Involvement of Histidine-21 in the pH-Induced Switch in Porin Channel Size

Jill C. Todt and Estelle J. McGroarty*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

Received April 3, 1992; Revised Manuscript Received July 21, 1992

ABSTRACT: Porin is a channel-forming protein in the outer membrane of Gram-negative bacteria. In the previous paper (Todt et al., 1992), we showed that the pH induced a switch in the channel size in vitro for the porins OmpF, OmpC, and PhoE. In the results presented here, His21 of OmpC and OmpF from *Escherichia coli* was chemically modified with diethyl pyrocarbonate. Functional analysis of these modified porins at different pHs suggested that this histidine is involved in the pH-induced switch in channel size. Secondary structure analysis of porins at various pHs using Fourier transform infrared spectroscopy indicated that there was no global change in structure accompanying the pH-induced switch in channel size.

The outer membrane of Gram-negative bacteria contains trimeric channel-forming proteins called porins through which influx of nutrients and antibiotics occurs. The structure and function of bacterial porins have been reviewed extensively (Benz, 1985; Nikaido & Vaara, 1985; Rosenbusch, 1990; Tommassen, 1988); however, the exact mechanism of the regulation of porin function has yet to be defined. In recent functional studies, we found that porins of *Escherichia coli* K-12 can be stabilized in at least two open-channel configurations in vitro: a small channel detected at acidic pH and a larger channel stabilized under basic conditions [see Todt et al. (1992)]. The pK_a of the channel-size switch suggested the involvement of the single histidine present in both OmpC and OmpF (His21). In this study, we present further evidence for the involvement of histidine in the pH-induced functional changes by chemically modifying the residue with diethyl pyrocarbonate (DEPC).[1] Furthermore, using Fourier transform infrared spectroscopy (FTIR), we show that this functional change does not involve a structural alteration of a global nature since there is no change in porin secondary structure with pH.

MATERIALS AND METHODS

Cell Growth. LPS-enriched OmpC and OmpF were isolated from *E. coli* K-12 strains ECB 621 ($ompF^-$, $lamB^-$; gift of S. Benson) and PLB 3261 ($ompC^-$, $lamB^-$; Benson & Decloux, 1985), respectively. Cultures were grown in 1% tryptone, 0.5% yeast extract, and 0.4% NaCl, pH 7.5, as described previously (Rocque & McGroarty, 1989, 1990; Xu et al., 1986). Cells producing OmpF were grown at 37 °C, while cells producing OmpC were grown at 30 °C. Cells were harvested in late-logarithmic phase.

Porin Isolation. Porins were isolated by the method of Lakey et al. (1985) with some modifications (Rocque & McGroarty, 1989; Xu et al., 1986). Cells were broken using a French pressure cell and treated with RNase and DNase. After the membranes were pelleted at 100000*g*, the inner and some outer membrane proteins were dissolved with sodium dodecyl sulfate (SDS) in Tris-HCl. After centrifugation, the pellet contained outer membrane proteins, primarily porin, bound to the peptidoglycan. The porins were solubilized with high NaCl concentrations in the presence of mercaptoethanol, Tris-HCl, and SDS. The solubilized porin was dialyzed and precipitated with 90% acetone. Homogeneity of porin was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using the method of Laemmli (1970). The levels of LPS bound to porin isolates were quantitated by the thiobarbiturate assay for 2-keto-3-deoxyoctulosonic acid (Droge et al., 1970). The final preparations were suspended in 1% SDS, 10 mM Tris-HCl, pH 6.8, and 0.02% sodium azide (standard buffer). To adjust the pH of the porin samples for FTIR studies, samples were dialyzed against 1.0% SDS containing either 10 mM phosphate (for pH 5.8) or 10 mM CHES (for pH 9.25). For bilayer lipid membrane (BLM) studies, the samples were dialyzed against 0.5% SDS and 20 mM phosphate, pH 6.0 (phosphate–SDS buffer). Porin was quantitated using the bicinchoninic acid protein assay (Pierce Chemical Co.), and porin trimer structure was confirmed by SDS–PAGE (Rocque et al., 1987).

Carbethoxylation and Decarbethoxylation of Porin. Carbethoxylation of porin with DEPC was performed in phosphate–SDS buffer using the method of Bindslev and Wright (1984) with modifications. A DEPC stock solution was prepared immediately prior to use by diluting an aqueous solution of DEPC (Sigma) with equal volumes of anhydrous ethanol and determining the DEPC concentration by quantitative dilution of a small aliquot (1–5 μL) into 3 mL of 10 mM imidazole, pH 7.5. The absorbance of this solution at 230 nm was converted to DEPC concentration using an extinction coefficient of 3000 cm^{-1} M^{-1}. OmpC and OmpF (1 mL of a 0.5–2 mg/mL solution) were modified by the addition of a small volume (1–2 μL) of DEPC stock solution to a final concentration of between 0.18 and 4 mM (18.5–80 mol of DEPC/mol of protein). Carbethoxylation of porin's histidine was detected at 246 nm and converted to concentration using an extinction coefficient of 3200 cm^{-1} M^{-1}. Tyrosine modification was monitored at 278 nm. Analysis was performed on modified porin only after determining by the absorbance at 246 nm that one residue was modified per monomer of porin. This modified residue was stable for at least 3 h at pH 5.6 and 8.0. The carbethoxy group was removed from the modified OmpC and OmpF by the addition of hydroxylamine (in 20 mM phosphate and 0.5% SDS, pH 6.8) to a final concentratino of 20 mM. The reaction was allowed to proceed until the number of residues modified (as detected at 246 nm) was close to 0 (2–10 min). The reaction was

* Correspondence should be addressed to this author. Phone: (517) 353–8949.

[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; DEPC, diethyl pyrocarbonate; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; BLM, bilayer lipid membrane assay.

0006-2960/92/0431-10479$03.00/0 © 1992 American Chemical Society

stopped by dilution of porin with phosphate–SDS buffer to a concentration of 10 μg/mL.

Bilayer Lipid Membrane Assay (BLM). Analysis of electrical conductance across a bilayer lipid membrane was used to measure the channel size of modified and unmodified porins. The electrical conductance was measured across a lipid bilayer comprised of 1% diphytanoylphosphatidylcholine (in *n*-decane). As described previously (Rocque & McGroarty, 1989, 1990), a small volume of porin suspended in standard buffer was added to the salt solution bathing the lipid membrane. This bathing solution contained 0.5 M NaCl and 0.5 mM buffer at an appropriate pH. It was difficult to maintain the pH precisely during the time period of the BLM analysis; however, the pH was controlled to within 0.1 pH unit of the original pH. Silver–silver chloride electrodes were placed on either side of the membrane, and a constant voltage was applied using a 1.5-V battery. Changes in current were amplified using a Keithley Model 614 electrometer and recorded. The changes in current were reported as the size parameter λ/σ (channel conductance increment/bathing solution's specific conductance) versus the probability of the occurrence of an event with a particular size. A statistically significant number of channels (≥200) were analyzed for each experimental condition.

FTIR. For FTIR measurements, porin samples were diluted to a concentration of 2 mg/mL and analyzed along with control samples lacking the protein but containing LPS at concentrations equivalent to the amount present in porin samples. The porin and control samples were dialyzed against either pH 5.8 or pH 9.25 buffer. The samples were then lyophilized and resuspended in D_2O. For FTIR analysis, samples were placed in a CaF_2 Harrick cell. FTIR spectra were recorded on a Nicolet 710 spectrophotometer, and an atmospheric background was recorded before sample scanning. Eight hundred interferograms were averaged, apodized with a triangular squared function, and Fourier-transformed to a resolution of 2 cm^{-1}. The spectrum of the control sample at the appropriate pH was subtracted from each sample spectrum. A BP Decon self-deconvolution program was used to deconvolute each background-subtracted spectrum and to obtain intensity values for each peak. Two constants required as computer input were σ, the estimated half-width at half-height of unresolved bands, and k, the resolution "efficiency" factor. Values entered for σ and k were 90 cm^{-1} and 2.3, respectively.

RESULTS

In previous work, we have found that a switch in porin channel size may be induced by the titration of a single group with an apparent pK_a of 7.2 for OmpF and of 6.5 for OmpC. Since these pK_a's are close to that of a histidine side chain (pK_a 6.0), of which OmpF and OmpC contain only one, porin was modified with DEPC, and the effects on function were examined. DEPC has been used to specifically modify histidine to study its function in proteins (Bindslev & Wright, 1984; Blanke & Hager, 1990; Miles, 1977; Sams & Matthews, 1988; Takeuchi et al., 1986). Unmodified and DEPC-modified OmpF (Figure 1) and OmpC (Figure 2) were analyzed at different pHs in the BLM apparatus, measuring channel conductance. As seen in the previous paper (Todt et al., 1992), there is an increase in the proportion of larger-size channels ($\lambda/\sigma \sim 3.1$–3.5 Å for OmpF and ~ 1.9–2.5 Å for OmpC) and a decrease in the relative number of small channels ($\lambda/\sigma \sim 1.55$ Å for OmpF and ~ 0.9 Å for OmpC) at basic pH. In contrast, histograms of DEPC-modified OmpC and OmpF showed a high proportion of larger-size channels at both acidic and basic pH, suggesting that the modified residue was involved with controlling the pH-induced switch in channel size. There was a slight increase in the number of small-size channels with DEPC-modified OmpF at pH 8.0 compared to wild type at pH 8.15 which was probably due to that fact that the pH was slightly lower. Removal of the carbethoxy group from histidine (with hydroxylamine) restored the small-channel configuration at low pH (Figures 1C and 2C).

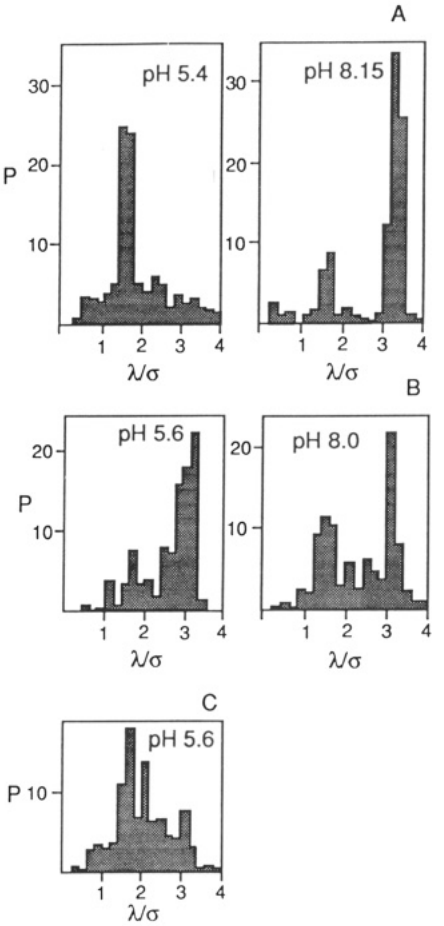

FIGURE 1: Probability distribution histograms of the size parameter λ/σ (Å) for OmpF (A), DEPC-modified OmpF (B), and DEPC-modified OmpF treated with 20 mM hydroxylamine (C) as measured in bilayer lipid membranes. The porins were added to bathing solutions of 05. M NaCl containing 0.5 mM sodium phosphate. Electrical conductance was measured using a transmembrane potential of 25 mV. λ is the channel conductance increment, and σ is the specific conductance of the bathing solution. P in arbitrary units is the relative number of events with a given size parameter range. Both opening and closing events are included in the histograms of ≥200 events.

To determine whether the pH-induced switch in channel size involved global structural changes, FTIR analysis of porins at various pHs was performed. FTIR spectra have been used to measure structural changes in proteins (Alvarez et al., 1987; Haris et al., 1989; Susi et al., 1967; Wantyghem et al., 1990). The secondary structure of porin can be measured by examining the amide I region (1620–1690 cm^{-1}) of the absorption spectra. Amide I peaks of OmpC and OmpF were analyzed at pH 5.8 and 9.25 after suspending the samples in D_2O. When porin samples at pH 5.8–9.25 were compared, the deconvoluted amide I peaks were essentially identical (Figure 3), indicating little change in secondary structure in this pH range.

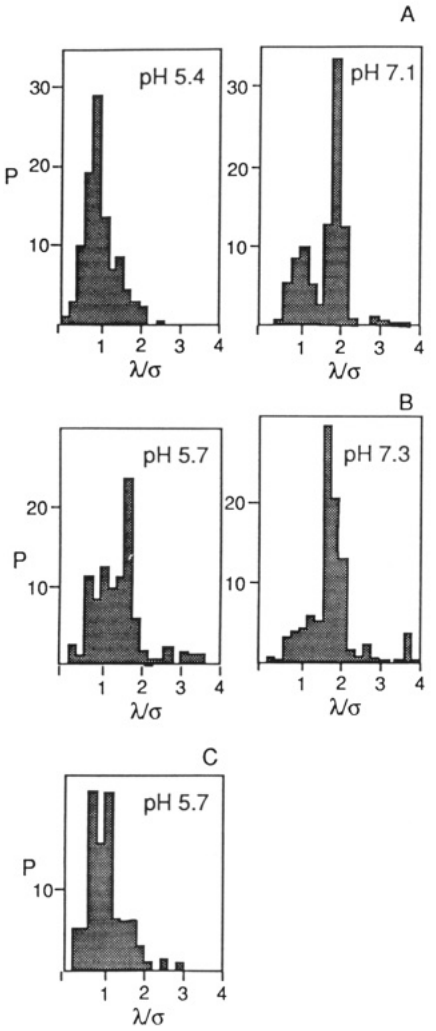

FIGURE 2: Probability distribution histograms of the size parameter λ/σ (Å) for OmpC (A), DEPC-modified OmpC (B), and DEPC-modified OmpC treated with 20 mM hydroxylamine (C) as measured in bilayer lipid membranes. The porins were added to bathing solutions, and electrical conductance was measured as described in Figure 1.

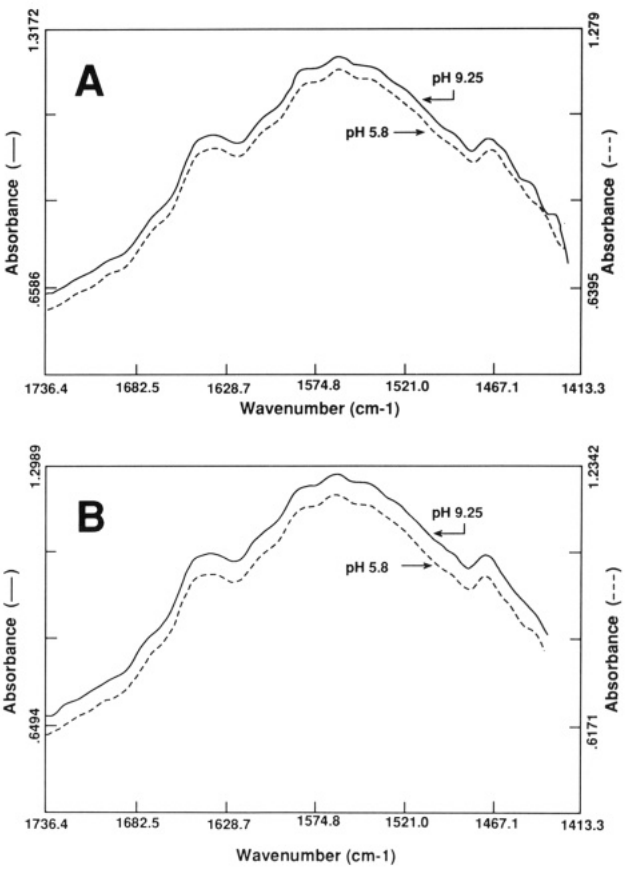

FIGURE 3: Amide I region of the FTIR spectra of LPS-enriched OmpF (A) or OmpC (B) suspended in 1% SDS and either 10 mM succinate, pH 5.8 (- - -), or 10 mM CHES, pH 9.25 (—). The spectra were recorded on a Nicolet 710 FTIR spectrometer and deconvoluted using the BP Decon package. Spectra of control samples containing the same buffer, detergent, and LPS as the porin samples were subtracted from each porin spectra. The spectra at pH 5.8 and 9.25 were offset.

DISCUSSION

These results corroborate our previous functional analysis of *E. coli* porins which showed that porins exist in at least two open-channel configurations: a small-size channel stable at acidic pH and a larger-sized set of channels stable at basic pH. Since the pK_a of the switch in channel size indicated the involvement of a histidine [see Todt et al. (1992)] and since OmpC and OmpF (as well as PhoE) contain one histidine residue (His 21), an attempt was made to specifically modify this histidine using DEPC and study the effect on function using BLM analysis. The results indicated that the DEPC-modified residue was involved in the pH-induced switch in channel size. Evidence that this modified residue was histidine includes the following: (1) only one residue per monomer was modified for OmpC and OmpF, and both porins contain only one histidine per monomer; (2) hydroxylamine addition, which is known to remove the carbethoxy group from modified histidyl and tyrosine groups (but not from modified lysyl or sulfhydryl groups) (Miles, 1977), reversed the effect of DEPC on OmpF and OmpC (Figures 1C and 2C, respectively); (3) there was no indication of tyrosine modification by DEPC since there was no drop in the absorbance at 278 nm (Burstein et al., 1974) and the addition of 20 mM hydroxylamine (a concentration too low to reverse tyrosine modification; Burstein et al., 1974) still reversed the effect of DEPC on porin. Thus, our results indicate that histidine is involved in the pH-induced switch in porin channel size.

Upon analyzing the amide I peak for changes in porin secondary structure, no differences were detected for either OmpF or OmpC between pHs 5.8 and 9.25. This indicated that any conformational alteration accompanying the pH-induced change in function does not involve a global alteration in structure.

In combination, these results indicate the involvement of His21 in a change in porin channel size with pH which results in a small conformational change but a fairly large alteration in function. Histidine has been found to be involved in the regulation of transport through a variety of other channels (Bertran et al., 1991; Cain & Simoni, 1988; Padan et al., 1985; Yamaguchi et al., 1991; Abrams et al., 1991; Pederzolli et al., 1991). Evidence for small changes in porin structure resulting in large changes in function has also been found by Benson and co-workers (Benson & Delcoux, 1985; Benson et al., 1988), who isolated mutants with single-residue changes in OmpF and OmpC which allowed the uptake of significantly larger maltodextrins presumably due to enlarged porin channels. Also, the presence of channel substates has been proposed for VDAC (Mannella et al., 1992), and similar substates in bacterial porin could explain some of the discrepancies among channel sizes reported by various investigators (Benz et al., 1984; Jap et al., 1991; Weiss et al., 1990; Xu et al., 1986).

The mechanism of the pH-induced switch in channel size may involve the movement of a protonated histidine toward a neighboring carboxyl at acidic pH which results in a reduction in channel size. Structural analysis of the *Rhodobacter capsulatus* porin has shown that this histidine would be in a positively-charged section lining the channel, close to the trimer center (Weiss et al., 1991). This would place histidine across from the large, negatively-charged loop between strands $\beta 5$ and $\beta 6$ which defines the exclusion limit for diffusing particles. Therefore, at acidic pH, the protonated histidine could cause this negatively-charged loop to move closer, causing a change in channel size. A more detailed description of porin molecular structure will be needed to further define the mechanism of the pH-induced switch in channel size.

REFERENCES

Abrams, C. K., Jakes, K. S. Finkelstein, S., & Statins, S. L. (1991) *Annu. Meet. Biophys. Soc. Abstr.*, 458.

Alvarez, J., Lee, D. C., Baldwin, S. A., & Chapman, D. (1987) *J. Biol. Chem. 262*, 3502–3509.

Benson, S. A., & Decloux, A. (1985) *J. Bacteriol. 161*, 361–367.

Benson, S. A., Occi, J. L. L., & Sampson, B. A. (1988) *J. Mol. Biol. 203*, 961–970.

Benz, R. (1985) *Crit. Rev. Biochem. 19*, 145–190.

Benz, R., Tokunaga, H., & Nakae, T. (1984) *Biochim. Biophys. Acta 769*, 348–356.

Bertran, J., Roca, A., Pola, E., Testar, X., Zorzano, A., & Palacin, M. (1991) *J. Biol. Chem. 266*, 798–802.

Bindslev, N., & Wright, E. M. (1984) *J. Membr. Biol. 81*, 159–170.

Blanke, S. R., & Hager, L. P. (1990) *J. Biol. Chem. 265*, 12454–12461.

Burstein, Y., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 205–210.

Cain, B. D., & Simoni, R. D. (1988) *J. Biol. Chem. 263*, 6606–6612.

Droge, W., Lehmann, V., Luderitz, O., & Westphal, O. (1970) *Eur. J. Biochem. 14*, 175–184.

Haris, P. I., Coke, M., & Chapman, D. (1989) *Biochim. Biophys. Acta 995*, 160–167.

Jap, B. K., Walian, P. J., & Gehring, K. (1991) *Nature 350*, 167–170.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lakey, J. H., Watts, J. P., & Lea, E. J. A. (1985) *Biochim. Biophys. Acta 817*, 208–216.

Mannella, C. A., Forte, M., & Colombini, M. (1992) *J. Bioenerg. Biomembr. 24*, 7–19.

Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442

Nikaido, H., & Vaara, M. (1985) *Microbiol. Rev. 49*, 1–32.

Padan, E., Sarkar, H. K., Vitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765–6768.

Pederzolli, C., Cescatti, L., & Menestrina, G. (1991) *Annu. Meet. Biophys. Soc., Abstr.*, 485a.

Rocque, W. J., & McGroarty, E. J. (1989) *Biochemistry 28*, 3738–3743.

Rocque, W. J., & McGroarty, E. J. (1990) *Biochemistry 29*, 5344–5351.

Rocque, W. J., Coughlin, R. T., & McGroarty, E. J. (1987) *J. Bacteriol. 169*, 4003–4010.

Rosenbusch, J. P. (1990) *Experientia 46*, 167–173.

Sams, C. F., & Matthews, K. S. (1988) *Biochemistry 27*, 2277–2281.

Susi, H., Timasheff, S. N., & Stevens, L. (1967) *J. Biol. Chem. 242*, 5460–5466.

Takeuchi, M., Asano, N., Kameda, Y., & Matsui, K. (1986) *J. Biochem. 99*, 1571–1577.

Todt, J. C., Rocque, W. J., & McGroarty, E. J. (1992) *Biochemistry* (preceding paper in this issue).

Tommassen, J. (1988) in *Membrane Biogenesis* (Op den Kamp, J. A. F., Ed.) pp 352–373, Springer-Verlag, New York.

Wantyghem, J., Baron, M., Picquart, M., & Lavialle, F. (1990) *Biochemistry 29*, 6600–6609.

Weiss, M. S., Wacker, T., Weckesser, J., Welte, W., & Schultz, G. E. (1990) *FEBS Lett. 267*, 268–272.

Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., & Schulz, G. E. (1991) *Science 254*, 1627–1630.

Xu, G., Shi, B., McGroarty, E. J., & Tien, H. T. (1986) *Biochim. Biophys. Acta 862*, 57–64.

Yamaguchi, A., Adachi, K. L., Akasaka, T., Ono, N., & Sawai, T. (1991) *J. Biol. Chem. 266*, 6045–6051.

Registry No. His, 71-00-1; sodium, 7440-23-5.