Structural transitions of porin, a transmembrane protein

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Received 23 May 1984

Conformational transitions of porin were monitored using 3 independent criteria: (i) oligomeric state as observed by SDS-polyacrylamide gel electrophoresis; (ii) spectroscopic titrations (ultraviolet and circular dichroism) and (iii) chemical modifications. Four pH-dependent transitions were observed with half-maximal changes occurring at pH values of 1.6, 3.5, 11.2 and 12.4. Two of these pH values differ significantly from intrinsic p*K* values of the constituent amino acids of this membrane protein. Since porin is very polar despite its location predominantly within the outer membranes, this may be due to ion pair formation in the hydrophobic environment of the membrane.

E.coli *outer membrane* *pH-dependent transition* *Chemical modification* *Spectroscopic titration* *Ion pairing*

1. INTRODUCTION

Porin, a protein forming channels across outer membranes of *Escherichia coli,* is unusually stable. It can be extracted by excess SDS at 70°C [1] and still be reconstituted into phospholipid bilayers in a functional state [2]. The extraction product consists of large porin aggregates which are resistant to organic solvents (e.g., benzene, toluene, ethers, alcohols), to chaotropic agents (e.g., 6 M guanidinium thiocyanate), as well as to ionic or non-ionic detergents (including SDS, and cetyltrimethylammonium bromide) at room temperature [3]. After removal of these agents, porin forms a hexagonal array [4] as well as its functional properties. If porin is solubilized and purified in octyl-POE (polydisperse octyloligooxyethylene), it exists as trimers in a monodisperse state [5]. Upon exposure to isooctane in the presence of diethylhexylsulfosuccinate (AOT), a system in which reverse micelles have been thoroughly investigated [6], the protein exists in a monodisperse state [7]. These characteristics suggest that both electrostatic and hydrophobic interactions contribute to the stability of this protein.

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2. EXPERIMENTAL

For the exposure of porin to a pH range from 1.4 to 12.5, the following solutions were used (pH range in parentheses). HCl (1.4–2.5 at appropriate concentrations), glycine–HCl (1.7–3.5 at 20 mM, as were all of the following buffers), sodium citrate (2.4; 3.0–4.3), sodium acetate (3.6–4.5), Hepes (6.5–8), glycine–NaOH (9–11.5), and phosphate (11–12.5). Porin solutions (1 mg/ml) were dialyzed (with 3 changes) in these buffers which sometimes contained 0.1 M NaCl and always 1% octyl-POE. Alternatively, concentrated porin solutions (20–50 mg) were diluted into these buffers to a final concentration of 1 mg/ml. All changes observed upon long incubations or dialysis (overnight) were also detected 5 min after dilution into the respective buffers. Incubations were at 20°C. Enzymatic hydrolysis with pepsin (pH range 1.4–3.5; also to 4.5) or pronase (pH range 4.5; 7–12.5; trypsin was

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00145793/84/$3.00

also used at pH 7.0 with results indistinguishable from pronase treatment; not shown) was performed at concentrations of 10 μg/ml, or 1% of porin by weight. Incubations were from 30 to 60 min at 37°C. Neutralisation after pH or protease treatments was performed by adding 1 M glycine–HCl (pH 3) to alkaline solutions, or 0.2 M NaP_i (pH 7.9) to acidic solutions. Incubations were for 30 min (variations from 5 min to 20 h did not affect the results). Before electrophoresis, SDS and 2-mercaptoethanol (5% each) were added to final concentrations of 1%. Treatments (37 or 95°C) were performed in a thermal block. All other procedures are described in the figure legends.

3. RESULTS

3.1. Analysis by gel electrophoresis

The ability of porin to retain its trimeric structure in detergents unless heated to temperatures above 75°C, and its resistance to proteolysis [1] have been used as criteria to monitor retention of the trimeric state (fig.1a, pH 7). The band with low mobility corresponds to the trimer (which persists even in the presence of SDS; A. Lustig and J.P. Rosenbusch, unpublished), while the band with higher mobility represents denatured, protease-sensitive monomers [1]. The observations shown in fig.1a (for details, see figure legend) are depicted diagrammatically in fig.1b, and may be summarized as follows. Across the transition at pH 3.5, the trimeric structure of porin is preserved in 1% octyl-POE (or octyl-glucoside; unpublished), but below that pH, it dissociates upon addition of 1% SDS and becomes sensitive to pepsin treatment. The two transitions at pH 1.6 and 12.4 are accompanied by irreversible dissociation and denaturation of porin trimers, as monitored by gel electrophoretic mobility and pronase sensitivity. Irreversibility is defined as the failure of the protein to assume a trimeric configuration upon readjustment of pH to neutral, and its sensitivity to pronase under the same conditions. A detailed analysis shows that the 3 transitions at pH values of 1.6, 3.5 and 12.4 occur over very narrow pH ranges. (<0.2 pH unit; fig.1a).

3.2. Analysis by circular dichroism and ultraviolet spectroscopy

Circular dichroism spectra of porin in 1% octyl-

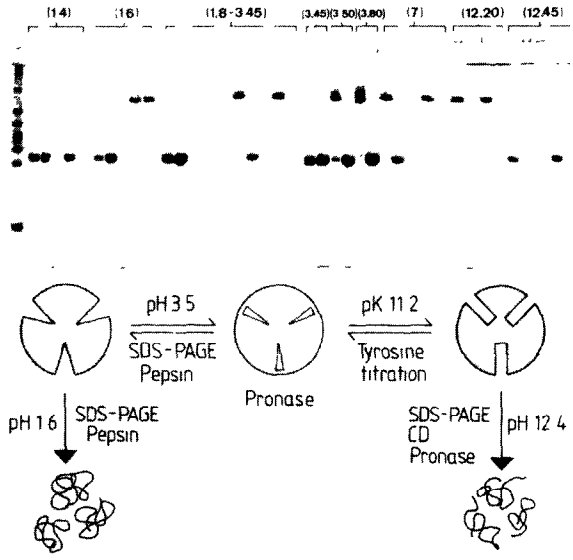

Fig.1. Electrophoretic mobility of porin as a function of pH. Upper panel (a) gives the experimental details on which the conclusions of the lower panel (b) are based. The first two lanes at each pH indicated represent untreated and heat-denatured (boiled) samples, respectively. At pH values of 1.4 and 1.6, lanes 3 represent porin treated with pepsin. In lanes 4, samples were readjusted to neutral pH before electrophoresis. Lanes 5 represent protein treated by pronase after readjusting the pH to 7.0. In the pH range 1.8–3.45, lanes 3 and 4 represent pepsin treatment before and after heat denaturation, respectively. Lanes 6 and 7 show samples of porin (no protease), readjusted to neutral pH after exposure to acidic conditions without and with heat treatment. Lanes 9 and 10 are samples treated with pronase after readjustment of pH to neutral. Sample 9 was not boiled before electrophoresis, while sample 10 was. Lanes 5 and 8 were left empty. At pH 7.0, the last two lanes (4,5) were treated with pronase and electrophoresed either without (lane 4) or with (lane 5) heat treatment. Lane 3 at pH 7.0 was left empty. At pH values 12.20 and 12.45, samples were applied without other treatment (lanes 1), or after heat denaturation (lanes 2 indicate complete, or nearly complete, alkaline hydrolysis). Samples treated with pronase were applied without (lane 3) and with heat treatment (lanes 4 and 5 at pH 12.20 and 12.45, respectively). In lane 3 (pH 12.45), the protein was heat-denatured before pronase treatment, while the sample in lane 4 was readjusted to neutral pH without boiling or pronase treatment. Standards (at left), in kDa were 95, 58, 49, ~45, 40, 35 and 13.4. SDS–polyacrylamide gel electrophoresis was performed as in [1]. Lower panel (b), see text.

POE exhibit a minimum at 218 nm in the pH range 2–11, characteristic for β-pleated sheet structure. At pH 12 and 13, the ellipticity decreases significantly (fig.2a). The spectrum of porin at low pH (2.05) and in octyl-POE retains the shape of the native protein, while that in SDS (1%) at pH 2.35 shows an alteration (fig.2b) characteristic of the structural changes produced by SDS [9]. At pH 7 and 9, spectra obtained in SDS (not shown) are indistinguishable from those in octyl-POE (fig.2a). The resistance of porin towards chaotropic reagents is shown quantitatively in fig.2c. It shows the ellipticity (monitored at 218 nm) as a function of guanidinium chloride concentration. Estimation of the half-maximal decrease of ellipticity yields a value >5.5 M of this chaotropic agent. No attempt has been made to interpret the 3 slopes observed.

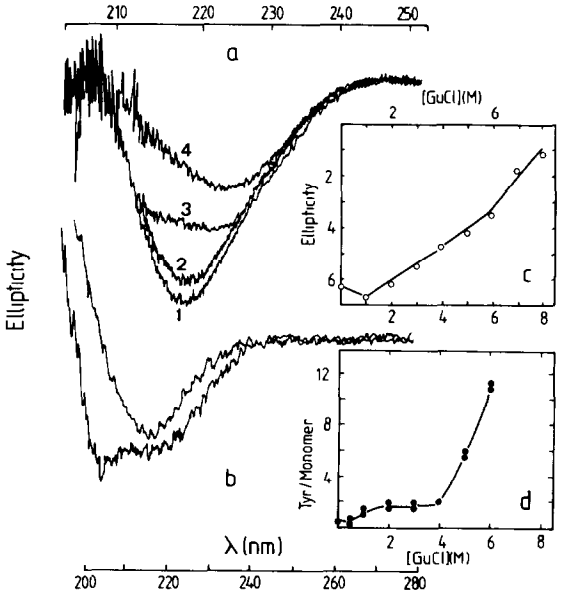

Fig.2. Circular dichroism of porin at various pH values. Panel a shows spectra obtained between pH 7 and 13. Spectra at pH 9 and 11 were indistinguishable (curve 1), as were scans in 1% SDS at these pH values. Spectra at pH 7, 12 and 13 are scans 2, 3 and 4, respectively. In panel b, spectra at acidic pH are displayed. The upper spectrum represents porin at pH 2.05 in 1% octyl-POE. The lower one is its spectrum at pH 2.35 in 1% SDS. In panel c, titration of porin in 1% detergent is shown in a function of guanidinium chloride concentration ([GuCl]). The (negative) ellipticity ($\times 10^{-3}$ deg · cm^2 · dmol^{-1}) at 218 nm was monitored as a function of [GuCl]. Ellipticity without GuCl is comparable to earlier measurements [1]. Panel d shows the number of titratable tyrosine residues as a function of [GuCl] at pH 10. Titration was performed as described in the legend to fig.3. CD measurements were obtained with a Cary Model 61 recording spectropolarimeter as in [8].

Spectrophotometric titration of porin in the absence of salt is shown in fig.3a. About half of the 25 tyrosyl residues present per polypeptide chain of porin [1] are normalized at pH 11.2. The presence of 0.2 M NaCl caused a small deviation (not shown) which does not appear significant in the present context. As is evident from fig.3a, at pH 10 less than 2 tyrosine residues are accessible. Porin conformation was also monitored by titrating tyrosinate groups at 295 nm as a function of guanidinium chloride concentration (fig.2d). At concentrations less than half molar, 0.5 tyrosinate group per monomer is observed at pH 10.0. Between 1 and 4 M guanidinium chloride, 1–2 and above 4 M groups, 2–11.5 tryosinate residues become exposed. Since the pK of normal tyrosine in

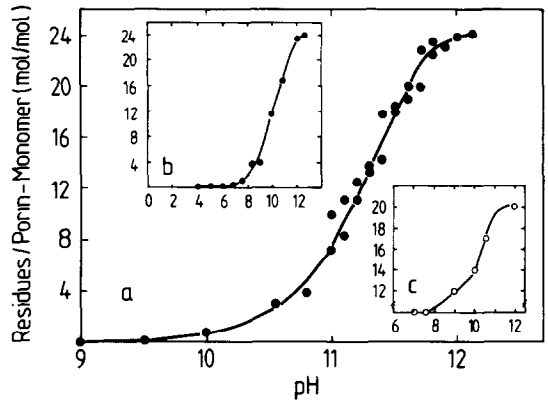

Fig.3. (a) Spectrophotometric titration of tyrosyl residues at 295 nm and chemical modification of tyrosyl and lysyl residues. Measurements employed a porin concentration of ~2.5 μM. Absorbance was followed between pH 7.5 and 12. Reference cells contained porin solutions at pH 7.5. The buffer used was 1 mM Hepes, 0.2 mM DTT and 1.0% octyl-POE. The pH was raised by direct addition of NaOH to the sample solution with total volume changes <5%. The difference spectra were recorded at λ_{295} in a Perkin Elmer double-beam spectrophotometer. The amount of tyrosinate ions was calculated using ϵ_{295} = 2300 M for tyrosinate [10]. Protein concentration was determined at pH 7.5 using $A_{280}^{0.1\%}$ = 1.4 mg/ml [1]. Panels b and c, tyrosyl and lysyl residues were converted to diiodotyrosine by KI-I_2, or modified with citraconic anhydride, respectively (in 1% β-octylglucoside) as in [12].

guanidinium chloride is 10.1 [11], half of the tyrosyl groups of porin are expected to be ionized at this pH. Thus, the values observed correspond to 1, 2–4 and 4–23 tyrosyl residues in the 3 concentration ranges.

3.3. *Chemical modification of tyrosyl and lysyl residues*

Iodination of tyrosine yields the same number of residues as in the titration (fig.3b). This indicates that most of the tyrosyl residues are normalized at pH 12 and confirms that tyrosines rather than tryptophans are monitored [10]. Apparent half-maximal modification occurs at pH 10, implying that iodination normalizes phenolic groups in the protein. Modification of the 18 lysyl residues and the α-amino group by citraconic anhydride [12] reached completion at pH 12 (fig.3c). The shape of the curve was drawn arbitrarily and shows that lysyl residues are heterogeneous in their reactivity towards this reagent.

4. DISCUSSION

Our results show that porin undergoes at least 4 pH-dependent transitions. Those at extreme pH values (1.6 and 12.4) are apparently accompanied by irreversible unfolding, while the transitions at pH 3.5 and 11.2 are reversible (porin acquires its original resistance to SDS and protease treatment upon reneutralizing the pH). Porin remains trimeric above pH 1.6 in octyl-POE, as determined by analytical ultracentrifugation (unpublished). A remarkable feature of all transitions except that at pH 11.2 is their completion within 0.2 pH units. This is unusual for titrating single groups [13], suggesting cooperative involvement of several residues. The ionization of tyrosyl residues (apparent pK 11.2) seems to affect most of the 25 tyrosyl residues present in the polypeptide. Although not proved, it appears likely that the other transitions also reflect ionization processes [14]. The apparent pK values at pH 1.6 and 3.5 are plausibly explained by protonations of carboxylate groups. The transition at pH 12.4 could reflect deprotonation of arginyl or possibly of some highly abnormal lysyl residues. (Preservation of the integrity of the polypeptide at extreme pH values is demonstrated in fig.1a.)

How many such groups could be involved in each of these transitions? Porin from *E.coli* B^E contains 18 lysyl, 12 arginyl and 44 carboxyl groups, apart from the α-amino and carboxyl groups. In a study investigating the accessibility of lysyl residues, we previously determined that 13 amino groups can be modified from the aqueous bulk phase using small probes [12]. Thus, 6 amino groups (which become accessible above pH 10.5) remained unaccounted for. In an investigation of the effects of charges on diffusion rates across channels, in which the number of amino groups determined by succinylation was 13–14 [15], modification of carboxyl groups yielded a value of 26 residues derivatized at pH 4.75. Assuming intrinsic pK values of free carboxyl groups [16] of 3.6–4.5, it seems likely that the groups exposed to the aqueous phase were modified, and that at least some of the remaining 18 carboxyl groups are buried. Previous experiments [12] have shown, moreover, that of the 25 tyrosyl residues, only two were accessible to small probes from the aqueous phase, while 22–24 were modified at pH 11 (after heat treatment) or at pH 12. These observations are in agreement with the abnormal apparent pK reported here. The quoted figures of residues potentially involved in electrostatic interactions in the core of the protein must naturally be regarded as upper limits. Due to the cooperativity of the transitions, it appears justified to postulate that several of the unaccounted residues (including a fraction of the 12 arginyl residues, which have not been assessed as yet) may reside in hydrophobic environments. Direct evidence is lacking with regard to their number as well as to their configuration. Clearly, their presence in locations inaccessible to the bulk aqueous phase would suggest that they occur in pairs, salt-bridged, or hydrogen-bonded. Considerations recently applied to ionizable groups buried in the interior of globular proteins (whose structures are known to high resolution) have pointed out that this type of arrangement indeed occurs [17]. Based on the stability of porin to high salt, apolar solvents, and detergents, as well as the observation that the bulk of porin is located within the membrane boundary [4,18], we suggest a mosaic arrangement of ion pairs and hydrogen bonds within hydrophobic areas which mutually exclude access of solvents and salts. Whether such pairs occur at subunit interfaces or within mono-

mers remains unknown. Such a hypothesis is at variance with the notion that transmembrane domains are predominantly hydrophobic and that α-helices are the prevalent secondary structure, as is often postulated on the basis of evidence obtained with proteins such as bacteriorhodopsin [19]. Porin, we surmise, is an example of a different type of transmembrane protein: it exists in β-pleated sheets [1,3,20] and contains more charged residues than can be accounted for by those found to be exposed to the aqueous bulk phase at the surface or within the channels of porin [12,15]. The occurrence of ion pairs is supported by the unusual shift of apparent pK values (particularly the concerted transition at pH 1.6) described here. The availability of 3-dimensional crystals [18] should soon allow a detailed understanding of such interactions.

ACKNOWLEDGEMENTS

Support from the Swiss National Science Foundation (Grant 3.656.80) and from NIH (grant GM 31707) is gratefully acknowledged. Our thanks for expert assistance and secretarial help go to M. Regenass and C. Barber.

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