Characterization of Purified, Reconstituted Site-directed Cysteine Mutants of the Lactose Permease of Escherichia coli

Iwaarden, Pierre R. van; Driessen, Arnold J.M.; Menick, Donald R.; Kaback, H. Ronald; Konings, Wilhelmus N.

Published in:
Biochemistry

Publication date:
1991

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 266, No. 24, Issue of August 25, pp. 15688–15692, 1991
Printed in U.S.A.

Characterization of Purified, Reconstituted Site-directed Cysteine Mutants of the Lactose Permease of *Escherichia coli**

(Received for publication, April 8, 1991)

Pierre R. van Iwaarden, Arnold J. M. Driessen, Donald R. Menick‡, H. Ronald Kaback§, and Wilhelmus N. Konings¶

From the Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands and the §Howard Hughes Medical Institute, Department of Physiology, Molecular Biology Institute, University of California, Los Angeles, California 90024-1570

***lac* permease mutated at each of the 8 cysteinyl residues in the molecule was solubilized from the membrane, purified, and reconstituted into proteoliposomes. The transport activity of proteoliposomes reconstituted with each mutant permease relative to the wild-type is virtually identical with that reported for intact cells and/or right-side-out membrane vesicles. Moreover, a double mutant containing Ser in place of both Cys^{148} and Cys^{154} exhibits significant ability to catalyze active lactose transport. The results provide strong confirmation for the contention that cysteinyl residues in *lac* permease do not play an important role in the transport mechanism. The effect of sulfhydryl oxidant 5-hydroxy-2-methyl-1,4-maphthoquinone on lactose transport in proteoliposomes reconstituted with wild-type or mutant permeases was also investigated, and the results indicate that inactivation is probably due to formation of a covalent adduct with Cys^{148} and/or Cys^{154} rather than disulfide formation. Thus, it seems unlikely that sulfhydryl-disulfide interconversion functions to regulate permease activity.**

The lactose (*lac*) permease of *Escherichia coli* is a hydrophobic polytopic cytoplasmic membrane protein that catalyzes concomitant translocation of β-galactosides and H^+ with a stoichiometry of 1:1 (*i.e.* lactose/H^+ cotransport or symport) (*cf.* Refs. 1–4 for reviews). Encoded by the *lacY* gene that has been cloned and sequenced, this prototype membrane transport protein has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and demonstrated to be solely responsible for β-galactoside transport. Based on circular dichroic measurements with purified permease and hydropathy analysis of the primary amino acid sequence (5), a secondary structure has been proposed in which the protein consists of 12 hydrophobic domains in α-helical conformation that traverse the membrane in zig-zag fashion, connected by hydrophilic segments with the N and C termini on the cytoplasmic surface (*cf.* Fig. 1). The general features of the model are consistent with other spectroscopic measurements (6),[1] chemical modification (7), limited proteolysis (8, 9), and immunological studies (10–16). Furthermore, recent studies (17) on an extensive series of *lac* permease-alkaline phosphatase (*lac-Y-phoA*) fusion proteins provide strong and exclusive support for the topological predictions of the 12-helix model.

As demonstrated initially by Fox *et al.* (18) and Kennedy *et al.* (19), the *lac* permease is irreversibly inactivated by *N*-ethylmaleimide, and protection is afforded by substrates such as β,D-galactopyranosyl-β,D-thiogalactopyranoside. On the basis of these findings, it was postulated (18) that a cysteinyl residue is at or near the substrate-binding site of *lac* permease. Beyreuther *et al.* (20) later showed that the substrate-protectable residue is Cys^{148}. In addition, the permease is reversibly inactivated by other sulfhydryl reagents such as *p*-chloromercuribenzenesulfonate or by sulfhydryl oxidants such as diamide (21) or plumbagin[2] (22), and β,D-galactopyranosyl-β,D-thiogalactopyranoside prevents inactivation by these reagents. Because of the latter observations, it was suggested (22, 23) that permease activity may be regulated by a redox process involving sulfhydryl-disulfide interconversion.

Given the importance attributed to sulfhydryl groups in *lac* permease (*cf.* Ref. 24 in addition to arguments presented above), particularly Cys^{148}, site-directed mutagenesis was used to replace Cys^{148} with Gly (25, 26) or Ser (27, 28). Surprisingly, although Cys^{148} is required for substrate protection against alkylation by *N*-ethylmaleimide, it is not important for lactose/H^+ symport. Subsequently, it was shown (2, 29) that replacement of Cys^{154} with Gly leads to complete loss of transport activity, although the permease retains the ability to bind the high affinity ligand *p*-nitrophenyl-α,D-galactopyranoside. Moreover, replacement of Cys^{154} with Ser or Val yields permease with 10% or 30%, respectively, of wild-type activity, indicating that, although Cys^{154} is important for full activity, it is not mandatory. Finally, Brooker and Wilson (30) replaced Cys^{176} or Cys^{234} with Ser, and Menick *et al.* (31) replaced Cys^{117}, Cys^{333} or Cys^{353} and Cys^{355} with Ser and observed little or no effect on permease activity. Therefore, out of a total of 8 cysteinyl residues in the permease, only Cys^{154} appears to be important for transport, and even this residue is not absolutely essential.

Although the results from site-directed mutagenesis provide strong evidence that cysteinyl residues in the permease do not play a direct role in the mechanism, alkylation of Cys^{148}

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Cardiology Division, Dept. of Medicine, and the Gazes Cardiac Research Institute, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425.

¶ To whom correspondence should be addressed.

[1] In addition to circular dichroism and laser Raman spectroscopy, Fourier transform infrared studies also show that purified *lac* permease is largely helical (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished work).

[2] The abbreviations used are: plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone; DTT, dithiothreitol.

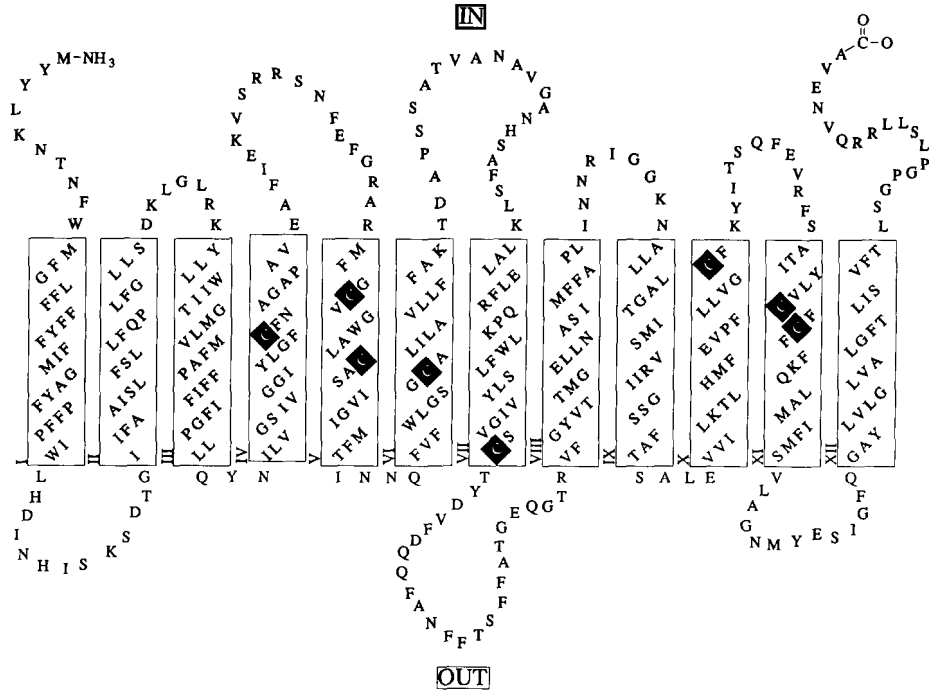

FIG. 1. **Secondary structure model of *lac* permease based on the hydropathy of the deduced amino acid sequence (5) with cysteinyl residues (C) highlighted.** The single-letter amino acid code is used.

or replacement of Cys[154] with Gly leads to inactivation. Furthermore, Cys[148] and Cys[154] are both located in putative transmembrane helix V (Fig. 1) and are predicted to be on the same face. Therefore, an attractive explanation for inactivation by sulfhydryl oxidants is catalysis of disulfide formation between these 2 residues. In order to test this possibility, we have solubilized and purified each site-directed Cys mutant in the permease, as well as a double mutant containing Ser in place of both Cys[148] and Cys[154], and studied transport activity and inactivation by plumbagin in reconstituted proteoliposomes. The results indicate that permease inactivation by the sulfhydryl oxidant results from formation of a covalent adduct with either Cys[148] or Cys[154] and not from disulfide formation.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]Lactose (57 mCi/mmol) was obtained from Amersham (Buckinghamshire, U. K.). All other reagents were obtained as described (32, 33) and were reagent grade.

Methods

Bacterial Strains and Plasmids—*E. coli* T206, *E. coli* T184, and plasmid pGM21 encoding wild-type *lac-Y* (34) or *lac-Y* with site-directed mutants in single Cys codons (25–31) have been described.

Construction of C148S/C154S[3] *Double Mutant*—Starting with the M13 mp18 (*lac-Y*) DNA containing a Ser codon at position 148 (25), the double mutant containing C148S/C154S was constructed as described by Menick *et al.* (29). Subsequently, mutations were confirmed by sequencing the entire *lac-Y* gene in single-stranded M13 mp18 DNA and the region containing the mutations in plasmid pGM21. In both cases, with the exception of the mutations described, the remainder of the *lac-Y* sequence was identical with that reported by Büchel *et al.* (35).

Cell Growth—*E. coli* T184 transformed with a given plasmid was grown and induced with isopropyl-1-thio-β,D-galactopyranoside as described by Teather *et al.* (34), with the exception that 0.2–0.5% casamino acid was used in place of methionine and threonine in the growth medium (36).

Purification and Reconstitution of lac Permease—Wild-type and mutant *lac* permeases were solubilized, purified, and reconstituted into proteoliposomes as described (32), except that the C148S and C148G permeases were reconstituted into proteoliposomes by overnight dialysis instead of detergent dilution. In addition, after freeze-thawing, the proteoliposomes were not sonified, but extruded through polycarbonate filters of 100-nm pore size, using an extrusion device (Lipex Biomembranes, Vancouver). This procedure yields proteoliposomes of a relatively uniform size distribution (37). The final preparations contained proteoliposomes at 37.5 mg of *E. coli* phospholipids/ml and 40–90 μg/ml of protein, as indicated, in 50 mM KP_i, pH 7.5, 1.0 mM DTT.

Transport Assays—Membrane potential (ΔΨ)-driven lactose transport in proteoliposomes reconstituted with purified permeases was assayed in the presence of a potassium diffusion potential (interior negative) as described (32).

Protein Determinations—Protein was determined by the method of Schaffer and Weissmann (38).

RESULTS

ΔΨ-Driven Lactose Accumulation—As demonstrated previously (39–42), proteoliposomes reconstituted with purified *lac* permease rapidly accumulate lactose against a concentration gradient in the presence of the potassium diffusion gradient ($K_{in} \rightarrow K_{out}$) and the ionophore valinomycin (Fig. 2). Under identical conditions, proteoliposomes reconstituted with purified C117S, C148S, C176S, C234S, C333S, or C353S/C355S transport lactose at initial rates to steady-state levels of accumulation that are comparable with proteoliposomes reconstituted with wild-type permease (*cf.* Table I for quantitation of initial rates relative to wild-type). In contrast, proteoliposomes reconstituted with C148G, C154S, or C154V transport lactose at initial rates that are about 15, 10, and 35% respectively, of wild-type proteoliposomes (Fig. 2 and Table I). In each instance, the level of accumulation continues to increase as the time course of the experiments is extended (Fig. 2; data not shown). Finally, proteoliposomes reconstituted with C148S/C154S permease transport at about 5% the rate of wild-type but continue to accumulate the disaccharide with time, whereas proteoliposomes reconstituted with C154G

[3] Site-directed mutants are designated as follows. The one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type *lac* permease. This is followed by a second letter denoting the amino acid replacement at this position (*e.g.* C148S designates that Cys[148] is replaced by Ser).

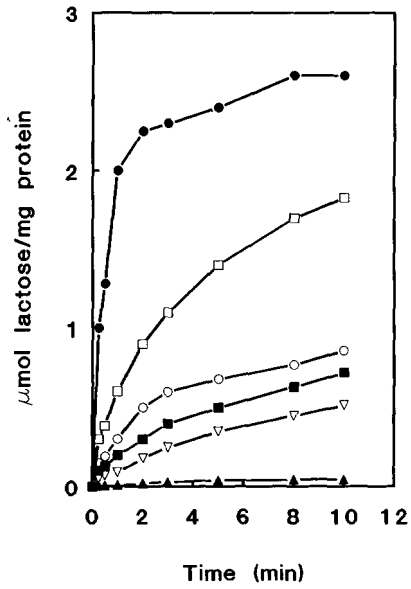

FIG. 2. **Membrane potential (ΔΨ)-driven lactose transport in proteoliposomes reconstituted with purified wild-type (●), C148G (○), C154G (▲), C154S (■), C154V (□) or C148S/C154S (▽) *lac* permease.** *lac* permease was purified from membranes of *E. coli* T206 or T184 transformed with the appropriate plasmid and reconstituted into proteoliposomes at a protein concentration of 85 μg/ml (wild-type), 75 μg/ml (C148G), 45 μg/ml (C154G), 70 μg/ml (C154S), 65 μg/ml (C154V), or 40 μg/ml (C148S/C154S). Lactose transport was measured as described (32) by diluting 1 μl of proteoliposomes containing 20 μM valinomycin into 200 μl of 50 mM sodium phosphate (pH 7.5) containing 35 μM [1-^{14}C]lactose (57 mCi/mmol). Control experiments (▲) were performed by diluting 1 μl of proteoliposomes into 50 mM potassium phosphate (pH 7.5).

TABLE I

Initial rate of ΔΨ-driven lactose transport in proteoliposomes reconstituted with wild-type or mutant lac permease

Permease	Initial rate of transport[a]
	% of wild-type activity
Wild-type[b]	100
C117S	75
C148S	85
C148G	15
C154S	10
C154G	<1
C154V	35
C148S/C154S	5
C176S	100
C234S	90
C333S	95
C353S/C355S	61

[a] Initial rates of transport were calculated from the initial linear portion of transport curves (*cf.* Fig. 2).

[b] The turnover number for wild-type permease in this preparation was 16 s^{-1}.

permease are essentially devoid of transport activity (Fig. 2; Table I).

Effect of Plumbagin—Treatment of right-side-out membrane vesicles with the sulfhydryl oxidant plumbagin at a concentration of 0.5 mM leads to complete inactivation of lactose transport in a manner that can be restored by 10 mM DTT (22). Similar phenomena are observed in proteoliposomes reconstituted with purified *lac* permease. Thus, ΔΨ-driven lactose transport is progressively inactivated by relatively low concentrations of plumbagin, and maximal inactivation is observed at about 0.5 mM (Figs. 3 and 4*A*). Moreover, when 10 mM DTT is added after treatment of the proteoliposomes with plumbagin, transport is restored to a great extent (Fig. 4*A*; Table II). Parenthetically, when the proteoliposomes are washed and resuspended in buffer without addition of DTT, *lac* permease is apparently slightly inactivated by air oxidation, as evidenced by the observation that addition of DTT by itself causes a small stimulation of transport activity.

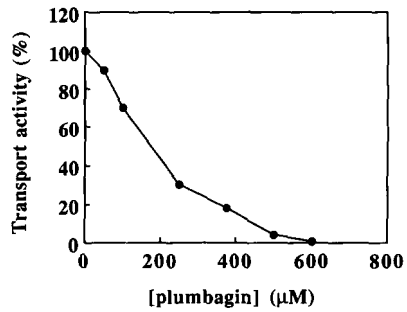

FIG. 3. **Titration of lactose transport with plumbagin in proteoliposomes reconstituted with purified wild-type *lac* permease.** Proteoliposomes were incubated at room temperature for 30 min with different concentrations of plumbagin. Subsequently, the initial rate of ΔΨ-driven lactose transport was measured as in Table I.

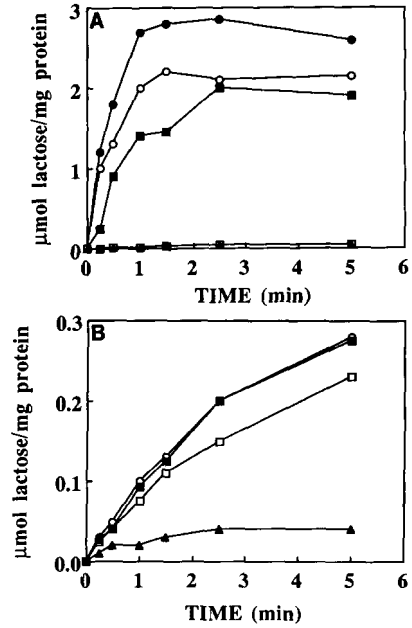

FIG. 4. **Effect of 0.5 mM plumbagin and 10 mM DTT on lactose transport in proteoliposomes reconstituted with purified wild-type permease (*A*) or C148S/C154S permease (*B*).** Proteoliposomes were incubated at room temperature for 30 min with or without 0.5 mM plumbagin and then incubated for 30 min with or without 10 mM DTT as indicated. Subsequently, ΔΨ-driven lactose transport was measured as in Fig. 2 with an external concentration of 35 μM [1-^{14}C] lactose (57 mCi/mmol). □, plumbagin; ■, plumbagin followed by DTT; ● 10 mM DTT; ○, no additions; ▲, control experiments, performed by diluting 1 μl of proteoliposomes into 50 mM potassium phosphate (pH 7.5).

Results of similar experiments carried out on proteoliposomes reconstituted with each mutant permease are summarized in Table II. The initial rate of ΔΨ-driven lactose transport in proteoliposomes reconstituted with C117S, C148S, C154V, C176S, C234S, C333S, or C353S/C355S is essentially completely inactivated by treatment with 0.5 mM plumbagin, and activity is restored by addition of 10 mM DTT in each instance. In contrast, C148G or C154S permease is inactivated by only about 50%, and strikingly, C148S/C154S permease

TABLE II

Inhibition of the initial rate of ΔΨ-driven lactose transport with 0.5 mM plumbagin in proteoliposomes reconstituted with mutant permeases and the restoration of activity by DTT

Initial rates of lactose transport were measured as in Table I. Proteoliposomes were treated with plumbagin and DTT as described in the legend to Fig. 4.

Permease	Residual activity after plumbagin inhibition	Restoration of activity by DTT
	%	%
Wild-type	0	90
C117S	8	85
C148S	0	95
C148G	48	70
C154S	50	80
C154V	7	100
C148S/C154S	80	100
C176S	0	90
C234S	0	85
C333S	0	78
C353S/C355S	0	100

(Fig. 4*B*) is only marginally inactivated by the sulfhydryl oxidant. Although the transport activity of C148G, C154S, or C148S/C154S permease is low, accumulation of lactose in proteoliposomes reconstituted with these permeases clearly increases with time before and after treatment with plumbagin (Fig. 4*B*). In contrast, proteoliposomes reconstituted with wild-type permease or any of the other mutants exhibit no accumulation of lactose after treatment with plumbagin (Fig. 4*A*).

DISCUSSION

The data presented in this paper provide strong confirmation of the conclusion that cysteinyl residues in *lac* permease do not play a direct role in the mechanism of lactose/H^+ symport (2, 25–31). Proteoliposomes reconstituted with purified permease molecules containing mutations at each individual cysteinyl residue exhibit transport activities relative to wild-type proteoliposomes that are virtually identical with those reported for intact cells and/or right-side-out membrane vesicles. Specifically, purified permease with C117S, C148S, C176S, C234S, C333S, or C353S/C355S exhibits small if any defects in ΔΨ-driven lactose accumulation, permease with C148G, C154S, or C154V exhibits reduced rates of lactose uptake, and C154G permease is devoid of transport activity. Importantly, the double mutant C148S/C154S exhibits low but significant activity. Clearly, therefore, although Cys^{148} is important for substrate protection against *N*-ethylmaleimide inactivation (18, 20), Cys^{154} is the only cysteinyl residue in the permease that is important for activity; but even this residue is not obligatory.

Although cysteinyl residues do not play a direct role in the mechanism of *lac* permease, based on the observation that sulfhydryl oxidants reversibly inactivate lactose/H^+ symport, it was suggested that sulfhydryl-disulfide interconversion may have a regulatory function (22). Since alkylation of Cys^{148} or replacement of Cys^{154} with Gly leads to inactivation and these residues should be on the same face of putative helix V, the effect of the sulfhydryl oxidant plumbagin on lactose transport by proteoliposomes reconstituted with each site-directed Cys mutant was investigated. As discussed, one possible mechanism of inactivation is that the sulfhydryl oxidant catalyzes disulfide formation between Cys^{148} and Cys^{154}. Alternatively, since modification of either Cys^{148} or Cys^{154} alone can lead to inactivation, it is possible that disulfide formation between either of these residues and another cysteinyl residue in the permease causes inactivation. Finally, sulfhydryl oxidants may undergo "half-reactions" with cysteinyl residues (21, 43, 44), thereby forming sulfhydryl adducts without catalyzing disulfide formation. Our results favor the last possibility for the following reasons. (i) Plumbagin treatment leads to a marked inactivation of C117S, C176S, C234S, C333S, or C353S/C355S permease, suggesting that none of these cysteinyl residues is involved in the phenomenon. Consequently, it is unlikely that mixed disulfide formation between Cys^{148} or Cys^{154} and any of the other cysteinyl residues in the permease is responsible for inactivation. (ii) The double mutant C148S/C154S, which has a low but significant activity, is resistant to inactivation by plumbagin, thereby demonstrating directly that Cys^{148} and/or Cys^{154} are the residues involved. (iii) The single mutants C148S and C154V are essentially completely inactivated by the sulfhydryl oxidant, whereas C148G or C154S permease are partially inactivated. Thus, a single cysteinyl residue at either of these positions is sufficient for inactivation by plumbagin, a result not expected if disulfide formation between the residues is required for inactivation. In conclusion, since it is unlikely that permease inactivation by sulfhydryl oxidants involves the formation of disulfide bonds, sulfhydryl-disulfide interconversion probably does not have a regulatory function in the activity of this transport protein.

Acknowledgments—We wish to thank Dr. J. S. Lolkema and Dr. M. G. P. Page for helpful discussions and M. Y. D. van Iwaarden for typing the manuscript.

REFERENCES

1. Kaback, H. R. (1983) *J. Membr. Biol.* **76,** 95
2. Kaback, H. R. (1989) *The Harvey Lect.* **83,** 77
3. Kaback, H. R. (1990) *Microbiol. Membr. Transp. Syst.* **326,** 425
4. Wright, J. K., Seckler, R. & Overath, P. (1986) *Annu. Rev. Biochem.* **55,** 225
5. Foster, D. L., Boublik, M. & Kaback, H. R. (1983) *J. Biol. Chem.* **258,** 31
6. Vogel, H., Wright, J. K. & Jähnig, F. (1985) *EMBO J.* **4,** 3625
7. Page, M. G. P., Rosenbusch, J. P. & Yamato, I. (1988) *J. Biol. Chem.* **263,** 15897
8. Goldkorn, T., Rimon, G. & Kaback, H. R. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3322
9. Stochaj, V., Bieseler, B. & Ehring, R. (1986) *Eur. J. Biochem.* **158,** 423
10. Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T. & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 6894
11. Carrasco, N., Viitanen, P., Herzlinger, D. & Kaback, H. R. (1984) *Biochemistry* **23,** 3681
12. 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
13. Seckler, R., Wright, J. K. & Overath, P. (1983) *J. Biol. Chem.* **258,** 10817
14. Seckler, R. & Wright, J. K. (1984) *Eur. J. Biochem.* **142,** 269
15. Seckler, R., Möröy, T., Wright, J. K. & Overath, P. (1986) *Biochemistry* **25,** 2403
16. Herzlinger, D., Viitanen, P., Carrasco, N. & Kaback, H. R. (1984) *Biochemistry* **23,** 3688
17. Calamia, J. & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4937
18. Fox, C. F. & Kennedy, E. P. (1965) *Proc. Natl. Acad. Sci. U. S. A.* **51,** 891
19. Kennedy, E. P., Rumley, M. K. & Armstrong, J. B. (1974) *J. Biol. Chem.* **249,** 33
20. Beyreuther, K., Bieseler, B., Ehring, R. & Müller-Hill, B. (1981) in *Methods in Protein Sequence Analysis* (Elzina, M., Ed.) pp. 139–148, Humana, Clifton, NY
21. Kaback, H. R. & Patel, L. (1978) *Biochemistry* **17,** 1640
22. Konings, W. N. & Robillard, G. T. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 5480
23. Robillard, G. T. & Konings, W. N. (1982) *Eur. J. Biochem.* **127,** 597

24. Kaback, H. R. & Barnes, E. M. (1971) *J. Biol. Chem.* **246,** 5523
25. Trumble, W. R., Viitanen, P. V., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1984) *Biochem. Biophys. Res. Commun.* **119,** 860
26. Viitanen, P., Menick, D. R., Sarkar, H. K., Trumble, W. R. & Kaback, H. R. (1985) *Biochemistry* **24,** 7628
27. Neuhaus, J.-M., Soppa, J., Wright, J. K., Riede, I., Blockere, H., Frank, R. & Overath, P. (1985) *FEBS Lett.* **185,** 83
28. Sarkar, H. K., Viitanen, P. V., Padan, E., Trumble, W. R., Poonian, M. S. & Kaback, H. R. (1986) *Methods Enzymol.* **125,** 214
29. Menick, D. R., Sarkar, H. K., Poonian, M. S. & Kaback, H. R. (1985) *Biochem. Biophys. Res. Commun.* **132,** 162
30. Brooker, R. J. & Wilson, T. H. (1986) *J. Biol. Chem.* **261,** 11765
31. Menick, D. R., Lee, J. A., Brooker, R. J., Wilson, T. H. & Kaback, H. R. (1987) *Biochemistry* **26,** 1132
32. Viitanen, P., Newman, M. J., Foster, D. J., Wilson, T. H. & Kaback, H. R. (1986) *Methods Enzymol.* **125,** 429
33. Sarkar, H. K., Menick, D. R., Viitanen, P. V., Poonian, M. S. & Kaback, H. R. (1986) *J. Biol. Chem.* **261,** 8914
34. Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Fürst, M., Aichele, G., Wilhelm, U. & Overath, P. (1980) *Eur. J. Biochem.* **108,** 223
35. Büchel, D. E., Groneborn, B. & Müller-Hill, B. (1980) *Nature* **283,** 541
36. Page, M. G. P. & Rosenbusch, J. P. (1988) *J. Biol. Chem.* **263,** 15906
37. Hope, M. J., Bally, M. B., Webb, G. & Cullis, P. R. (1985) *Biochem. Biophys. Acta* **812,** 55
38. Schaffner, W. & Weissmann, C. (1973) *Anal. Biochem.* **56,** 502
39. Newman, M. J., Foster, D., Wilson, T. H. & Kaback, H. R. (1981) *J. Biol. Chem.* **256,** 11804
40. Foster, D. L., Garcia, M. L., Newman, M. J., Patel, L. & Kaback, H. R. (1982) *Biochemistry* **21,** 5634
41. Viitanen, P., Garcia, M. L., Foster, D. L., Kaczorowski, G. J. & Kaback, H. R. (1983) *Biochemistry* **22,** 2531
42. Viitanen, P., Garcia, M. L. & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1629
43. Page, M. G. P. & West, I. C. (1986) *Biochem. Biophys. Acta* **858,** 67
44. Nickerson, W. J., Falcone, G., and Strauss, G. (1963) *Biochemistry* **2,** 537–545