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Pore-forming peptide of *Entamoeba histolytica* Significance of positively charged amino acid residues for its mode of action

Jörg Andrä, Matthias Leippe*

Department of Molecular Biology, Bernhard Nocht Institute of Tropical Medicine, 20359 Hamburg, Germany

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Abstract Amoebapore is a 77-residue pore-forming peptide from *Entamoeba histolytica* with antibacterial and cytolytic properties. It contains eight lysine residues and one histidine residue. Chemical modifications of amoebapore with various reagents affecting either both types of cationic residues or lysine and histidine residues separately resulted in virtually complete loss of pore-forming activity. The activity was restored by reversal of modifications. Whereas amoebapore was no longer capable of binding to phospholipid vesicles when its lysine residues were modified, the modification of the single histidine primarily affected oligomerization of the peptide upon membrane association.

Key words: Amoebiasis; Amoebapore; Chemical modification; Membranolytic peptide; Pore formation; *Entamoeba histolytica*

1. Introduction

The most prominent pathogenic feature of the protozoan parasite *Entamoeba histolytica* is the powerful ability to kill eukaryotic cells, which may result in massive tissue destruction in the human host [1]. In vitro, the killing event itself is rapid, contact dependent, and potentially directed against almost all cells [2,3]. The primary candidate for mediating the extraordinary cytolytic activity of *E. rhistolytica* is a peptide that forms ion channels in phospholipid membranes (for review see [4]). The peptide, named amoebapore, was purified from amoebic extracts [5] and its primary and secondary structure elucidated [6]. Amoebapore consists of 77 amino acid residues and has an all-α-helical conformation. The purified peptide was found to be cytotoxic to human cell lines and also to kill Gram-positive bacteria by permeabilizing their cytoplasmic membranes [7].

Biophysical data from planar lipid bilayer experiments [8] indicated that amoebapore creates ion channels in membranes by oligomerization according to the 'barrel stave' model [9]. Stable transmembrane pores inserted into a cell membrane would affect its function as a permeability barrier and may eventually result in lysis of the target cell. Amoebapore is predicted to be composed of four mostly amphipathic helices and is stabilized by three disulfide bonds, providing a relatively rigid structure [6,10]. Synthetic peptides corresponding to the structural elements proposed to permeate membranes, i.e. the amphipathic helices 1 and 3, exhibited pore-forming activity [7].

In addition to amphipathic domains, positively charged residues are considered critical for membranolytic peptides [11,12]. This is reflected by selective binding of many of such molecules to negatively charged phospholipids, and by their increased activity at low pH ([11,13] and references therein). Likewise, amoebapore was found to insert preferentially in liposomes composed of acidic phospholipids and to be optimally active at pH 5.2 [5]. The peptide contains nine positively charged residues that might be crucial for activity, i.e. eight lysine residues interspersed along the entire sequence and one histidine residue near the C terminus (Fig. 1). Amoebapore does not possess any arginine residues.

Here we describe the reversible inhibition of the pore-forming activity of amoebapore by chemical modification of its lysine residues and of its single histidine residue. Furthermore, we try to link the effects of both modifications to two steps that may be studied separately when a peptide forms a pore through the barrel stave mechanism, i.e. membrane association (binding/insertion) and self-assembly.

2. Materials and methods

2.1. Chemicals

Trifluoroacetic acid and acetonitrile, both of HPLC grade, were obtained from Applied biosystems. Diethylpyrocarbonate (DEPC), citraconic anhydride, and mixed phospholipids (phosphatidylcholin type II-S) from soybean are products from Sigma. Dithiobis(succinimidylpropionate) (DSP) was from Pierce, and manoalide was from Biomol. Other chemicals were purchased from Merck and were of analysis grade.

2.2. Purification of amoebapore

Amoebapore was purified from pathogenic *E. histolytica* strain HM-1:IMSS essentially as described [5]. Final purification was achieved by reverse-phase high-performance liquid chromatography using an Aquapore Butyl 300 column (2.1 × 220 mm; Brownlee) connected to a 130 A separation system (Applied biosystems). Elution was done with a linear gradient of 0–70% acetonitrile in water acidified with 0.1% trifluoroacetic acid. The flow rate was 250 μl/min, and the effluent was monitored by absorbance at 214 nm. Fractions of 250 μl were collected and assayed for pore-forming activity. Active fractions corresponding to a single peak were pooled and apparent homogeneity was confirmed by Tricine-SDS/PAGE. Purified peptide was lyophilized and stored at −20°C.

2.3. Assay for pore-forming activity

Pore-forming activity was determined by monitoring the dissipation of a valinomycin-induced diffusion potential in liposomes [5].

2.4. Modification with DEPC

Reaction of amoebapore (5 μM) with DEPC was performed essentially according to Miles [14]. Briefly, amoebapore was dissolved in 50 mM sodium phosphate buffer, pH 6.0 or 8.5. DEPC was added from a freshly prepared stock solution in anhydrous ethanol to reach a final concentration of 1 mM. The mixture was allowed to react for 1 h at 20°C. Aliquots were taken at different time intervals to measure their pore-forming activity.

*Corresponding author. Fax: (49) (40) 3118 2512.

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2.5. Reversal of DEPC modification by treatment with hydroxylamine

DEPC-treated amoebapore (5 μM) was dialyzed against 100 mM sodium phosphate buffer, pH 7.0, in tubings with a molecular weight cut-off of 1,000 (Spectra/Por 6; Spectrum Industries, Los Angeles). Subsequently, hydroxylamine (adjusted with KOH to pH 7.0) was added to modified amoebapore to reach a final concentration of 0.8 M, and the mixture was incubated at 20°C for up to 1 h. The pore-forming activity was monitored after the dialysis step and after various periods of incubation with hydroxylamine.

2.6. Modification with citraconic anhydride

Treatment of amoebapore with citraconic anhydride was performed according to Atassi and Habeeb [15]. Amoebapore (5 μM) was dissolved in 50 mM sodium phosphate buffer, pH 8.5. Citraconic anhydride was added dropwise from a freshly prepared stock solution in anhydrous ethanol to reach a final concentration of 1 mM. The mixture was allowed to react for 1 h at 20°C. Aliquots were taken at different time intervals and their pore-forming activity was monitored.

2.7. Reversal of citraconic anhydride modification by treatment with HCl

Modified amoebapore (5 μM) was dialyzed against 50 mM sodium phosphate buffer, pH 8.5, to remove remaining citraconic anhydride. After dialysis the pore-forming activity of the sample was determined. HCl was added dropwise to the modified amoebapore to adjust the pH to 4. The mixture was incubated at 37°C and aliquots were removed at different time intervals to determine their pore-forming activity.

2.8. Modification with manoalide

Manoalide [16,17] was added from a 3 mM stock solution in anhydrous ethanol to an amoebapore solution (5 μM) in 50 mM sodium phosphate buffer, pH 8.5, to reach a final concentration of 125 μM (unless otherwise specified). Subsequently, the reaction mixture was incubated for up to 1 h at 20°C. Aliquots were taken at different time intervals to measure their pore-forming activity.

2.9. Amino acid analysis

Peptide samples (100 pmol) were hydrolized in 6 M HCl, 1% phenol at 110°C for 20 h in vacuo and subjected to amino acid analysis (421 amino acid analyzer; Applied biosystems)

2.10. Enzyme-linked immunosorbent assay (ELISA)

The assay was performed in radiated, flat-bottom microtiter plates (F-plates; Greiner, Germany). The wells of the plates were coated with various concentrations of the peptides in 100 mM sodium carbonate buffer, pH 9.5, for 12 h at 4°C followed by blocking with 5% milk powder in phosphate-buffered saline (PBS) for 30 min at 20°C. Subsequently, the plates were incubated with rabbit antiserum to amoebapore [5] at a 1:100 dilution in 2.5% milk powder in PBS for 2 h at 20°C. After three washes with PBS containing 0.1% Tween 20, the plates were incubated with an anti-rabbit immunoglobulin antibody-peroxidase conjugate (Dako, Denmark) for 2 h at 20°C, washed with PBS/Tween, and the assay was developed using *o*-phenylendiamine and H_2O_2 as substrates. The resulting colour reaction was stopped with 2 M sulfuric acid after 2–5 min and the absorbance at 492 nm was measured with an ELISA plate reader (Titertek Multiskan Plus/MK II; ICN Flow).

2.11. Gel-electrophoresis and blotting

Tricine-SDS/PAGE [18] in 13% separation gels and subsequent immunoblotting were carried out as described [5].

2.12. Association of peptides with liposomes

Natural and modified amoebapore (5 μM) was diluted ten times in liposome buffer (50 mM Tris maleate, 50 mM potassium sulfate, 0.5 mM ethylenediamine tetraacetic acid, 0.02% sodium azide), adjusted with KOH to various pH, and incubated with vesicles of mixed soybean phospholipids for 1 h at 4°C by permanently shaking. These liposomes were prepared by dissolving dry phospholipids in chloroform (1 mg/ml), the solvent was evaporated under a stream of argon and the lipids were stored under vacuum overnight; the phospholipids were resuspended in the same volume of liposome buffer, pH 5.2, by vortex mixing. After the incubation period, liposomes and liposome-associated peptides were separated from peptides in solution by centrifugation at 24 psi (≈ 100,000 × *g*) for 30 min using an Beckmann airfuge centrifuge. The resulting pellet was washed with liposome buffer, and supernatant and washes were subjected to the ELISA. Since the washes showed absorbance always <5% of that of the supernatants, in the following only absorbance values of the latter represent peptides not associated with liposomes. These values were expressed as the percent-

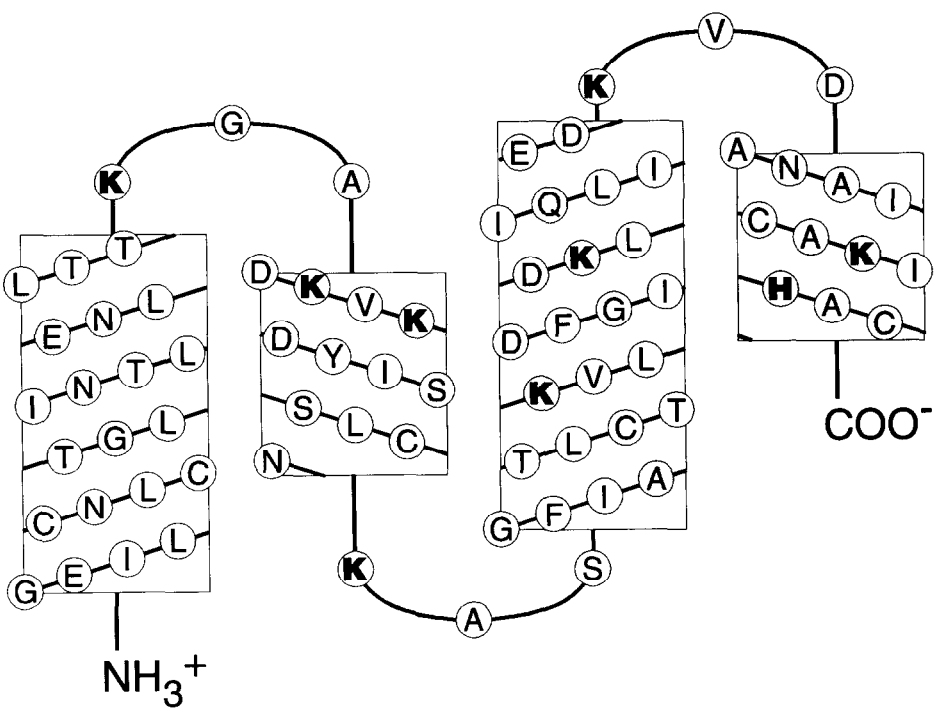

Fig. 1. α-Helical net diagram of the amoebapore sequence. The four predicted α-helical regions are represented as opened and flattened cylinders viewed from the side. Residues are displayed therein to give approximately 3.6 residues per turn of the helix. Positively charged residues are highlighted by bold-faced letters. The one-letter notation of amino acids is used.

age of the values of the controls, i.e. the same peptides adsorbed directly to the microtiter plates.

2.13. Crosslinking of liposome-associated peptides

Sedimented liposomes and liposome-associated peptides were washed and resuspended in 50 mM sodium phophate buffer, pH 7.0. For crosslinking interacting peptides, DSP [19] was added from a freshly prepared stock solution in dimethylsulfoxide to reach a final concentration of 100 μM. After 30 min at 20°C the reaction was stopped by adding an excess of Tris, and eventually the sample was subjected to Tricine-SDS/PAGE and immunoblotting.

3. Results and discussion

3.1. Pore-forming activity

Chemical modification is a widely used method to study the role of amino acid residues for the function of peptides and proteins [20–22]. Since cationic residues were considered critical for the pore-forming activity of amoebapore, we used various reagents for modifying these residues: (i) DEPC at pH 8.5 to modify both, histidine and lysines; (ii) the same reagent at pH 6.0 to specifically modify histidine; and (iii) citraconic anhydride or manoalide to modify lysines only. All of the treatments resulted in nearly complete loss of pore-forming activity (Fig. 2). The activity of histidine- as well as lysine-modified amoebapore was virtually restored by reversal of the chemical modifications (Fig. 2) indicating that the effects observed were not due to irreversible side reactions or peptide denaturation.

Whereas DEPC at pH 6.0 specifically modifies histidine [14], at higher pH a variety of residues, such as lysines, arginines, cysteines, tyrosines, and tryptophanes, are targets for side reactions [14,20,23]. Since amoebapore does not contain any arginine and tryptophane residues and the six cysteine residues of the peptide are all involved in disulfide bonds [6], the candidate residues affected by DEPC treatment at pH 8.5 in addition to histidine and lysines can be limited to the single tyrosine residue. Citraconic anhydride [15] and manoalide [16,17] are known to be specific for primary amino groups and their inhibiting effects on pore formation strengthen the notion that lysine residues are critical for the peptide's activity. In particular manoalide, a sesterterpenoid from the marine sponge *Luffariella variabilis* [24], is known to react irreversible with lysine residues and therefore is suitable to determine the number of residues modified by amino acid analysis [16,17]. Treatment of amoebapore with various amounts of this reagent revealed that the resulting loss of pore-forming activity correlates with the number of lysine residues modified (Fig. 3). The peptide lost 50% of its activity when four of its eight lysine residues were affected by an equimolar amount of manoalide (relative to the primary amino groups present) as evidenced by amino acid analysis. A fivefold molar excess of the reagent results in a considerably more pronounced inhibition but led to the modification of one additional lysine only. This finding suggests that the residual three lysine residues are not accessible to modification by manoalide. Since most of the treatments affect primary amino groups we can not exclude the possibility that the α-amino group of the N-terminus is modified also. However, modifications of residues other than lysines including the N-terminal glycine were not detected by amino acid analysis, amd the aforementioned results suggest that the modification of the N-terminus is negligible.

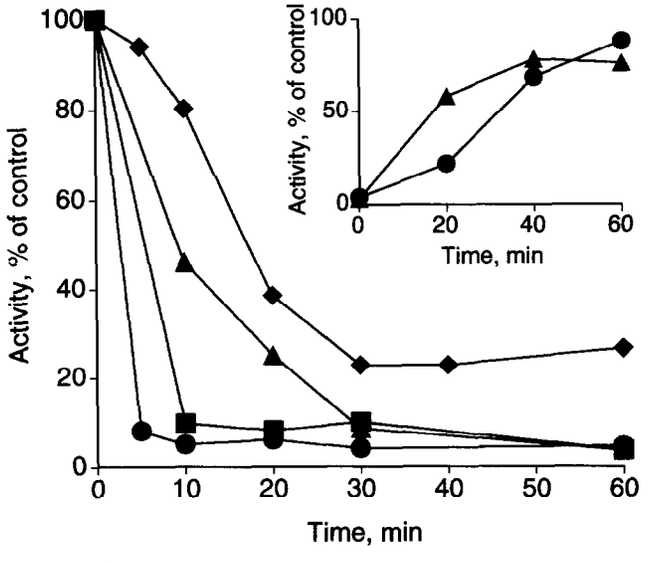

Fig. 2. Effects of chemical modifications and of their reversal on the pore-forming activity of amoebapore. Pore-forming activity was determined by measuring the dissipation of a liposome membrane potential. As controls, amoebapore samples were incubated without modifying reagents. Amoebapore was treated with DEPC at pH 6.0 (▲) and pH 8.5 (■) to specifically modify the histidine residue and histidine plus lysine residues, respectively. Citraconic anhydride (●), and manoalide (◆) were used to modify lysine residues only. The loss of activity during incubation with modifying reagents is shown as a function of time. (Inset) Pore-forming activity was virtually restored by reversal of modifications. The histidine-modified amoebapore (▲), treated with DEPC at pH 6.0, was reconstituted by hydroxylamine. Lysine-modified amoebapore (●), treated with citraconic anhydride, was reconstituted by addition of HCl. Experiments were done in duplicate.

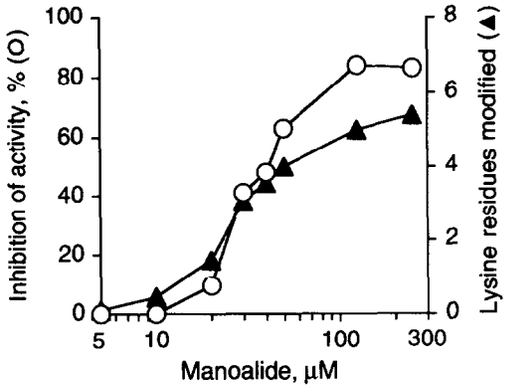

Fig. 3. Correlation between inhibition of pore formation and the number of lysine residues modified. Aliquots of amoebapore (5 μM) were incubated for 1 h at 20°C in the presence of various concentrations of manoalide. For each sample the residual pore-forming activity was measured by liposome depolarization. The percent inhibition of activity was calculated from the values obtained after incubation of amoebapore with or without manoalide under the same conditions. The number of modified lysine residues was determined by subjecting the samples to amino acid analysis and comparing the data for the manoalide-treated samples to those for the untreated control. Residues other than lysines were not found to be affected. Experiments were done in duplicate.

3.2. Effects of pH

The remarkable effects of the chemical modifications suggested that the interaction of amoebapore with negatively charged phospholipids via its cationic residues is impaired. To test this assumption, the association of natural amoebapore

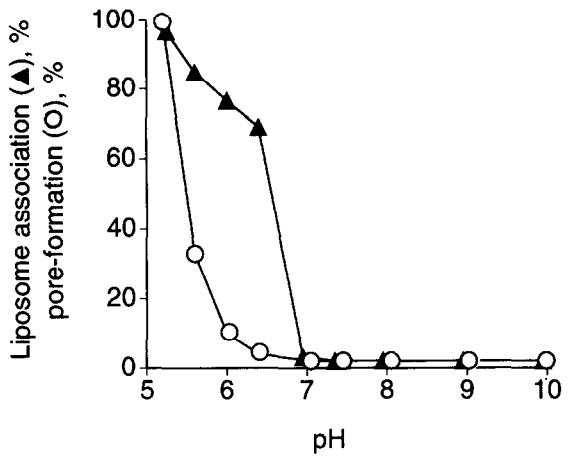

Fig. 4. Effect of pH on liposome association and pore formation. Liposome association was determined by measuring the amount of non-adsorbed amoebapore after incubation of the peptide (0.5 μM) with liposomes for 1 h at 4°C using an ELISA with anti-amoebapore. Pore-forming activity of amoebapore (0.5 μM) was determined by measuring liposome depolarization. Both properties at pH 5.2 were set as 100%. Experiments were done in duplicate.

with membranes of liposomes, i.e. its binding and insertion, was measured in comparison to its pore-forming activity at various pHs. When pH was raised and hence the cationic side chains of the peptide were deprotonated, the percentage of membrane-associated peptide diminished, but the decrease in membrane association did not coincide with that in pore formation (Fig. 4). On the contrary, a considerable discrepancy in the pH dependence was found between these two properties; e.g. whereas the majority of molecules were associated with liposomes at pH 6.0, pore formation was found here to be virtually abolished. This finding suggests that, in addition to the interaction of amoebapore with phospholipids, another event involved in pore formation, probably the peptide–peptide interaction, is affected by raising the pH. Consequently, we investigated separately the effects of the aforementioned chemical modifications on membrane association and self-assembly of amoebapore.

3.3. Membrane association

To estimate the effects of histidine and lysine modifications on the ability of amoebapore to associate with membranes, we measured by ELISA the amount of modified peptide which was not bound to phospholipid vesicles compared to that of natural amoebapore. In preliminary experiments it became evident that the treatment of amoebapore with the modifying reagents altered its reactivity with anti-amoebapore to a different extent (Fig. 5). Whereas the lysine-modified peptides are considerably less reactive than the natural amoebapore in the ELISA, the histidine-modified peptide was readily recognized by the antiserum, most likely because just one residue is affected. To rule out any influence that would be due to those differences in reactivity with anti-amoebapore or to a variability in binding to microtiter plates, the corresponding modified peptide was used as a control in each assay.

When the single histidine residue was modified by treatment of amoebapore with DEPC at pH 6.0, approximately 70% of the molecules were still found to be associated with liposomes (Fig. 6). The same reagent has a remarkable effect on the ability of the peptide to associate with the lipid vesicles at pH 8.5, at which it leads to neutralization of all cationic residues, suggesting that lysine residues play the major role in membrane interaction compared to histidine. The inhibiting effect on lipid association is even more pronounced when lysine residues were modified specifically by treatment with citraconic anhydride or manoalide. Here one has to bear in mind that these reagents in contrast to DEPC convert the charge of the lysine residues. The newly generated negative charges may repulse the peptide from its preferred targets, the negatively charged phospholipids.

3.4. Self-assembly

Natural amoebapore tends to undergo self-association, particularly after insertion into a membrane, suggesting peptide–peptide interactions relevant for pore formation [5]. Since histidine modification hardly diminished the ability of amoebapore to interact with phospholipids but abrogated its pore-forming activity, we examined whether the molecular organization of the peptide after its association to liposomes is affected by this treatment.

After incubation with liposomes and crosslinking with DSP, natural amoebapore formed homooligomers that were detected by immunoblotting as reactive higher-molecular mass entities (Fig. 7). The self-association is reversible since without crosslinking the peptide showed monomeric behaviour upon SDS/PAGE. The same experiment performed with histidine-modified amoebapore revealed that a large amount of the peptide was in the monomeric state after crosslinking and appeared to form oligomers to a much lesser extent. Although the migration behaviour of the amoebapore monomer upon SDS/PAGE do not represent its actual molecular mass, it may be estimated from the immunoblot analysis that natural peptide oligomerized at least up to hexamers whereas the histidine-modified amoebapore appeared to preferentially form smaller entities. To explain the seemingly uncomplete prevention of self-assembly by the histidine modification, it is worth noting that amoebapore is known to aggregate also to some extent in solu-

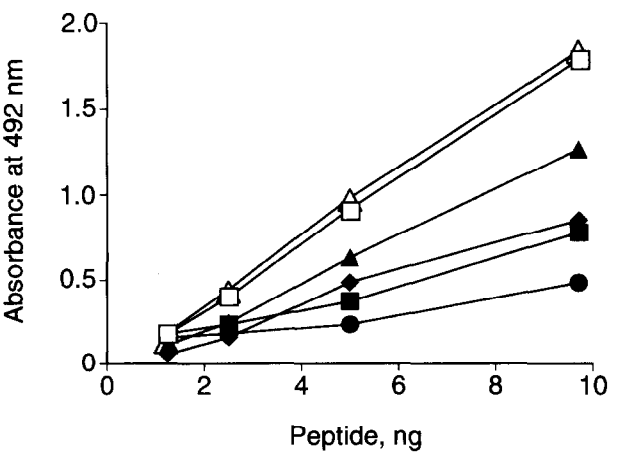

Fig. 5. ELISA calibration for natural and modified amoebapore. Serial dilutions of peptides were measured for absorbance after reaction with anti-amoebapore. Open symbols represent amoebapore incubated without reagent at pH 6.0 (△) or pH 8.5 (□). Filled symbols represent amoebapore treated with DEPC at pH 6.0 (▲), DEPC at pH 8.5 (■), citraconic anhydride (●) or manoalide (◆) for 30 min. Experiments were done in duplicate.

tion [5]. It may be argued that either in such spontaneously formed oligomers the peptide is not accessible for the action of DEPC, or preformed oligomers are stable even after histidine modification. The virtually total loss of activity indicates that DEPC-treated amoebapore cannot form a transmembrane channel although oligomers exist. This implies either that oligomers do not insert into membranes, i.e. do not form pores, or, if the peptide does insert, in whatever state it is in, that a critical size of the oligomers has to be reached to be active which is inhibited by the histidine modification.

The finding that histidine is implicated in the self-association of amoebapore is in good agreement with results of analogous functional studies on the α-toxin from *Staphylococcus aureus* [24] and on aerolysin from *Aeromonas hydrophila* [25,26]. For these pore-forming toxins, it was shown by chemical modification with DEPC or by site-directed mutagenesis that histidine residues are crucial for the oligomerization process. The significance of the single histidine residue at position 75 for the function of amoebapore is emphasized by the recent finding that, in cytoplasmic granules of the amoebae, two additional isoforms of amoebapore exist, in which this residue is conserved despite considerable sequence divergence [27]. The number of all other cationic residues in the three isoforms are virtually the same, but varied in their positions.

The results presented here may have implications as to the mode of action of amoebapore. According to the barrel stave model we suggest that (i) amoebapore binds to negatively charged phospholipids via its protonated lysine residues; (ii) the negative membrane potential of the target membrane drives the peptide into the lipid bilayer; and (iii) self-assembly of the peptide occurs within the membrane to form the ion channel. In the last step His^{75} may play a direct role in the interaction with another monomer via its involvement in hydrogen bonds.

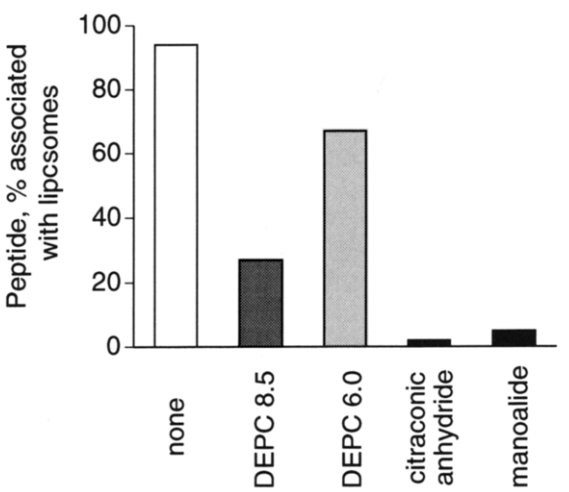

Fig. 6. Association of natural and modified amoebapore with liposomes. Liposome association was estimated by detecting unbound peptide with anti-amoebapore by ELISA after allowing the peptides (0.5 μM) to interact with phospholipid vesicles for 1 h at 4°C. Whereas the untreated amoebapore is represented by the open bar, modification of histidine and lysine residues is indicated by shaded and filled bars, respectively; the darker shading indicates that all cationic residues were modified. The modifying reagent is shown underneath each column; DEPC was used at pH 6.0 or pH 8.5. As controls, the same materials were adsorbed directly to the ELISA plates. Experiments were done in triplicate.

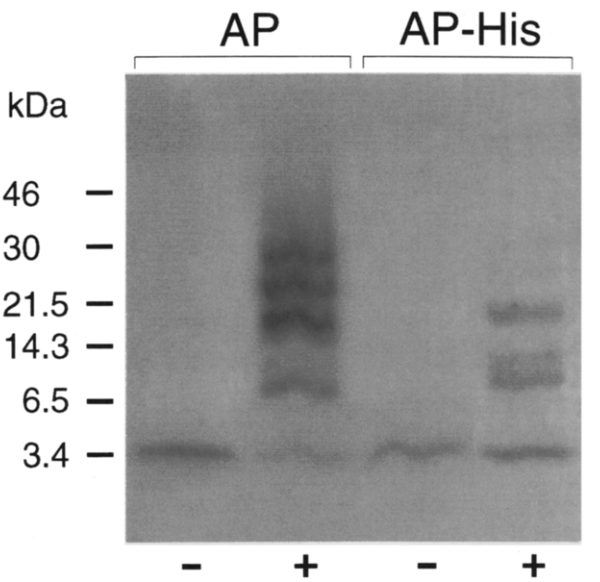

Fig. 7. Reversible oligomerization of natural amoebapore (AP) and histidine-modified amoebapore (AP-His) associated with liposomes. Peptide (0.5 μM) was allowed to interact with phospholipid vesicles for 1 h at 4°C and aliquots of the mixture (0.4 μg peptide) were subjected to immunoblot analysis with anti-amoebapore. Comparison of the same material with (+) and without (−) crosslinking after membrane association using DSP reveals that the histidine-modified peptide exists primarily in its monomeric state, whereas the natural peptide had nearly exclusively formed homooligomers of higher molecular mass.

Alternatively, the histidine residue may be important in stabilizing the predicted fourth α-helix, as experiments with synthetic peptides suggest [28] and it would thereby rather indirectly promote oligomerization. We have to admit that it can not be deduced from our data whether a variable number of preinserted monomers assemble after lateral diffusion in the phospholipid bilayer, as proposed, or oligomerization primarily occurs prior to insertion into the membrane.

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