyl-5, mtl-1, supE44,* λ^-/F^-] (Boyer & Roulland-Dussoix, 1969) was used as carrier for the plasmids described. *E. coli* T184 [$lacI^+O^+Z^-Y^-(A)$, *rpsL, met*$^-$, *thr*$^-$, *recA, hsdM, hsdR/F*$'$, $lacI^qO^+Z^{D118}(Y^+A^+)$] (Teather et al., 1980) harboring plasmid pT7-5/*lacY* with given mutations in the *lacY* gene was used for expression from the *lac* promoter by induction with isopropyl 1-thio-β-D-galactopyranoside (IPTG). A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (van Iwaarden et al., 1991) containing the *lac* promoter/operator was used for all *lacY* gene manipulations.

Oligonucleotide-Directed Site-Specific Mutagenesis. The cassette *lacY* gene encoding C-less permease was inserted into plasmid pT7-5 and used as a template for PCR mutagenesis. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers, the sequences of which are given in Table 1. As a general principle of design, fXa sites were constructed using native Ile, Glu, Gly, and Arg residues whenever possible (Figure 1). Thus, fXa-255 was "built" around Glu255 by inserting an Ile residue before and a Gly-Arg doublet after Glu255. Recognition site fXa-259 was constructed by insertion of the sequence Ile-Glu-Gly in front of Arg259. In mutant fXa-283, Asn284 was replaced by Glu and Gly, and in mutant fXa-286, Gly287 was changed to Glu, and Lys289 to Arg (G287E/K289R). Finally, fXa-341 permease was constructed by replacing Phe341 with Ile and Val343 with Gly (F341I/V343G). The PCR products were digested with *Age*I and *Bcl*I (fXa-255), *Kpn*I and *Eco47*III (fXa-259), *Bcl*I and *Eco*47III (fXa-283 and fXa-286), or *Spe*I and *Sty*I (fXa-341) and then ligated into pT7-5/*lacY* encoding C-less permease that had been digested with the same enzymes (Figure 1). Constructs with two or three fXa sites in tandem at position 255 were created using synthetic linkers (Table 1). Phosphorylated linkers were ligated between the *Age*I and *Kpn*I sites of pT7-5/*lacY*/C-less (Figure 1). After propagation in *E. coli* HB101, selected clones were sequenced. In addition to the site of mutation(s), the entire sequence between the restriction sites used for subcloning was verified by DNA sequencing.

DNA Sequencing. Double-stranded plasmid DNA prepared by Magic Minipreps (Promega) was sequenced by using the dideoxynucleotide termination method (Sanger et al., 1977; Sanger & Coulson, 1978) and synthetic sequencing primers after alkaline denaturation (Hattori & Sakaki, 1986).

Active Transport. Lactose transport was measured in *E. coli* T184 (Z^-Y^-) transformed with a given plasmid. Fully grown overnight cultures of cells were diluted 10-fold and grown aerobically for 1.5 h at 37 °C in the presence of 10 μg/mL streptomycin and 100 μg/mL ampicillin. Expression of lac permease was then induced by addition of IPTG (0.5

Table 1: DNA Sequence Analysis of fXa Site Insertion Mutants[a]

fXa-255 sense: TTTGCTACCGGT**ATT**GAA**GGTAGG**CAGGGTACCCGCGTATTTGGC

fXa-259 sense: GGTGAACAGGGTACC**ATTGAAGGTCGC**GTATTTGGCTACGTAACG

fXa-283 sense: TTTGCGCCACTGATCATT**GAAGGTCGC**ATCGGTGGGAAGAATGCC

fXa-286 sense: GCGCCACTGATCATTAATCGC**ATCGAAGGGAGG**AATGCCCTGCTG

fXa-341 sense: TATATTACTAGTCAG**ATT**GAA**GGGCGT**TTTTCAGCGACG

2fXa-255 sense: CCGGT**ATT**GAA**GGTCGAATCGAGGGACGG**CAGGGTAC

2fXa-255 antisense: CCTG**CCGTCCCTCGATTCGACC**TTC**AAT**A

3fXa-255 sense: CCGGT**ATT**GAA**GGTCGAATCGAAGGCCGTATCGAGGGACGG**CAGGGTAC

3fXa-255 antisense: CCTG**CCGTCCCTCGATACGGCCTTCGATTCGACC**TTC**AAT**A

[a] Sequences of mutagenic primers and linkers are presented in the 5′→3′ order with mutations in boldface type and the sequences encoding fXa sites underlined.

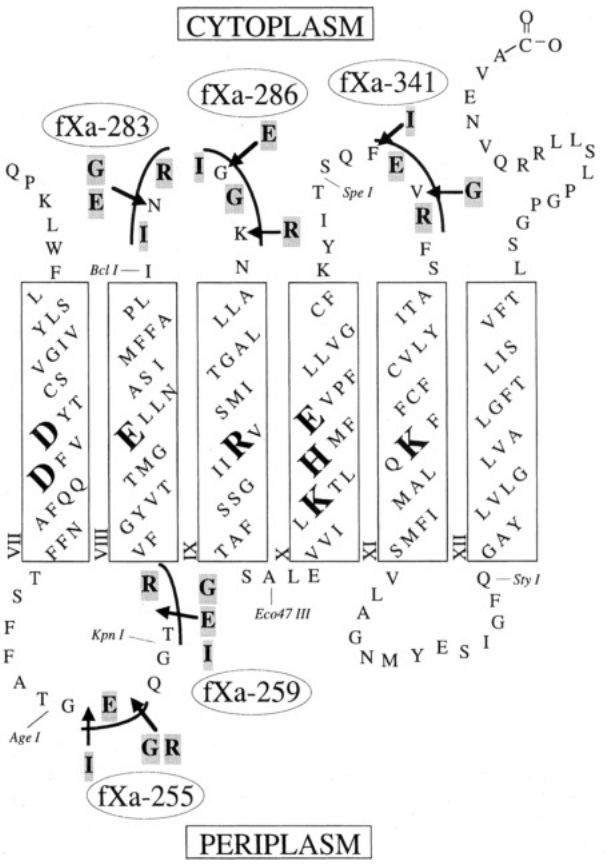

FIGURE 1: Secondary structure model of the C-terminal half of lac permease. The single-letter amino acid code is used, hydrophobic transmembrane helices are shown in boxes, and intramembrane charged amino acid residues are highlighted. The positions of single fXa restriction sites (Ile-Glu-Gly-Arg) engineered into hydrophilic loops 7, 8, and 10 are indicated. Also shown are the restriction endonuclease sites used for construction of mutants.

mM, final concentration), and the cultures were grown for an additional 2 h. Cells were harvested by centrifugation, washed with 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM $MgSO_4$, and assayed by rapid filtration (Consler et al., 1991).

Preparation of Membranes. Crude membrane fractions from *E. coli* T184 harboring plasmids with given mutations were prepared as described (Sahin-Tóth & Kaback, 1993a).

Proteolysis with fXa Protease and Immunological Analyses. Isolated membrane fractions were resuspended in 50 μL of fXa digestion buffer [100 mM NaCl/50 mM Tris-HCl (pH 8.0)/1 mM $CaCl_2$] containing 1% dodecyl β-D-maltoside (DM) unless stated otherwise. Lyophilized fXa enzyme (Boehringer Mannheim) was freshly reconstituted with distilled water and 3 μg per sample was added. Digestions were performed overnight (16−20 h) at 4 °C. Subsequently, 50 μL of 2× electrophoresis loading buffer was added, and samples were subjected to sodium dodecyl sulfate (Na-$DodSO_4$)−polyacrylamide gel electrophoresis (Newman et al., 1981). Proteins were electroblotted, and immunoblots were probed with site-directed polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984b).

Protein Determinations. Protein was assayed in the presence of $NaDodSO_4$ (Peterson, 1977).

RESULTS

Construction of fXa Sites. The four amino acid fXa protease recognition sequence Ile-Glu-Gly-Arg was engineered into hydrophilic loop 7 (fXa-255 and fXa-259), 8 (fXa-283 and fXa-286), or 10 (fXa-341) in the C-terminal half of C-less lac permease (Figure 1) as described in Materials and Methods. The nomenclature of the mutants denotes the position of the Ile residue in the polypeptide chain.

Cleavage Conditions. Previously (Consler et al., 1993), permease constructs containing an fXa site immediately preceding a biotin acceptor domain in hydrophilic domain 6 (L6XB) or at the C terminus (CXB) were described. In these chimeras, the fXa site is readily cleaved when the cytoplasmic face of the membrane is exposed to the protease. In the experiments presented in Figure 2, L6XB permease was used as a control to monitor activity of the fXa protease under the conditions used. To ensure maximal accessibility and efficiency of cleavage, proteolysis was carried out with

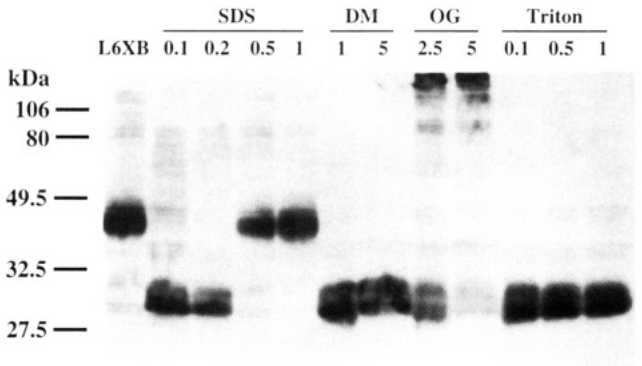

FIGURE 2: Effect of various detergents on the proteolysis of L6XB permease by fXa protease. *E. coli* T184 expressing L6XB permease (Consler et al., 1993) were induced with IPTG. Membranes were prepared, and approximately 100 μg of membrane protein was solubilized with the indicated detergent and incubated in the presence of fXa as described in Materials and Methods. Samples were subjected to $NaDodSO_4$−polyacrylamide gel electrophoresis and electroblotted, and the nitrocellulose paper was incubated with anti-C-terminal antibody. After incubation with horseradish peroxidase conjugated protein A followed by a short incubation with fluorescent substrate (Amersham), the nitrocellulose paper was exposed to film for 1 min. The first lane contains undigested L6XB permease (M_r ca. 48 kDa). SDS, sodium dodecyl sulfate; DM, dodecyl β-D-maltoside; OG, octyl β-D-glucopyranoside; Triton, Triton X-100. Numbers indicate final detergent concentrations in percent.

detergent-solubilized membrane preparations from cells expressing a given mutant, and various detergents were tested. Below the critical micelle concentration (0.1−0.2%), complete cleavage is observed in the presence of $NaDodSO_4$, but the detergent inactivates fXa above the critical micelle concentration (0.5−1%). High concentrations of DM (up to 5%) or Triton X-100 (up to 1%) do not affect the proteolytic activity of fXa. Although digestion is observed at 2.5% octyl β-D-glucoside, the detergent promotes strong aggregation, and minimal cleavage product is observed at 5%. On the basis of the observations, all subsequent fXa digestions were carried out on cytoplasmic membrane preparations solubilized in 1% DM (final concentration).

Properties of Mutants with Single fXa Sites. All five mutants with single fXa sites exhibit significant steady-state levels of lactose accumulation ranging from 30% to 70% of C-less permease (Figure 3). Furthermore, immunoblots on membrane fractions from T184 expressing the mutants reveal that all five are expressed at levels comparable to that of L6XB permease (Figure 4) or C-less permease (see Figure 5). The observations demonstrate that introduction of fXa cleavage sites into hydrophilic loop 7, 8, or 10 does not abolish activity and has essentially no effect on membrane insertion or stability of the permease. However, none of the mutants is proteolyzed to an appreciable extent by fXa protease under conditions where L6XB permease is almost completely cleaved. As shown on the overexposed immunoblot (Figure 4, arrow) traces of a C-terminal proteolytic fragment are detected when mutant fXa-255 is digested with fXa, indicating that this site is accessible, but the efficiency of proteolysis is extremely low. Increasing the DM concentration to 5% or supplementing the reaction mixture with 1% Triton X-100 and/or noninhibitory concentrations of $NaDodSO_4$ (see Figure 2) does not yield more cleavage product. Similarly, increasing the digestion time to 24 h, the temperature to 37 °C, or the fXa concentration to 5 μg/100 μg of membrane protein has no significant effect on the efficiency of proteolysis.

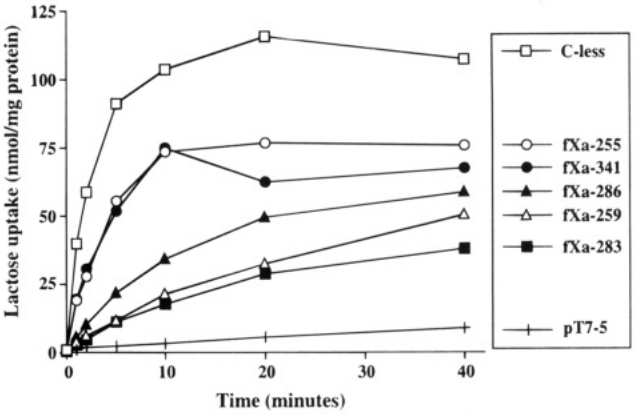

FIGURE 3: Transport of lactose by *E. coli* T184 harboring plasmids encoding C-less permease or mutants containing single fXa sites at positions 255, 259, 283, 286, and 341. Cells were grown at 37 °C, as described in Materials and Methods. Aliquots of cells (50 μL) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by the addition of [1-^{14}C]lactose (5 mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched at the times given by addition of 3.0 mL of 100 mM KPi (pH 5.5)/100 mM LiCl, and the reaction mixtures were rapidly filtered through Whatman GF/F filters.

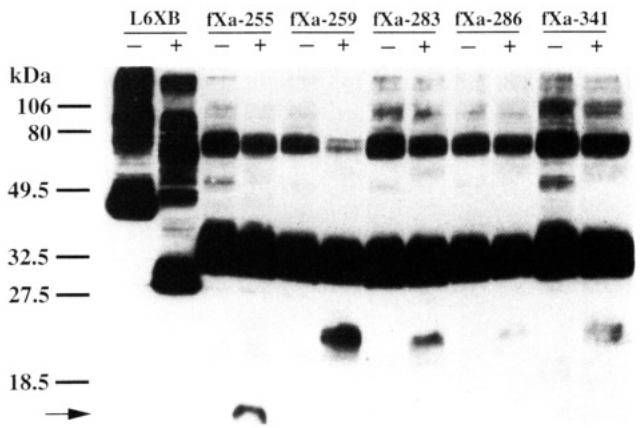

FIGURE 4: Effect of fXa treatment on mutants with single fXa sites. *E. coli* T184 expressing the given fXa site mutants were induced with IPTG. Membranes were prepared, and approximately 100 μg of membrane protein was solubilized with 1% DM (final concentration) and incubated in the presence (+) or the absence (−) of fXa as described in Materials and Methods. $NaDodSO_4$−polyacrylamide gel electrophoresis and Western blotting were performed as described in Materials and Methods and Figure 2. Uncut L6XB permease (lane 1) migrates at an M_r of approximately 48−50 kDa (Consler et al., 1993), and its C-terminal fXa cleavage product (lane 2) exhibits an apparent M_r of 28−30 kDa. Immunoreactive material migrating at higher M_r is the result of aggregation [see Consler et al. (1993)]. The broad intense band at an M_r of about 33 kDa in the remaining lanes is monomeric lac permease, and the immunoreactive bands observed with decreasing intensities at about 70 and over 100 kDa, respectively, are dimeric and trimeric aggregates; the bands at 22−24 kDa are nonspecific proteolytic fragments. The blot has been overexposed to demonstrate a specific fXa cleavage product (arrow) in lane 4.

Properties of Mutants with Tandem Recognition Sites at Position 255. A likely explanation for the inability of fXa to proteolyze the mutants with single fXa sites is inaccessibility of the cleavage sites to the enzyme. Therefore, two or three fXa sites in tandem were engineered into hydrophilic loop 7 at position 255. As shown in Figure 5, mutant 2fXa-255, which has two fXa sites in tandem, exhibits substantial

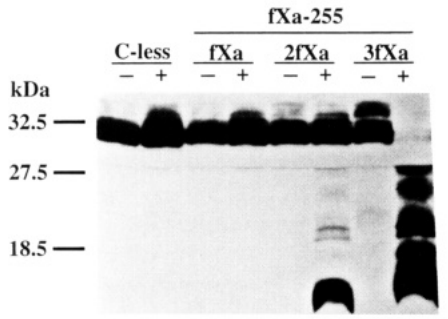

FIGURE 5: Site-specific proteolysis of permease mutants with single (fXa-255), double tandem (2fXa-255), or triple tandem (3fXa-255) fXa recognition sites at position 255. *E. coli* T184 expressing C-less permease or the given fXa site mutants were induced with IPTG. Membranes were prepared, and approximately 100 μg of membrane protein was solubilized with 1% DM (final concentration) and incubated in the presence (+) or the absence (−) of fXa as described in Materials and Methods. $NaDodSO_4$−polyacrylamide gel electrophoresis and Western blotting were performed as described in Materials and Methods and Figure 2.

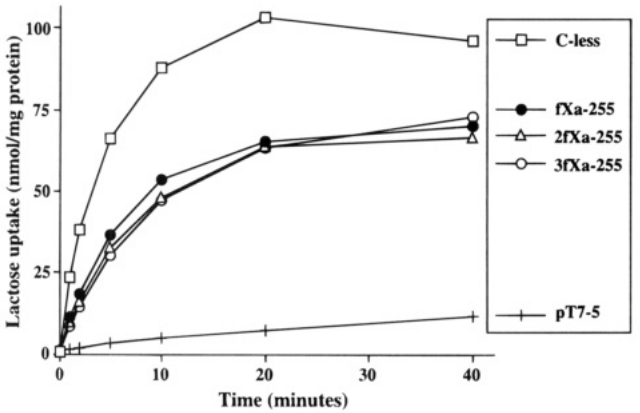

FIGURE 6: Active transport of lactose in *E. coli* T184 harboring plasmids encoding C-less permease or permease with single (fXa-255), double tandem (2fXa-255), or triple tandem (3fXa-255) fXa sites at position 255. Cells were grown and assayed as described in Figure 3.

proteolysis when treated with fXa under conditions where C-less permease or the single fXa-site mutant fXa-255 is not cleaved to any extent whatsoever. More dramatically, 3fXa-255 permease, a mutant with three fXa sites in tandem, is completely proteolyzed. Thus, upon treatment with fXa protease, essentially all of the full-length species at ca. 32.5 kDa is converted into cleavage products. Importantly, moreover, mutants with one, two, or three fXa sites at position 255 exhibit equally high transport activity, with rates approximating 50% of that of C-less permease and steady-state levels of accumulation of about 70% of control (Figure 6).

DISCUSSION

In this paper, the properties of mutants containing fXa recognition sites in hydrophilic loop 7, 8, or 10 of C-less lac permease are described. As demonstrated by insertional mutagenesis (McKenna et al., 1992), most of the hydrophilic loops in lac permease tolerate insertion of two or six His residues without drastic alteration in transport activity or expression. In agreement with these observations, mutants with fXa sites in hydrophilic loop 7, 8, or 10 exhibit significant transport activity, and levels of expression are comparable to that of C-less permease. Surprisingly, however, none of the mutants with a single fXa site are proteolyzed significantly by fXa protease, despite solubilization in various detergents, as well as efforts to optimize conditions by varying enzyme concentration, incubation time, or temperature. Insertion of two or particularly three fXa sites in tandem at position 255 (loop 7), however, results in markedly increased cleavage efficiency. There are at least two possible explanations for the observations; either or both of the following may apply: (i) The local substrate concentration for fXa (i.e., the number of recognition sites) is increased, resulting in an increased probability of cleavage. (ii) Multiple recognition sequences for fXa extend the size and hydrophilicity of the loop, making it more accessible to the aqueous phase and exposing the cleavage sites to the protease. Interestingly, a fortuitous 3fXa-255 mutant, in which the middle fXa site of the three is destroyed by a change of Arg to Ser, is readily digested by fXa (data not shown), indicating that increased accessibility is probably a key factor for efficient proteolysis. This conclusion is also consistent with the observation that L6XB permease, which contains a single fXa site followed by a hydrophilic biotin acceptor domain, is completely cleaved.

The primary aim of this work was to achieve controlled proteolysis of hydrophilic loops in lac permease, an approach which could greatly facilitate the study of helix−helix interactions by oxidation or chemical cross-linking of appropriately placed pairs of Cys residues. In addition, insertion of fXa sites in loops may be useful for probing surface topology. However, the possibility that hydrophilic loops in polytopic membrane proteins such as lac permease are buried and inaccessible to protease clearly poses a potentially serious problem. For example, it was reported recently (Hresko et al., 1994) that single fXa sites engineered into hydrophilic domains of the erythroid glucose facilitator GLUT1 are resistant to cleavage by fXa protease, and it was concluded that the approach is not viable for topological studies. In this respect, the use of multiple fXa sites in tandem offers a simple, straightforward solution to the problem and may provide a useful tool for structural studies on membrane proteins.

REFERENCES

Boyer, H. W., & Roulland-Dussoix, D. (1969) *J. Mol. Biol. 41,* 459−472.

Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 4937−4941.

Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79,* 6894−6898.

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

Carrasco, N., Viitanen, P., Herzlinger, D., & Kaback, H. R. (1984b) *Biochemistry 23,* 3681−3687.

Consler, T. G., Tsolas, O., & Kaback, H. R. (1991) *Biochemistry 30,* 1291−1298.

Consler, T. G., Persson, B. L., Jung, H., Zen, K., Jung, K., Privé, G. G., Verner, G. E., & Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 6934−6938.

Costello, M. J., Escaig, J., Matsushita, K., Viitanen, P. V., Menick, D. R., & Kaback, H. R. (1987) *J. Biol. Chem. 262,* 17072−17082.

Dornmair, K., Corin, A. S., Wright, J. K., & Jahning, F. (1985) *EMBO J. 4,* 3633−3638.

Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993a) *Biochemistry 32*, 3139−3145.

Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 12644−12650.

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

Frillingos, S., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Biochemistry 33*, 8074−8081.

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

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232−238.

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

Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221−229.

Hresko, R. C., Kruse, M., Strube, M., & Mueckler, M. (1994) *J. Biol. Chem. 269*, 20482−20488.

Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273−12277.

Jung, K., Jung, H., Colacurcio, P., & Kaback, H. R. (1995) *Biochemistry 34*, 1030−1039.

Kaback, H. R. (1989) *Harvey Lect. 83*, 77−105.

Kaback, H. R. (1992) in *International Review of Cytology* (Friedlander, M., & Mueckler, M., Eds.) pp 97−125, Academic Press, Inc., New York.

Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G., & Zen, K. (1993) *J. Bioenerg. Biomembr. 25*, 627−636.

Kaback, H. R., Frillingos, S., Jung, H., Jung, K., Privé, G. G., Ujwal, M. L., Weitzman, C., Wu, J., & Kaback, H. R. (1994) *J. Exp. Biol.* (in press).

King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177−186.

Lee, J.-I., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758−20764.

McKenna, E., Hardy, D., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11954−11958.

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

Page, M. G. P., & Rosenbusch, J. P. (1988) *J. Biol. Chem. 263*, 15906−15914.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346−356.

Poolman, B., & Konings, W. N. (1993) *Biochem. Biophys. Acta 1183*, 5−39.

Sahin-Tóth, M., & Kaback, H. R. (1993a) *Protein Sci. 2*, 1024−1033.

Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 10027−10035.

Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547−10551.

Sahin-Tóth, M., Persson, B., Schwieger, J., Cohan, P., & Kaback, H. R. (1994a) *Protein Sci. 3*, 240−247.

Sahin-Tóth, M., Lawrence, M. C., & Kaback. H. R. (1994b) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5421−5425.

Sahin-Tóth, M., Frillingos, S., Bibi, E., Gonzalez, A., & Kaback, H. R. (1994c) *Protein Sci.* (in press).

Sanger, F., & Coulson, A. R. (1978) *FEBS Lett. 87*, 107−110.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5468.

Seckler, R., & Wright, J. K. (1984) *Eur. J. Biochem. 142*, 268−279.

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

Seckler, R., Möröy, T., Wright, J. K., & Overath, P. (1986) *Biochemistry 25*, 2403−2409.

Stochaj, V., Bieseler, B., & Ehring, R. (1986) *Eur. J. Biochem. 158*, 423−428.

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

van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991) *Biochemistry 30*, 9595−9600.

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

BI942438F