Membrane Fusion Is Induced by a Distinct Peptide Sequence of the Sea Urchin Fertilization Protein Bindin*

(Received for publication, February 10, 1998, and in revised form, April 2, 1998)

Anne S. Ulrich‡‡, Marlies Otter‡, Charles G. Glabe§, and Dick Hoekstra¶

From the ‡Institute of Molecular Biology, University of Jena, Winzerlaer Strasse 10, 07745 Jena, Germany, the 
§Department of Physiological Chemistry, University of Groningen, Den Dolderstraat 30, 9714 AV Groningen, The Netherlands, 
¶Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

Fertilization in the sea urchin is mediated by the membrane-associated acrosomal protein bindin, which plays a key role in the adhesion and fusion between sperm and egg. We have investigated the structure/function relationship of an 18-amino acid peptide fragment “B18,” which represents the minimal membrane binding motif of the protein and resembles a putative fusion peptide. The peptide was found to mimic the behavior of its parent protein bindin with respect to (a) its high affinity for lipid bilayers, (b) the ability to aggregate and fuse vesicles, (c) the binding of Zn2+ by a histidine-rich motif, (d) the tendency to self-assemble, and (e), as indicated earlier, the adhesion to cell surface polysaccharides. Fluorescence and light scattering assays were used here to monitor peptide-induced lipid mixing, leakage, and aggregation of large unilamellar sphingomyelin/cholesterol vesicles. For these activities, B18 requires the presence of Zn2+ ions, with which it forms oligomeric complexes and assumes a partially α-helical conformation, as observed by circular dichroism. We conclude that aggregation and fusion involves a “trans-complex” between peptides on apposing vesicles that are connected by Zn2+ bridges.

Membrane fusion is a ubiquitous event in numerous intracellular and intercellular processes, such as vesicular trafficking (1, 2) and the infectious entry of viruses (3–5). It also constitutes the committing step that allows sperm and egg to merge their genetic material (6–9). Fertilization has traditionally been studied using sea urchin gametes, and much attention has focused on sperm proteins that become exocytosed upon contact with the egg jelly coat. The major acrosomal protein, bindin, is recognized as a key mediator of sperm-egg adhesion and fusion (10, 11). Its species-specific binding to the egg receptor, presumably via interactions with sulfated polysaccharides (12, 13), has been well documented in vivo and in vitro (14–16). Furthermore, the direct involvement of bindin in the fusion event between the membranes has been suggested from observations with lipid vesicles as model systems (17–21). To unravel the mechanisms underlying sperm-egg fusion and, in particular, to investigate the structure/function relationship of bindin in the overall process, reconstitution would be the approach of choice. However, structural analysis of bindin has been frustrated thus far, because in its native state the protein is extensively self-aggregated within the acrosomal vesicle or it is closely associated with the sperm membrane.

Given the interaction of bindin with lipid membranes as well as cell surface carbohydrates, there is much evidence that the protein plays a dual functional role during fertilization. A similar multiple involvement in cell recognition (adhesion or penetration) and fusion has been proposed for other proteins, too, like fertilin (PH-30) (6, 22, 23), abalone sperm proteins (8, 24, 25), or viral proteins (3–5). In many instances, the fusogenic activity of such a protein has been attributed to a short fusion peptide or hydrophobic patch, which could then be characterized in detail with regard to its membrane interactions and secondary structure (22, 23, 26–28). Here, we have identified the minimum membrane binding peptide of the sea urchin fertilization protein bindin, and we investigate its fusogenic and structural behavior in solution and on the membrane. Most experiments with this peptide are directed by the extensive knowledge about the interactions of the native parent protein bindin with its putative binding partners.

Previous work by Glabe and co-workers revealed that native and recombinant bindin binds peripherally to lipid vesicles, presumably as a monomolecular layer (17, 18, 20). Because the protein displays no preference for charged lipid head groups, its association appears to be mediated by hydrophobic interactions (18). An unusual feature is its specific affinity for membranes in the gel phase or enriched in cholesterol (17, 29). Moreover, bindin is able to induce the fusion of lipid vesicles, which proceeds only slowly with dipalmitoylphosphatidylcholine/cholesterol but within seconds when sphingomyelin/cholesterol (SM/Chol) is used (19, 21). The enrichment of sphingomyelin and cholesterol in the outer plasma membrane appears to be physiologically significant because of their formation of detergent-insoluble patches (30). Sphingolipids have also been described as relevant for the fusion mechanism of viral proteins (31).

The functionally important interactions of the 24-kDa protein bindin with the membrane are attributed to its highly conserved central domain, consisting of 70–80 amino acids (14, 17, 20, 21). By truncation experiments and using overlapping synthetic peptides of this region, we have located the minimal membrane binding motif “B18” (20, 21). These 18 amino acids (LGLLLRHLRHHSNLLANI) are perfectly conserved among all known sea urchin species, and the sequence bears some resemblance to viral fusion peptides. Interestingly, the same region

---

*This work was supported by the Fonds der Chemischen Industrie (Liebig Stipendium, to A. S. U.), by SFB 197 from the Deutsche Forschungsgemeinschaft, and by the German-American Academic Council. 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.

‡To whom correspondence should be addressed. Tel.: 49-3641-657572; Fax: 49-3641-657520.

§The abbreviations used are: SM, sphingomyelin; Chol, cholesterol; NBD-PE, N-7-nitrobenz-2-oxa-1,3-diazole-4-yolphosphatidylethanolamine; Rh-PE, N-lysisamine rhodamine B sulfonylphosphatidylethanolamine; ANTS, 8-amino-naphthalene-1,3,6-trisulfonic acid sodium salt; DPX, p-xylenebis(pyridinium)bromide; LUV, large unilamellar vesicle(s).

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
also appears to participate in receptor binding, and related peptide fragments have been shown to inhibit fertilization in vitro (32, 33). Hence we reasoned that B18 represents an attractive model system to simulate the lipid-protein interactions during fertilization. To this end, we have used fluorescence assays to investigate whether this amphiphilic peptide is capable of inducing vesicle aggregation, membrane fusion, and destabilization, as monitored by lipid mixing and leakage. Specifically, we have examined whether these processes are affected by Zn$^{2+}$, which is found in the native protein and presumably binds to the histidine-rich motif contained in the B18 peptide (34–36). In complementary circular dichroism experiments, structural changes of the peptide were monitored and correlated with its functional features. The results indicate that B18 may be regarded as an appropriate model for various aspects of lipid-protein interactions and membrane fusion during fertilization, because its behavior is in many respects comparable with that of the native protein bindin.

**EXPERIMENTAL PROCEDURES**

**Synthetic Peptide**—The peptide B18 (LGLLLRHLRHHSNLLANI), corresponding to the carboxy-terminal part of the current zona protein of the sea urchin *Strongylocentrotus purpuratus*, was synthesized semi-automatically using solid phase resin and Fmoc (N-(9-fluorenylmethoxycarbonyl) protecting groups (37). The crude peptide was purified by reverse phase high pressure liquid chromatography on a water/acetonitrile gradient with 0.1% trifluoroacetic acid. The purity and mass of the product (2090 g/mol) were checked by electrospray mass spectrometry, and the amount of lyophilized peptide was determined gravimetrically. Stock solutions were prepared by dissolving B18 at typically 1 mM in water, giving a pH of ~4 where it is fully soluble and does not self-aggregate, as otherwise slowly occurs at pH > 7.

**Buffers**—Buffers for fusion, leakage, and aggregation assays were made with 10 mM HEPES (usually pH 7.4), Bis-tris propane, or acetate. They contained 140 mM NaCl unless salt effects were to be examined. CD measurements were carried out without salt to avoid distortion in the far-UV. Stock solutions of ZnCl$_2$, CuCl$_2$, CoCl$_2$, CaCl$_2$, MgCl$_2$ (or the corresponding SO$_4$) were prepared by dissolving B18 at typically 1 mM in water, giving a pH of ~4 where it is fully soluble and does not self-aggregate, as otherwise slowly occurs at pH > 7.

**Lipid Mixing Assay**—To remove unencapsulated dye, the vesicles were washed right after exposure to B18 and Zn$^{2+}$, followed by monitoring the relief of fluorescence resonance energy transfer between B18 and Zn$^{2+}$. The time dependence of fluorescence was monitored with 1-s resolution on a spectrophotometer (SLM, Aminco Bowman Series 2 Luminescence, Urbana) at excitation and emission wavelengths of 465 and 530 nm, respectively. The temperature was maintained at 30 °C (unless stated otherwise) in a thermostated cuvette holder equipped with a magnetic stirrer. The labeled vesicles were suspended in a final incubation volume of 2 ml buffer together with a 3-fold excess of nonlabeled vesicles, giving a total lipid concentration of 200 μM. Fusion between the vesicles was followed upon adding the peptide, metal ions, or EDTA from their stock solutions with a Hamilton microsyringe. Most experiments were carried out with a peptide concentration of 5 or 10 μM and a Zn$^{2+}$ concentration of 40 μM to induce fusion or 80 μM to induce aggregation. The fluorescence scale was calibrated by setting the base line of the initial background signal to 0%. Infinite probe dilution, corresponding to 100% fluorescence, was determined after disrupting the vesicles in 0.5% (v/v) Triton X-100. The initial rate of fusion was analyzed by curve-fitting the signal with the Enzfitter software and expressed as the percentage of fluorescence change (relative to the level obtained upon infinite dilution) per second (% max/s).

**Leakage of Vesicle Contents**—The release of aqueous contents from the LUVs was monitored by the fluorescence decoupling of ANTS by DPX (42), both entrapped in the SM/Chol vesicles as described above. For resonance energy transfer measurements, the ANTS excitation was set at 284 nm, and emission was set at 530 nm. Sample concentrations, experimental conditions, and data analysis were the same as in the lipid mixing assay above.

**Vesicle Aggregation Assay**—Aggregation of the LUVs was monitored by turbidity measurements. The absorbance at 400 nm was recorded on a thermostated Hamamatsu spectrophotometer, using conditions as for lipid mixing and leakage. The rates of aggregation were calculated from the absorbance per minute (A/min) (38). The average diameter of the initial LUVs and of the aggregated/fused vesicles were measured on a Nicomac particle size analyzer (model 370).

**Circular Dichroism Spectroscopy**—CD spectra were recorded with a Jasco 710 spectropolarimeter over the wavelength range from 185 to 250 nm (43). The temperature was maintained at 5 °C, the scan rate was 50 nm/min, the step resolution was 0.5, the response time was 1 s, and 5–10 runs were accumulated per spectrum. The peptide was measured at pH 7.5, using different concentrations of 5 μM B18 in 0.5 mM HEPES, 50 μM B18 in 5 mM HEPES, or 500 μM B18 in 50 mM HEPES in a 10–, 0.1-mm cuvette, respectively.

**Electrospray Mass Spectrometry**—Noncovalent interactions of the peptide with various lipid mixtures were investigated on samples of 50 μM B18 in 250 mM NH$_4$HCO$_3$ buffer at pH 9.0. Metal ions were added at a ratio of 1:1 or 8:1, respectively, to B18. In view of the tendency of B18 to aggregate at this pH, fresh samples were prepared for each measurement.

**RESULTS**

**Interaction of the Peptide with SM/Chol Vesicles**—As shown in Fig. 1, the peptide B18 induces vesicle aggregation and lipid mixing when added to large unilamellar vesicles consisting of SM/Chol (80/20). For these effects, Zn$^{2+}$ must be included in the incubation medium. This divalent cation is known to be contained in the native bindin protein under physiological conditions (11, 32). Furthermore, the data show that vesicle leakage occurs upon peptide binding, which interestingly does not require the presence of Zn$^{2+}$, unlike aggregation and fusion.

It is illustrated in the top row of Fig. 1 that the peptide alone does not lead to vesicle aggregation (Fig. 1A), but turbidity does increase when B18 is added in the presence of Zn$^{2+}$ (Fig. 1B). By addition of EDTA, vesicle aggregation is arrested and only partially reversed (approximately 20% decrease in turbidity; not shown). This indicates the formation of larger structures, presumably because of B18-Zn$^{2+}$-induced vesicle fusion. The occurrence of fusion is further supported by the data presented in Fig. 1C, which illustrates the particle size distribution before and after exposure to B18 and Zn$^{2+}$. The average diameter of the SM/Chol vesicles (or vesicular clusters) increases from approximately 150 nm by more than an order of magnitude to 3000 nm. The subsequent addition of EDTA leads to a final size of around 1500 nm.

Membrane fusion is accompanied by the mixing of membrane lipids. As shown in the middle row of Fig. 1, it is apparent that neither the peptide on its own (Fig. 1D) nor Zn$^{2+}$ induces any lipid mixing. However, when both B18 and Zn$^{2+}$ are present, rapid lipid mixing is observed (Fig. 1E). This process is instantaneously interrupted upon chelation of Zn$^{2+}$.
bonds specifically to Zn\(^{2+}\) and H. W. Meyer, submitted for publications.

Electron microscopy was used to confirm that EDTA is not merely capable of chelating Zn\(^{2+}\) and H. W. Meyer, submitted for publications.

The addition of B18 is indicated by an arrow labeled B, the addition of Zn\(^{2+}\) is indicated by an arrow labeled Z, the addition of EDTA is indicated by an arrow labeled E, and the final disruption of vesicles with Triton X-100 is marked with an asterisk. The particle size distribution curves (panel C) are labeled correspondingly.

by EDTA (Fig. 1F). The fusion data, monitored by the NBD/Rh-PE lipid mixing assay, thus show the same Zn\(^{2+}\) dependence as observed for aggregation. Electron microscopy was used to confirm the formation of large fused vesicles.

The requirement of B18 for reasonably low concentrations of Zn\(^{2+}\) to induce vesicle aggregation and lipid mixing suggests that the peptide becomes “activated” by the cation. Little difference is observed when reversing the order of addition to the vesicles, but prior incubation of B18 with Zn\(^{2+}\) decreases their combined activity. This observation suggests that the peptide binds specifically to Zn\(^{2+}\), which may induce a structural change or cause the peptide monomers to aggregate. In the native parent protein, the Zn\(^{2+}\) ion is presumably bound via the same histidine-rich motif as in the B18 peptide, because there are no further conserved histidines or cysteines in the remaining parts of the bindin sequence (10, 11, 32).

Binding of the peptide to the SM/Chol vesicles evidently destabilizes the bilayer, as shown by the release and fluorescence dequenching of ANTS/DPX (Fig. 1, bottom row). Interestingly, addition of B18 on its own already induces a substantial release of contents (Fig. 1G), which indicates a distinct affinity of the water-soluble peptide for the uncharged membrane. Furthermore, this observation implies that B18 does not require exogenously added Zn\(^{2+}\) to interact with the bilayer as such, although the presence of Zn\(^{2+}\) further enhances the rate and extent of leakage (Fig. 1H). In fact, the data reveal that the metal ion must fulfill a structural role when binding to B18, conveying functional properties to the peptide by triggering vesicle aggregation and fusion. These properties are apparently not expressed when B18 is interacting with the bilayer in a Zn\(^{2+}\)-independent mode. It is in this context relevant to note that EDTA is not merely capable of chelating Zn\(^{2+}\). Unexpectedly, the presence of EDTA was observed to abolish the Zn\(^{2+}\)-independent mode of peptide-induced leakage (Fig. 1I). This observation implies that EDTA interacts directly with B18 and thereby prevents it from destabilizing the membrane.

The pH and temperature dependence of peptide-Zn\(^{2+}\)-induced fusion and aggregation of SM/Chol vesicles is shown in Fig. 2. The observed pH optimum near 7.6 (Fig. 2A) supports the notion that the histidine residues in B18 coordinate the metal ion. These side chains are deprotonated and available for Zn\(^{2+}\) binding when the pH is raised beyond the typical histidine pK\(_a\) of around 6 to 7. At an even higher pH above 8, on the other hand, the low solubility of Zn(OH)\(_2\) becomes the limiting factor for complex formation, and the aggregation and fusion activities are seen to decrease again accordingly.

A temperature optimum at around 30 °C is observed for both lipid mixing and aggregation as demonstrated in Fig. 2B. By differential scanning calorimetry we found that the pure (mixed chain) brain sphingomyelin has a relatively broad transition, with an onset near 30 °C, a maximum at 40 °C, and an end point around 50 °C. When mixed with 20% cholesterol, the differential scanning calorimetry signal is further broadened, and the onset shifted to a lower temperature. Nevertheless, it appears that in the mixed SM/Chol system, aggregation and fusion display their optimum temperature near the onset of the original melting point of pure SM. The possibility of a temperature-induced conformational change of the peptide is unlikely, according to our CD measurements in solution (see below).

Interaction of the Peptide with Zn\(^{2+}\)—To further define the role of Zn\(^{2+}\) in the B18-induced vesicle aggregation and fusion process, its concentration dependence was examined in Fig. 3. With increasing amounts of Zn\(^{2+}\), the rates of fusion (Fig. 3A) and aggregation (Fig. 3B) are initially found to increase, as expected. After passing through a maximum, however, the activity of the peptide decreases again, suggesting that it becomes blocked by excess Zn\(^{2+}\). To check whether the interaction between Zn\(^{2+}\) and B18 is influenced by the law of mass

\(^2\text{A. S. Ulrich, W. Tichelaar, G. Förster, O. Zschörnig, S. Weinkaut, and H. W. Meyer, submitted for publications.}\)

\(^3\text{H. W. Meyer, H. Bunjes, and A. S. Ulrich, manuscript in preparation.}\)
action, we measured the Zn\(^{2+}\) dependence of lipid mixing and aggregation at 5 and 10 \(\mu M\) peptide concentration, respectively (Fig. 3, A and B). Within error limits, the amount of Zn\(^{2+}\) required for a maximum response is independent of peptide concentration. Note, however, that more Zn\(^{2+}\) is required for optimum aggregation than for optimum fusion, suggesting that the Zn\(^{2+}\)-B18 complexes involved in aggregation and fusion are not necessarily identical.

Fig. 4 summarizes the lipid mixing and aggregation kinetics as a function of peptide concentration. According to Fig. 3, where the optimum Zn\(^{2+}\) concentration was found to be independent of peptide concentration, it is justified here to use a constant aliquot of Zn\(^{2+}\) to trigger fusion or aggregation (40 or 60 \(\mu M\), respectively). Lipid mixing and aggregation kinetics are displayed in the same plot on different scales (Fig. 4A) to allow a comparison of the two effects. Both profiles show essentially the same dependence on B18 concentration, suggesting that the membrane becomes saturated with peptide in an approximately linear fashion. Saturation occurs at a lipid/peptide ratio of around 15:1. Assuming that essentially all peptide binds to the bilayer, the available surface area on the outer vesicle leaflet would be approximately 300 \(\AA^2/\text{peptide}\), based on a lipid area of 40 \(\AA^2\). The molecular area of the 18-amino acid peptide is also close to 300 \(\AA^2\) when modeled either as a \(\beta\)-sheet or an \(\alpha\)-helix. This good correlation suggests that the bilayer surface may become completely covered by a monomolecular layer of peptide, at which point the maximum rate of aggregation and lipid mixing is reached.

The concentration dependence of leakage in the presence of Zn\(^{2+}\) is included in Fig. 4 as a dotted line. Compared with lipid mixing, contents release occurs about twice as fast, and the maximum effect is already reached at a much lower lipid/peptide ratio. To resolve the concentration dependence in more detail, the leakage data are illustrated on an expanded scale in Fig. 4B, both in the presence and in the absence of Zn\(^{2+}\). Judging by the first few data points, a sigmoidal character may be attributed to these curves, which would be indicative of a cooperative binding event (28). This interpretation, however, does not imply the formation of a transmembrane pore but rather that a limited number of peptides are sufficient to destabilize a vesicle such that it discharges its entire load.

Interaction of the Peptide with Other Ions—The ability of Zn\(^{2+}\) to trigger vesicle aggregation and lipid mixing is attributed to its interaction with the histidine side chains of the peptide. To further define the specificity and the functional consequences of this complex, electrospray mass spectrometry was used to check whether the peptide could also bind to any other metal ions, such as Cu\(^{2+}\), Co\(^{2+}\), Mg\(^{2+}\), Cu\(^{2+}\), and Na\(^{+}\). Only the transition elements gave a positive signal at the combined mass of the peptide plus metal ion, which reverted to the peptide mass alone under acidic denaturing conditions. This confirms that, next to Zn\(^{2+}\), the peptide can also form a complex with Cu\(^{2+}\) and Co\(^{2+}\). However, in contrast to Zn\(^{2+}\), neither Cu\(^{2+}\) nor Co\(^{2+}\) was able to stimulate the peptide to induce vesicle aggregation or fusion. More significant still is the observation that leakage, which is caused by the peptide per se (Fig. 1D) is suppressed in the presence of either Cu\(^{2+}\) or Co\(^{2+}\) (data not shown). Therefore, binding of Cu\(^{2+}\) or Co\(^{2+}\) has different structural and functional consequences on B18 than the binding of Zn\(^{2+}\).

To quantify the inhibitory effects of Cu\(^{2+}\) and Co\(^{2+}\), competition experiments were carried out using vesicles that were triggered to fuse by the addition of 10 \(\mu M\) peptide in the presence of 40 \(\mu M\) Zn\(^{2+}\). Fig. 5A illustrates how the rate of lipid mixing becomes progressively reduced when an increasing amount of Cu\(^{2+}\) or Co\(^{2+}\) is present in the incubation solution. Cu\(^{2+}\) is capable of suppressing fusion almost completely, its effect being much more pronounced than that of Co\(^{2+}\). It is
Fertilization: A Bindin-derived Peptide Fuses Lipid Vesicles

![Graph A](image1.png)

**FIG. 5.** To illustrate the binding affinities of various ions to B18, fusion is triggered as usual by the addition of 10 μM B18 and 40 μM Zn²⁺ (or 60 μM Zn²⁺ for aggregation). In A, increasing concentrations of Cu²⁺ (filled circle), Co²⁺ (filled triangle), or excess Zn²⁺ (dotted line) are present in the incubation solution. B shows the inhibitory effect of EDTA on the rates of fusion (solid line) and aggregation (dotted line).

![Graph B](image2.png)

![Graph C](image3.png)

**FIG. 6.** Circular dichroism reveals the structural changes of B18 (500 μM) that are induced by Zn²⁺ (panels A and B) and Cu²⁺ (panel C). The addition of Zn²⁺ initially leads to the formation of a partially α-helical B18-Zn²⁺ complex (panel A), followed by precipitation (panel B). The binding of Cu²⁺, on the other hand, induces a β-turn in the peptide (panel C) and is also followed by precipitation (not shown).

![Graph D](image4.png)

![Graph E](image5.png)

![Graph F](image6.png)

### Structural Consequences of Ion Binding

For vesicle aggregation and fusion to occur, a specific Zn²⁺-mediated assembly of B18 has to take place, as documented above. To determine the structural features of this complex, the conformation of the peptide and its tendency to oligomerize were investigated by circular dichroism. Because sphingomyelin gives a pronounced CD signal at wavelengths below 220 nm, measurements were carried out with the peptide alone in aqueous solution in the absence of any lipid. The peptide tends to self-aggregate slowly when kept as a millimolar solution above pH 7, but samples were prepared freshly from an acidic stock. Under these conditions, B18 is well soluble and largely unstructured at pH 7.5, even at a concentration of 500 μM. CD measurements revealed a slight increase in “secondary structure” over the pH range from 3.0 to 9.0, which amounts to less than 10% as judged by the signal amplitude at 222 nm (data not shown).

A series of Zn²⁺ titrations was carried out at pH 7.5, using different peptide concentrations to assess oligomerization effects. At 5 μM peptide concentration, the addition of Zn²⁺ has hardly any effect on its random coil conformation (data not shown). At 50 μM B18, a weak precipitation of the peptide sets in with increasing amounts of Zn²⁺, as judged by the decrease in signal intensity because of light scattering (data not shown). At an even higher peptide concentration of 500 μM B18, however, substantial structural changes are revealed in the CD spectra, which are summarized in Fig. 6 (A and B). Initially, the addition of about half an equivalent of ions (250 μM Zn²⁺, note the stoichiometry w.r.t. 500 μM B18) induces a partially α-helical peptide conformation, according to the double minimum in the line shape at 207 nm and close to 222 nm (Fig. 6A (43)). The addition of further Zn²⁺ decreases approximately linearly with EDTA concentration, as expected for a successive removal of free Zn²⁺ ions from the solution. The rate of lipid mixing, on the other hand, decreases more dramatically and is completely suppressed already at much lower EDTA concentration. Therefore, it appears that EDTA interferes with the formation of the B18-Zn²⁺ fusion complex, for which more strict structural criteria have to be met than for mere vesicle aggregation. The most likely side chains on the peptide to interact with EDTA are the two arginine residues.

### Structural Consequences of Ion Binding

For vesicle aggregation and fusion to occur, a specific Zn²⁺-mediated assembly of B18 has to take place, as documented above. To determine the structural features of this complex, the conformation of the peptide and its tendency to oligomerize were investigated by circular dichroism. Because sphingomyelin gives a pronounced CD signal at wavelengths below 220 nm, measurements were carried out with the peptide alone in aqueous solution in the absence of any lipid. The peptide tends to self-aggregate slowly when kept as a millimolar solution above pH 7, but samples were prepared freshly from an acidic stock. Under these conditions, B18 is well soluble and largely unstructured at pH 7.5, even at a concentration of 500 μM. CD measurements revealed a slight increase in “secondary structure” over the pH range from 3.0 to 9.0, which amounts to less than 10% as judged by the signal amplitude at 222 nm (data not shown).

A series of Zn²⁺ titrations was carried out at pH 7.5, using different peptide concentrations to assess oligomerization effects. At 5 μM peptide concentration, the addition of Zn²⁺ has hardly any effect on its random coil conformation (data not shown). At 50 μM B18, a weak precipitation of the peptide sets in with increasing amounts of Zn²⁺, as judged by the decrease in signal intensity because of light scattering (data not shown). At an even higher peptide concentration of 500 μM B18, however, substantial structural changes are revealed in the CD spectra, which are summarized in Fig. 6 (A and B). Initially, the addition of about half an equivalent of ions (250 μM Zn²⁺, note the stoichiometry w.r.t. 500 μM B18) induces a partially α-helical peptide conformation, according to the double minimum in the line shape at 207 nm and close to 222 nm (Fig. 6A (43)). The addition of further Zn²⁺ then leads to the precipitation of B18, as seen in Fig. 6B (representing the continuation of the titration in Fig. 6A). The visibly cloudy precipitate could be redissolved by adding EDTA or by acidifying the solution. This CD analysis suggests (and a more detailed discussion will be published elsewhere), that initially a soluble peptide-Zn²⁺ complex assembles with a 2:1 stoichiometry of B18:Zn. Further

---

4 L. Magdaleno-Maiza, O. Zschöning, and A. S. Ulrich, manuscript in preparation.
addition of Zn\(^{2+}\) then leads to the formation of a 1:1 precipitate of (B18-Zn\(^{2+}\))\(_n\). Both in the soluble and precipitated Zn\(^{2+}\) complexes, B18 has a partially helical structure. It appears that Zn\(^{2+}\) connects the peptides via their histidine residues, and the resulting conformational change may expose some hydrophobic regions that promote aggregation further.

Whereas Zn\(^{2+}\) was shown to activate the peptide, Cu\(^{2+}\) binds competitively and causes an inhibition of vesicle leakage, aggregation, and fusion. Fig. 6C illustrates the changes in the spectral line shape when Cu\(^{2+}\) is added to a 500 μM peptide solution, suggesting that a local β-turn is formed (43). Precipitation starts to set in at higher Cu\(^{2+}\) concentrations but with a different overall line shape than the Zn\(^{2+}\) precipitate (data not shown). Similar to the inhibitory effect of Cu\(^{2+}\), we also observed that EDTA prevents the peptide from destabilizing the membrane, possibly by binding to the two arginine side chains. The structural change induced by EDTA is weak, and the resulting CD line shape resembles that observed with Cu\(^{2+}\), again reminiscent of a β-turn (data not shown). Hence, it appears that the binding of Cu\(^{2+}\) or EDTA to certain peptide side chains induces a different conformation than Zn\(^{2+}\), thus rendering the peptide inactive toward the membrane on the time scale of the fusion measurements.

**DISCUSSION**

We have demonstrated that the peptide B18 is able to induce aggregation and fusion of uncharged lipid vesicles, thus mimicking the function of its parent protein bindin. The native sea urchin acrosomal sperm protein (236 amino acids) is supposedly involved in sperm-egg adhesion as well as membrane fusion during fertilization (10, 11), and its interactions with lipid vesicles have been well characterized (17–20). Here, we describe some remarkably similar interactions of the 18-amino acid peptide B18, which may be relevant for the peripheral anchoring of bindin onto the acrosomal membrane and which may even play a role in the fusion event between sperm and oocyte. Both the peptide and the protein display a high affinity toward SM/Chol vesicles (21), which may represent the acrosomal sperm membrane and possibly the egg membrane. Like bindin, B18 is also able to trigger the aggregation and fusion of artificial model membranes (19, 21). This functional property of the peptide is exclusively expressed in a Zn\(^{2+}\)-dependent manner. Similarly, the native fertilization protein is known to contain one equivalent of this particular cation (32).

To gain further insight into the mechanism of fusion, multiple interactions need to be taken into account between all the participating molecules in this model system, namely the B18 peptide, Zn\(^{2+}\) cations, and SM/Chol vesicles. First, the membrane binding mode of the peptide per se requires some attention. We found that B18 destabilizes the bilayer and causes substantial leakage from the large unilamellar vesicles (Fig. 1G). The high affinity for the uncharged lipid must be attributed to hydrophobic interactions, rather than an initial long range electrostatic attraction. Although the amphiphilic peptide carries two positively charged arginines in its center, many hydrophobic side chains are clustered at each end of the molecule (Fig. 7A). There is some indication that leakage may proceed as a cooperative event (Fig. 4B) (28).

Upon binding to the lipid vesicles, the peptide is able to trigger their aggregation and fusion, but for these activities it needs to be stimulated by Zn\(^{2+}\) (Fig. 1E). Before evaluating the ternary membrane system, first consider the peptide-Zn\(^{2+}\) interactions in the absence of any lipid vesicles. Our CD analysis showed that Zn\(^{2+}\) induces a partially α-helical conformation of B18 and leads to the formation of oligomeric metallo-peptide complexes. The coordination must involve the histidine residues of the motif HLRHH, but their spacing is too close to allow

---

**Fig. 7. Summary of the interactions of B18 with different ions and with the membrane.** In A, the local conformation around the histidine-rich motif is correlated with fusogenic activity, showing the putative side chains involved in complex formation. The schematic picture (B) illustrates the postulated sequence of events involved in membrane fusion. Following the accumulation of B18 at the membrane surface, which is accompanied by leakage, the vesicles become aggregated via a trans-complex. This active fusion intermediate presumably consists of a Zn\(^{2+}\) bridge that connects two peptides on apposing surfaces.

---

A key step in vesicle aggregation and fusion must be the assembly process or the resulting molecular conformation of the peptide-Zn\(^{2+}\) oligomers in the presence of the membrane. Complex formation in solution was found to be favored only at high peptide concentration (500 μM B18), whereas vesicle fusion was accomplished with much lower amounts (typically 10 μM). Apparently, the membrane recruits the water-soluble peptides from the bulk solution, and the high local concentration promotes their self-assembly with Zn\(^{2+}\) either near the vesicle surface or once they are partially immersed in the bilayer. As indicated above (Fig. 1F), the peptide interacts with the membrane almost instantaneously, and leakage is even more pronounced in the presence of Zn\(^{2+}\). The binding of Zn\(^{2+}\) appears to promote a fusogenic peptide conformation, possibly by cross-linking the monomers to one another. By analogy to the mechanism of Cu\(^{2+}\)-induced fusion of acidic phospholipid vesicles (41), we suggest that a so-called “trans-complex” may be formed between two apposing membranes. As illustrated in Fig. 7B, a
central Zn$^{2+}$ ion may be complexed from either side by two B18 molecules that are associated with separate vesicles. Part of the function of such a membrane-bound complex would be to mediate vesicle aggregation. The subsequent fusion process will then be facilitated through the concerted destabilization of the bilayers by the hydrophobic side chains. In Fig. 7B we have drawn the peptide in the Zn$^{2+}$ complex with a partially helical structure, based on the CD data in solution. On the other hand, we have no information about its conformation when bound to the membrane on its own without Zn$^{2+}$. Neither does this drawing take into account the possibility that Zn$^{2+}$ bridges may also form between peptides in-plane of the membrane rather than between apposing vesicles.

Various additional observations underscore the specificity and subtlety of the Zn$^{2+}$-B18 complex formed, which is involved in the actual fusion step. At a fixed peptide concentration, titration experiments demonstrate that fusion and aggregation are inhibited by excess Zn$^{2+}$ (Fig. 3). Similarly, preincubation of B18 with Zn$^{2+}$ reduces their combined fusion activity. We thus conclude that the dimeric B18-Zn$^{2+}$-B18 complex is the active fusogenic agent, whereas further oligomerization deteriorates its potency. The saturation of all histidine residues with Zn$^{2+}$ or the formation of extended oligomeric chains may sterically interfere with the membrane fusion process. Apparently, an excess of Zn$^{2+}$ has a less perturbing effect on aggregation than on fusion, and vesicular aggregates as part of the fusion complex could be dispersed again with EDTA (Fig. 1C).

When the histidine residues are deprotonated, the peptide can bind to various transition metal ions, which eventually leads to precipitation. B18 also tends to aggregate slowly by itself in solution. Yet, peptide aggregation or complex formation per se do not provide the molecular specificity or the necessary kinetics required for membrane fusion. In contrast to Zn$^{2+}$, the addition of Cu$^{2+}$ or Co$^{2+}$ to B18 does not induce any vesicle aggregation or fusion. On the contrary, Cu$^{2+}$ and Co$^{2+}$ compete rapidly and efficiently with Zn$^{2+}$, and their mere presence in the incubation mix inhibits the Zn$^{2+}$-induced membrane fusion (Fig. 5A). In solution, the peptide is folded by Cu$^{2+}$ into a local β-turn (Fig. 6C). We therefore suggest that the initial binding site may be different for Cu$^{2+}$ than for Zn$^{2+}$. It very likely involves the histidine pair His$^{109}$ and His$^{112}$, whose spacing $i$ and $i + 3$ is characteristic of metal binding sites with a β-turn (Fig. 7A) (34–36).

A similar conformational or steric block appears to be the reason why EDTA prevents the interaction of B18 with the membrane (Fig. 1I) and actively inhibits fusion (Fig. 5B). A bidentate complex between EDTA and the two arginine side chains (Arg$^{108}$ and Arg$^{111}$) would be entropically favored and energetically stabilized by hydrogen-bonded interactions between the guanido- and carboxyl-groups (Fig. 7A). Such binding mode was in fact proposed to explain the adhesion of bindin to the sulfate esters of certain cell surface polysaccharides on the putative sea urchin bindin receptor (12, 13). Remarkably, a nonapeptide (LRHLRHHSN), derived from B18 with the same arginine/histidine motif, was shown to be a potent inhibitor of fertilization in vitro, and it expressed its optimum binding affinity only in the presence of Zn$^{2+}$ (32). These two observations, namely (a) that B18 requires Zn$^{2+}$ to trigger membrane fusion and (b) that the related nonapeptide requires Zn$^{2+}$ to bind to the sulfate groups of cell surface carbohydrates, emphasize the specific structural role of this ion to promote an active local conformation in the peptide backbone. The function of the conserved protein domain around the sequence of B18 thus appears to be involved in the binding of several partners, i.e. the Zn$^{2+}$ cation, the acrosomal membrane, and the egg cell receptor.

Finally, it is remarkable that fusion occurs at all with the SM/Chol model membranes, given that they are not in the fluid phase but in a liquid ordered state. A similar gel phase preference has also been reported for the vesicle binding and fusion activity of the native fertilization protein with other lipids (17, 29). A broad transition temperature range was determined for the mixed SM/Chol system used here, with a maximum at 40 °C. Nevertheless, the optimum for B18-induced fusion coincides with the onset of the phase transition of pure SM around 30 °C (Fig. 2B). Accordingly, it is tempting to suggest that B18-mediated fusion may be accomplished via its interaction with locally enriched SM domains in the mixed SM/Chol bilayers (30, 31). Because the lipid packing is strongly perturbed during the melting process, this would favor any local or temporary peptide penetration. In line with numerous previous studies concerned with structural features of fusogenic synthetic or natural peptides, penetration into the bilayer is particularly facilitated by a helical structure (26, 44). Indeed, B18 tends to assume a partially α-helical conformation in the presence of Zn$^{2+}$, at least in the aqueous environment where we could study complex formation directly by CD. Peptide self-assembly as a mechanism for membrane perturbation and fusion has also been described in the literature, when it involves a β-sheet structure (22, 23, 28). Oligomerization is in fact a recognized feature in the fusion mechanism of viral proteins, which may even involve the hydrophobic fusion peptides once they get exposed (3–5, 27). Here, we have described a Zn$^{2+}$-mediated self-assembly of B18, which correlates with its fusogenic activity.

In summary, the minimum membrane-binding peptide B18, derived from the sperm protein bindin, represents an attractive model system to study lipid-protein interactions during fertilization. Membrane binding, adhesion to sulfated polysaccharides, and self-association appear to be regulated by the formation of specific metallo-complexes, which in turn determine the local protein conformation. The functionality of the full size protein will surely depend on numerous other factors that are not accessible by this model. Fusion between sperm and egg, for instance, is presumably a nonleaky process, but the peptide induces substantial perturbations on the membrane. Neither can the mechanism of species-specific recognition be addressed using the short conserved B18 sequence. Nevertheless, our studies reveal the very likely involvement of this peptide in membrane binding. Whether it acts as a genuine fusion peptide or simply serves as a peripheral membrane anchor remains to be established.

**Acknowledgments**—We are very grateful to Matthias Wilm (EMBL) for the mass spectrometry, to Heike Bunjes (University of Jena) for the differential scanning calorimetry measurements, to Leticia Magdaleno-Maiza (University of Jena) for additional CD data, and to Dr. Schmokele (Heidelberg) for the sincere discussions.

**REFERENCES**

1. Rothman J. E., and Wieland, F. T. (1996) Science 272, 227–234
2. Vidal, M., and Hoekstra, D. (1995) J. Biol. Chem. 270, 17823–17829
3. Hernandez, L. D., Hoffman, L. R., Wolfsberg, T. G., and White, J. M. (1996) Annu. Rev. Cell Dev. Biol. 12, 627–661
4. Hughson, F. M. (1995) Curr. Opin. Struct. Biol. 5, 507–513
5. Hoekstra, D. (1999) J. Biomed. Biophambr. 23, 121–135
6. Havelia, A. P. J., Almeida, E. A. C., and White, J. M. (1996) Curr. Opin. Cell Biol. 8, 692–699
7. Wassarman P. M. (1995) Curr. Opin. Cell Biol. 7, 658–664
8. Lennarz, W. J. (ed) (1994) Semin. Dev. Biol. 5 (special issue)
9. Aris, E. G. J. M., Kuklen, J., Jager, S., and Hoekstra, D. (1993) Eur. J. Biochem. 217, 1001–1009
10. Vaqueiro, V. D., Swanson, W. J., and Hellberg, M. E. (1995) Dev. Growth Differ. 37, 1–10
Fertilization: A Bindin-derived Peptide Fuses Lipid Vesicles

11. Hofmann, A., and Glabe, C. G. (1994) Semin. Dev. Biol. 5, 233–242
12. DeAngelis, P. L., and Glabe, C. G. (1990) Biochim. Biophys. Acta 1037, 169–165
13. DeAngelis, P. L., and Glabe, C. G. (1998) Biochemistry 37, 8189–8194
14. Lopez, A., Miraglia, S. J., and Glabe, C. G. (1993) Dev. Biol. 156, 24–33
15. Stears, R. L., and Lennarz, W. J. (1997) Dev. Biol. 187, 200–208
16. Mauk, R., Jaworski, D., Kamei, N., and Glabe, C. G. (1997) Dev. Biol. 184, 31–37
17. Kennedy, L., DeAngelis, P. L., and Glabe, C. G. (1989) Biochemistry 28, 9153–9158
18. Glabe, C. G. (1985) J. Cell Biol. 100, 794–799
19. Glabe, C. G. (1985) J. Cell Biol. 100, 806–806
20. Miraglia, S. J., and Glabe, C. G. (1993) Biochim. Biophys. Acta 1145, 191–198
21. Miraglia, S. (1993) Structure-Function Analysis of the Membrane Binding Domain of Bindin, and the Potential Role of Bindin in Plasma Membrane Fusion. Ph. D. Thesis, University of California, Irvine
22. Muga, A., Neugebauer, W., Hirama, T., and Surewicz, W. K. (1994) Biochemistry 33, 4444–4448
23. Niidome, T., Kimura, M., Chiba, T., Ohmori, N., Mihara, H., and Aoyagi, H. (1997) J. Peptide Res. 49, 563–569
24. Hong, K., and Vacequier, V. D. (1986) Biochemistry 25, 543–549
25. Swanson, W. J., and Vacequier, V. (1995) Biochemistry 34, 14202–14208
26. Brasseur, R., Pillot, T., Lins, L., Vanderkerckhove, and Rosseneu, M. (1997) Trends Biol. Sci. 22, 167–171
27. Kliger, Y., Aharoni, A., Rappaport, D., Jones, P., Blumenthal, R., and Shai, Y. (1997) J. Biol. Chem. 272, 13486–13505
28. Nieva, J. L., Nir, S., Muga, A., Geni, F. M., and Wilschut, J. (1994) Biochemistry 33, 3201–3209
29. Loihi-Stahlhufen, A., Ulrich, A. S., Kaufmann, S., and Bayerl, T. M. (1996) Eur. Biophys. J. 25, 151–153
30. Slotte, P. (1995) Biochim. Biophys. Acta 1235, 419–427
31. Moesby, L., Corver, J., Kumar, R., Bittman, R., and Wilschut, J. (1995) Biochemistry 34, 10319–10324
32. DeAngelis, P. L., and Glabe, C. G. (1990) Peptide Res. 3, 1–7
33. Minor, J. E., Britten, R. J., and Davidson, E. H. (1993) Mol. Biol. Cell 4, 375–387
34. Matthews, D. J. (1995) Curr. Opin. Biotechnol. 6, 419–424
35. Regan, L. (1995) Trends Biol. Sci. 20, 280–285
36. Arnold, F. H., and Haymore, B. L. (1991) Science 252, 1796–1797
37. Glabe, C. (1990) Technique 2, 138–146
38. Mayer, L., Hoope, M. J., and Cullis, P. R. (1986) Biochim. Biophys. Acta 858, 161–168
39. Fromm, N., and Wilschut, J. (1993) Methods Enzymol. 220, 3–14
40. Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
41. Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Biochemistry 20, 4093–4099
42. Ellens, H., Bentz, J., and Szoka, F. C. (1985) Biochemistry 24, 3099–3106
43. Johnson, W. C. (1990) Proteins Struct. Funct. Genet. 7, 205–214
44. Pecheur, E.-I., Martin, J., Ruyschaert, J.-M., Bienvenue, A., and Hoekstra, D. (1998) Biochemistry 37, 2361–2371
Membrane Fusion Is Induced by a Distinct Peptide Sequence of the Sea Urchin Fertilization Protein Bindin

Anne S. Ulrich, Marlies Otter, Charles G. Glabe and Dick Hoekstra

J. Biol. Chem. 1998, 273:16748-16755.
doi: 10.1074/jbc.273.27.16748

Access the most updated version of this article at http://www.jbc.org/content/273/27/16748

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 8 of which can be accessed free at http://www.jbc.org/content/273/27/16748.full.html#ref-list-1