Role of Basic Amino Acids in the Interaction of Bindin with Sulfated Fucans†

Paul L. DeAngelis and Charles G. Glabe*
Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717
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ABSTRACT: Bindin, the acrosomal sperm adhesion protein of the sea urchin *Strongylocentrotus purpuratus*, binds specifically and with high affinity ($\bar{K}_d = 10^{-8}$ M) to egg sulfated fucans in the high ionic strength milieu of seawater (0.55 M salt). Previous studies indicated that the negatively charged sulfate groups of the polysaccharide are critical for binding which suggested a binding mechanism involving basic residues of bindin. We found that the binding of fucan to bindin or polyarginine is stable at the ionic strength of seawater, whereas the binding of fucan to polylysine or polyhistidine is inhibited by 50% or more at this ionic strength. Group-specific modification of either arginine, lysine, or histidine residues of bindin results in a substantial inactivation of fucan binding activity. Preincubation of bindin with fucan can almost completely protect bindin from inactivation by arginine-specific reagents, butanedione and phenylglyoxal, but only moderately slowed the inactivation by the histidine reagent diethyl pyrocarbonate. In contrast, prior fucan binding could not prevent loss of activity by the reaction of citraconic anhydride with lysine residues. Other sulfated polysaccharides which do not interact strongly with bindin did not protect binding from phenylglyoxal-mediated inactivation when 800–3000-fold more polysaccharide than fucan was used during the preincubation before modification. We found that the larger and more hydrophobic arginine-modifying reagents, camphorquinone-10-sulfonic acid and cyclohexanedione, fail to inactivate fucan binding, suggesting that essential arginine residues may reside in an environment with restricted accessibility to these reagents. Parallel kinetic studies monitoring [^{14}C]phenylglyoxal incorporation and fucan binding inactivation indicate that several of the four total arginine residues may be critical for fucan binding. We suggest that the bindin arginine guanido moieties may form cyclic, resonating hydrogen-bonding systems with sulfate esters and the complementary orientation of the arginine residues in relationship to the sulfate esters on the various polysaccharide structures may determine the observed specificity of polysaccharide binding to bindin.

Bindin is a 24-kilodalton (kDa) protein that mediates the species-specific adhesion of sperm to the egg surface during fertilization (Vacquier & Moy, 1977; Glabe & Vacquier, 1978; Glabe & Lennarz, 1979). Isolated bindin particles preferentially agglutinate homologous eggs in comparison to eggs from other species of sea urchins (Glabe & Vacquier, 1977; Glabe & Lennarz, 1979). The high-affinity ($\bar{K}_d = 10^{-8}$ M) egg surface receptors for bindin appear to be sulfated fucose-containing polysaccharides which are found on egg surface proteoglycans (Glabe & Vacquier, 1978; Glabe et al., 1982; Rossignol et al., 1984). Bindin displays a high degree of specificity for sulfated fucans (Glabe et al., 1982; DeAngelis & Glabe, 1987). Other sulfated polysaccharides of equal or higher charge density have affinities for bindin that are several orders of magnitude lower than those observed for sulfated fucans. This led to the initial interpretation of the binding of sulfated fucans by bindin as a lectin-like interaction (Glabe et al., 1982). More recent work indicates that bindin may recognize the spatial positioning of sulfate esters on the polysaccharide rather than directly recognizing the saccharide moieties per se (DeAngelis & Glabe, 1987). Desulfated fucans do not bind to bindin while other highly sulfated polysaccharides and polymers, such as dextran sulfate and poly(vinyl sulfate), bind avidly to bindin. The observation that sulfate esters are critical for the binding of polyanions to bindin suggested that basic amino acid residues of bindin may play a role in the selective binding mechanism, based on the assumption that the primary adhesive forces may be electrostatic. A critical role for basic amino acid residues in the interaction of numerous proteins with a variety of anionic polymers has been established (Riordan, 1979; Peterson et al., 1987; Malebran & Cardemil, 1987; Sprang et al., 1987).

In the present study, we have investigated the functional groups of bindin which are responsible for its interaction with sulfated fucans by analyzing the effect of group-specific chemical modification of the basic amino acids of bindin. In addition, characterization of the ionic strength and pH dependence of fucan binding of bindin and various polyamino acids (arginine, histidine, and lysine) was used as an independent method for analyzing types of basic amino acids involved in the interaction. Our results indicate that basic amino acids, particularly arginine residues, are critical for the binding of sulfated fucans to bindin.

Experimental Procedures

Chemical Modification of Basic Residues. Bindin was isolated as described by Vacquier and Moy (1977). Histone H1 of *Strongylocentrotus purpuratus* was the generous gift of Dr. V. D. Vacquier. All reagents were from Sigma unless otherwise noted. Phenylglyoxal (Takahashi, 1968; Cheung & Fonda, 1979), cyclohexanedione (Patthy & Smith, 1975), butanedione (Riordan, 1973), and camphorquinone-10-sulfonic acid (CQS)[1] (Pande et al., 1980; obtained from Pierce Chemicals, Rockford, IL) were used to modify arginine residues. Chemical modification with these reagents was done

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* Address correspondence to this author.

[1] Abbreviations: DEPC, diethyl pyrocarbonate; CA, citraconic anhydride; HA, hydroxylamine; CQS, camphorquinone-10-sulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, 10 mM sodium phosphate/0.15 M NaCl; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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at 1–75 mM reagent in 0.15 M NaCl with 50–200 mM sodium borate or sodium bicarbonate pH 7.5–9.0 as noted in the figure legends. Phenylglyoxal and cyclohexanedione were dissolved in a small amount of ethanol or methanol to facilitate dispersal into aqueous solution. Final alcohol concentration never exceeded 2%. Butanedione was used as supplied or freshly distilled (bp 88 °C). DEPC was used to modify histidine groups (Church et al., 1985). DEPC was added to reaction mixtures of 25 mM MES, pH 6.0, or PBS, pH 7.4, from freshly diluted stocks in absolute ethanol (1:5 or 1:10 v/v, DEPC/ethanol). The final concentration of DEPC ranged from 0.5 to 10 mM. In some cases, the modification reaction was quenched with 10 mM histidine in the same reaction buffer. CA was reacted with primary amino groups (Atassi & Habeeb, 1972; Dixon & Perham, 1968). CA was used at a concentration of 1–4 mM either in 0.2 M sodium borate, pH 8.0, and maintained with 1 M NaOH addition or in saturated sodium bicarbonate. CA was added directly with thorough mixing of reaction mixtures.

^{125}I-Fucoidan Binding Assay. At various reaction time points, the ^{125}I-fucoidan binding activity of bindin was determined as previously described (DeAngelis & Glabe, 1987). Reagents and reaction buffers were diluted 5–150-fold in various assay buffers as noted in the figure legends (0.15 or 0.54 M NaCl, 20 mM sodium borate or Tris, pH 8.0, or PBS, pH 7.4), and ^{125}I-fucoidan [1×10^4 cpm/ng, $(1–2.5) \times 10^5$ cpm/point] was added and allowed to incubate for 8–10 min at 25 °C. Bindin was present in limiting concentrations. The bindin-associated fucan was separated from free fucan by filtration through Whatman GF/C filters followed by 3×5 mL washes of buffer. The amount of ^{125}I-fucoidan bound was quantitated by γ counting in a Beckman Gamma 5500 counter. Specific binding [typically $(15–30) \times 10^3$ cpm] was determined in the presence and absence of modifying reagent and is expressed as the total binding minus the appropriate nonspecific background. Nonspecific background binding [typically $(1–5) \times 10^3$ cpm] was determined in the presence and absence of a specific modifying reagent for each experiment and is defined as the amount of fucan retained on the filter in the absence of bindin. The same value for nonspecific binding is obtained in the presence of a 100-fold excess of unlabeled fucan. The reagent background never differed by more than 2-fold from the background in the absence of reagent. Residual binding is defined as specific binding in the presence of modification reagent divided by the specific binding in the absence of reagent.

For protection experiments with polysaccharides, radiolabeled fucoidan or other unlabeled anionic polysaccharides were added to bindin in modification buffer and allowed to incubate on ice for 15–60 min before reagent or plain buffer addition. After chemical modification with nonradioactive polysaccharide present, the reaction mix was diluted 5-fold with 0.54 M NaCl/20 mM sodium borate, pH 8.0, before ^{125}I-fucoidan addition and allowed to incubate an additional 8 min before filtration. Residual binding was defined as the specific cpm bound by bindin in the presence of polysaccharide plus reagent divided by the specific cpm bound in the presence of polysaccharide. Nonspecific binding for the reagent and fucan was determined in the absence of bindin. The presence of the unlabeled polysaccharides heparin, galactan, hyaluronic acid, and chondroitin sulfate caused negligible inhibition (0–10%) of binding compared to bindin controls without these polysaccharides.

The salt dependence of fucan binding to polyamino acids or bindin was determined in buffers containing 5 mM Tris, pH 8.0, and 0–2 M NaCl. The polyamino acids were present in limiting concentrations. The pH dependence of fucan binding was characterized in buffers containing 0.15 M NaCl and one of the following buffers: 50 mM sodium borate (pH 10–8), 50 mM MOPS (pH 8–6), 50 mM sodium acetate (pH 6–4), or 10 mM sodium phosphate (pH 3–12).

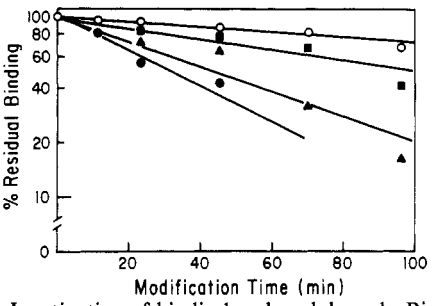

FIGURE 1: Inactivation of bindin by phenylglyoxal. Bindin (3 μM final concentration) was incubated in 0.1 M sodium bicarbonate/0.15 M NaCl, pH 7.8, at 24 °C in the dark with the following concentrations of phenylglyoxal: 5 (●); 2 (▲); 1 (■); 0.5 (○) mM. Fucan binding was measured in duplicate after 8-fold dilution in 20 mM sodium borate/0.54 M NaCl, pH 8.0. The logarithm of residual binding is presented as a function of time of incubation of bindin with phenylglyoxal. The binding activity is eventually completely inactivated at longer incubation times than shown in the figure.

Incorporation of [^{14}C]Phenylglyoxal into Bindin. Radiolabeled [7-^{14}C]phenylglyoxal (specific activity 18 mCi/mmol; Amersham, Arlington Heights, IL) was used to measure incorporation of reagent into bindin. Parallel kinetic measurements of the inactivation of fucan binding were done under identical conditions. The radiolabeled phenylglyoxal was diluted 20-fold with unlabeled phenylglyoxal and added to the reaction mixture at a final concentration of 2 mM. At various times, unincorporated phenylglyoxal was removed by filtration through GF/C filters in the same manner as used for the ^{125}I-fucoidan binding assays. After the washes with buffer, a final 5-mL wash of 95% ethanol was used to reduce background due to free reagent before collection for liquid scintillation counting. The filters were air-dried, and the amount of incorporated radiolabel was determined by scintillation counting in minivials with 4.5 mL of Ecoscint (National Diagnostics, Highland Park, NJ) using a Beckman LS 7500 scintillation counter.

RESULTS

Effect of Arginine Modification. The bindin polypeptide contains 4 arginine residues, 2 lysine residues, and 5 histidine residues out of a total of 236 amino acids (Gao et al., 1987). Most of the basic amino acids are concentrated in the middle one-third of the molecule from positions 77 to 126. Two of the arginines and three of the histidine residues are located in a contiguous segment from residues 108 to 113. Reaction of bindin with phenylglyoxal inactivated the sulfated fucan binding capacity of bindin as shown in Figure 1. Kinetic studies of the inactivation of fucan binding by phenylglyoxal revealed that the loss of binding activity obeys second-order kinetics with an observed rate constant of 3.5 M^{-1} min^{-1} and appeared to be the result of a single class of reactive arginines. The inactivation of fucan binding by butanedione exhibited a reproducible lag period (8 min at 25 mM reagent) before a linear reduction in fucan binding was observed as shown in Figure 2. In contrast, the bulkier reagents, cyclohexanedione and CQS, caused very slow or no loss of fucan binding as shown in Figure 2. After extensive incubations with high concentrations of cyclohexanedione (75 mM for 100 min), bindin exhibited a 50% decrease in binding activity (data not

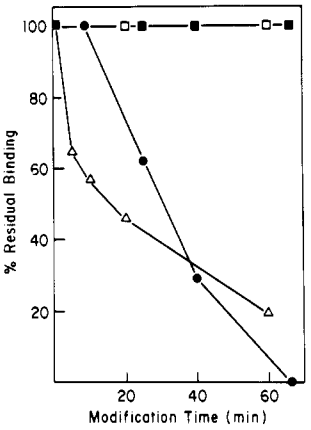

FIGURE 2: Effect of arginine-specific reagents on the fucan binding of bindin and histone. Bindin (6 μM final concentration) was incubated in 0.1 M sodium borate/0.15 M NaCl, pH 8.3, at 24 °C in the dark with butanedione at 50 mM (●) or cyclohexanedione at 50 mM (■). Fucan binding was measured in duplicate after 8-fold dilution in 20 mM sodium borate/0.54 M NaCl, pH 8.0. Bindin (□) and histone (△) (0.8 and 0.015 μM final concentration, respectively) were incubated in 0.2 M sodium borate, pH 9.0, at 24 °C in the dark with 280 mM CQS. Fucan binding was measured in duplicate after 8-fold dilution into PBS, pH 7.4. Residual binding is presented as a function of time of incubation of the proteins with modification reagent.

shown). The results shown in Figure 2 show that high concentrations of CQS (560 mM) failed to decrease the fucan binding activity of bindin by more than 2.5% after 1 h of incubation. The failure to inactivate fucan binding by CQS appears to be due to inaccessibility of the bindin arginine residues to this reagent, since the fucan binding activity of sea urchin sperm histone H1 was rapidly destroyed under the same conditions whereas bindin remains unaffected.

We found that the fucan binding activity of bindin could be protected from inactivation by phenylglyoxal and butanedione if the radiolabeled fucan was preincubated with bindin before modification. Butanedione-mediated disruption of fucan binding was prevented by formation of the bindin/fucan complex before addition of 50 mM reagent (99% protection with a 50-min modification time). The majority of the binding activity was also protected from inactivation caused by treatment with 5 mM phenylglyoxal (90% protection). Other anionic polysaccharides such as heparin, hyaluronic acid, chondroitin sulfate, and galactan sulfate, which bind only weakly to bindin, could not prevent substantial inactivation of binding activity even when present at 800–3000-fold higher concentrations than fucoidan (50–8% protection).

Comparison of the rate of fucan binding inactivation and the rate of incorporation of radiolabeled phenylglyoxal, shown in Figure 3, suggests that arginine modification and loss of fucan binding are strongly correlated for the first phase of the reaction. Inspection of the plot suggested that two to three of the four possible arginines are involved in binding fucan. There is some uncertainty in this estimate, however, due to the possible formation of the 2:1 phenylglyoxal/arginine complex (Takahashi, 1968) even though a 1:1 ratio was deemed sufficient to inactivate bindin in our kinetic studies.

Effect of Histidine Modification. The histidine-specific reagent DEPC also rapidly inactivated fucan binding quickly under mild conditions as shown in Figure 4. Prior incubation of bindin with fucan could partially protect bindin from inactivation by DEPC treatment. Preincubation of bindin with fucan slowed the initial rate of inactivation by approximately 12-fold (data not shown; 20-min reaction with 6 mM DEPC in PBS, pH 7.4). In this experiment, the residual binding with fucan protection was 65.6% of the maximum binding versus

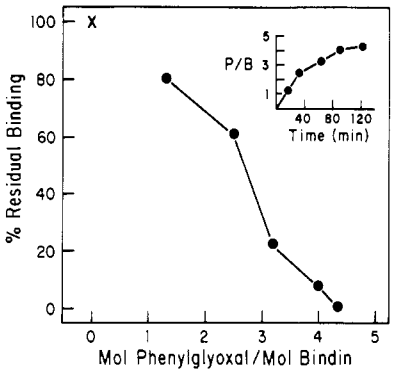

FIGURE 3: Incorporation of [^{14}C]phenylglyoxal into bindin. Bindin was modified as described in Figure 1 and under Experimental Procedures. Specific incorporation was 480–1960 cpm, and nonspecific background was 53 cpm. The correlation between incorporation of reagent into bindin and inactivation of fucan binding is illustrated. The X marks the hypothetical starting point. The inset shows the time course of incorporation of [^{14}C]phenylglyoxal into bindin in the same units as the x axis of the larger figure.

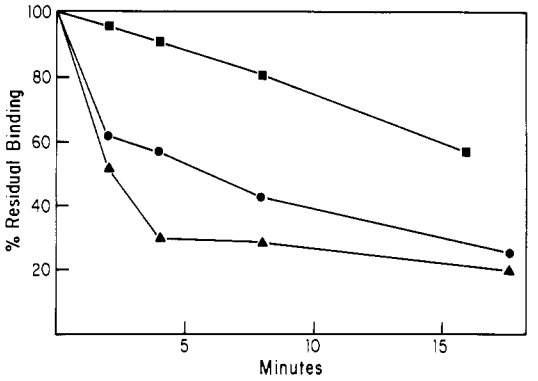

FIGURE 4: Inactivation of bindin by diethyl pyrocarbonate. Bindin (0.8 μM final concentration) was incubated in 25 mM MES, pH 6.0, at 24 °C with 6 (▲), 3 (●), and 1 (■) mM diethyl pyrocarbonate. Fucan binding was measured after quenching with free histidine and 3-fold dilution into PBS, pH 7.4. Residual activity is presented as a function of the time of incubation of bindin with reagent.

5.5% residual binding without the fucan preincubation step. The amount of residual fucan binding in the preincubation experiment above was reduced to 33% after a further 10-min incubation with DEPC.

Effect of Lysine Modification. CA was used to test the role of amino groups of bindin in binding fucan. Incubation of bindin with CA rapidly inactivated the fucan binding activity of bindin (data not shown). Reaction of bindin in saturated sodium bicarbonate solution with 1 mM CA resulted in 88.2% residual binding while 4 mM CA completely inactivated bindin. Preincubation of bindin with radiolabeled fucan did not protect bindin against inactivation by CA under similar conditions that resulted in protection of arginine mentioned earlier.

Ionic Strength Dependence of Fucan Binding to Polyamino Acids. The fucan binding ability of bindin or various polyamino acids was characterized over a range of salt concentrations between 0 and 2 M. The fucan binding ability of bindin has been previously shown to be relatively insensitive to salt concentration over the range of 0.1–0.6 M NaCl and is inhibited by 50% at approximately 1.2 M NaCl (DeAngelis & Glabe, 1987). These studies also showed that the binding of fucan to polylysine was disrupted at a much lower concentration of salt. In the present study, we also compared the ionic strength dependence of the binding of fucan to polyhistidine and polyarginine, and the results are presented in

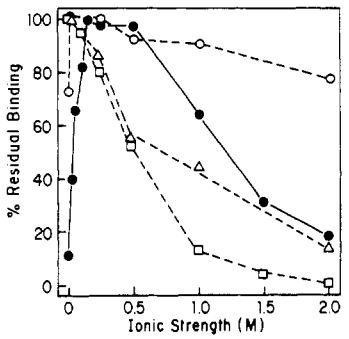

FIGURE 5: Ionic strength dependence of fucan binding of various polypeptides. ^{125}I-fucoidan filter binding assays with 1 μg of bindin (●), 10 ng of polyarginine (M_r 70 000) (○), 18 ng of polylysine (M_r 17 000) (□), or 100 ng of polyhistidine (M_r 18 000) (△) were performed in 0.2 mL of 5 mM Tris, pH 8.0, with increasing amounts of NaCl. Binding is expressed as the percent of specific binding at the ionic strength optima (graphed as 100%) for that polypeptide. Specific binding ranged from 15 000 to 50 000 cpm for the various proteins at their optima, and nonspecific binding increased linearly with higher salt from 1000 to 5000 cpm.

Figure 5. The binding of ^{125}I-fucoidan to polyhistidine was inhibited by 50% at a NaCl concentration of 0.75 M. In contrast, the binding of fucoidan to polyarginine was extremely resistant to disruption by NaCl. The binding of fucan to polyarginine was inhibited by only 20% at 2.0 M NaCl. As was observed for bindin, the binding of ^{125}I-fucoidan to polyarginine exhibited a broad plateau of activity between 0.1 and 0.6 M NaCl.

pH Dependence of Fucan Binding to Bindin. We determined the pH dependence of fucan binding over the range of pH 3–12 as shown in Figure 6. The extent of fucan binding increased very little from pH 11 to 8, over the ionization range of the side chain amino group of lysine and the free α-amino terminus of bindin. A large increase in the extent of fucan binding was observed between pH 6 and 5 corresponding to the typical histidine pK_a. As the pH was lowered to 3, a further increase in fucan binding was observed. This observation may arise from neutralization of bindin carboxylate residues resulting in the loss of electrostatic repulsive forces between bindin and sulfated fucans. The previous observation of the weak binding of fucoidan to bindin in low salt may be a reflection of the need for salt ions to screen such a repulsive force.

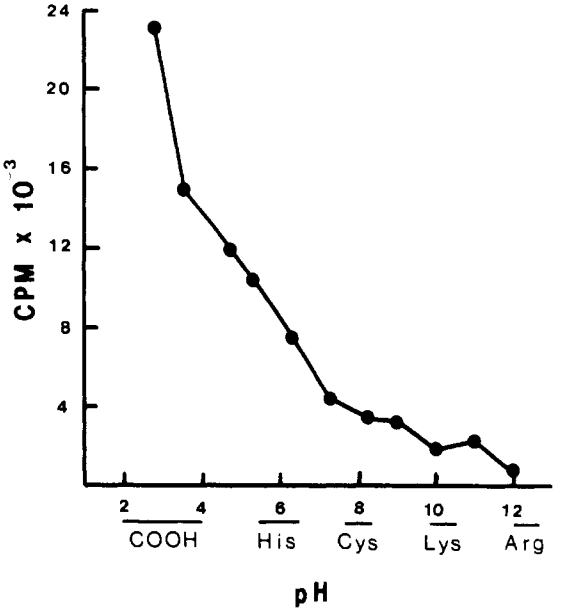

FIGURE 6: pH dependence of fucan binding to bindin. Fucan binding was determined by ^{125}I-fucoidan binding in PBS buffer of various pHs at isoionic strength (0.15 M). Binding is expressed as the specific cpm bound for a given pH. Nonspecific binding was determined with no protein present in every buffer for the assay and was 10% or less of specific binding. Common pK_a values of the amino acid side chains are marked.

Discussion

In this study, we have attempted to identify the types of amino acid residues of bindin which are critical for sulfated fucan binding by group-specific chemical modification of basic amino acids and characterization of the dependence of binding on pH and ionic strength. We chose conditions previously shown to maximize the specificity of the chemical reaction for a single type of amino acid (Lundblad & Noyes, 1984). We found that modification of arginine residues with phenylglyoxal or butanedione quantitatively inactivated the fucan binding activity of bindin. Similarly, we found that reaction of histidine residues with diethyl pyrocarbonate or modification of lysine residues with citraconic anhydride also substantially inactivated the binding activity of bindin.

Correlation of reagent reaction with amino acids and the destruction of biological activity are important in judging whether the chemical modification is actually probing the phenomenon of interest (Lundblad & Noyes, 1984). The physical nature of bindin presents a problem for interpreting quantitative measurements of the modification of amino acids; bindin is largely insoluble in the aqueous buffers used in these experiments. Previous studies indicate that sulfated fucans bind to the surface of bindin particles (Glabe et al., 1982). Thus, the bindin polypeptides in the interior of the granule may not be equally exposed to the solvent, fucan, or the chemical modification reagent as the bindin polypeptides on the periphery of the bindin particle. Measurements of the rate of reaction of a particular type of amino acid (commonly involving amino acid analysis after acid hydrolysis) will be an average of the residues that are exposed to the reagent and those that are not. Attempts to solubilize a significant amount of bindin with urea or detergent (4–7 M urea and/or 0.1–1% Chaps) were only partially successful. More bindin molecules were exposed (as measured by increased ^{125}I-fucan binding and resistance to sedimentation by centrifugation) by these treatments, but the polysaccharide selectivity of binding is reduced. Therefore, we did not pursue chemical modification with such altered bindin preparations. The quantitation of chemical modification was difficult in all but one reaction.

In the case of arginine modification, quantitation was afforded by use of radiolabeled phenylglyoxal. The same method of filtration through glass fiber filters was used for both detection of ^{125}I-fucan binding and measurement of incorporation of [^{14}C]phenylglyoxal into bindin to maximize the kinetic consistency of the data. The rate of incorporation and inactivation are strongly correlated. Our results suggest that several arginines are essential for fucan binding. In other systems such as nucleotide and anionic sugar binding proteins, more than one positively charged residue complexes with the ligand (Peterson et al., 1987; Malebran & Cardemil, 1987; Sprang et al., 1987). Although butanedione also completely inactivates bindin, the inactivation by butanedione demonstrates a reproducible lag phase before a linear reduction in fucan binding occurs. The explanation for this difference in inactivation kinetics is not obvious. One possibility is that butanedione reacts with at least two arginines possessing different modification reaction rates, in contrast to the apparent single class of reaction with phenylglyoxal.

Another criterion for determining the credibility of chemical

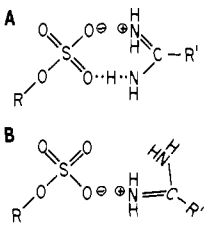

FIGURE 7: Proposed structure of the arginine/sulfate bond. The bidentate, cyclic bond is sensitive to the orientation of its substituents.

modification as an accurate indication of essential residues is the protection of activity from chemical inactivation by bound ligand. Either the actual physical blocking of the binding site or alteration of the conformational state of the polypeptide upon ligand binding prevents modification involved in inactivation; observations of either mechanism provides useful information for identifying important residues. The three types of basic amino acid residues of bindin exhibit a range of fucan protection effectiveness. Arginine residues are almost completely protected by preincubation with fucan from inactivation by butanedione and phenylglyoxal under rigorous conditions. Other sulfated polysaccharides that weakly bind to bindin do not significantly protect bindin from inactivation by phenylglyoxal. This result argues that arginine is directly involved in the specific interaction with fucan at the active site of bindin. Other data from this study suggest that these critical arginine residues may be located in a cleft or pocket that has restricted accessibility. The bulkier and more hydrophobic arginine-modifying reagents, CQS and cyclohexanedione, were observed to have no significant effect on the binding activity of bindin under conditions where the binding activity of histone H1 was rapidly inactivated. Other systems do not always exhibit such a difference in the effectiveness of CQS or cyclohexanedione as compared to phenylglyoxal or butanedione (Davidson & Flynn, 1979; Mautner et al., 1981). Restricted access to essential arginine residues may also partially account for the observed strong protection effect produced by preincubation with sulfated fucans before chemical modification.

In contrast to our results with arginine-modifying reagents, the rate of inactivation of bindin by the reaction of histidine residues with DEPC was slowed only moderately by the presence of fucans. This result suggests that histidine interacts rather weakly with the fucan or that histidine is not directly involved in the active-site binding mechanism. Evidence for the importance of histidine in the binding mechanism is the large increase in fucan binding at pH 5–6 which is in the range of the pK_a of the typical histidine residue. The physiological relevance of this observation is not obvious in view of the fact that the pH of seawater is 7.8–8.0. The extracellular egg jelly coat matrix, however, is composed of anionic polysaccharides that could provide a low-pH microenvironment where the increased binding affinity may be utilized for increased adhesion of the sperm. The failure of fucan to protect against the loss of the binding activity from modification of lysine residues by citraconic anhydride suggests that these residues may not be directly involved in polysaccharide binding but rather may play another essential role, such as maintaining an active conformation of the polypeptide.

The restoration of binding activity upon reversal of the reaction of reagent with amino acid is frequently used as a criterion for assessing the specific effects of chemical modification (Lundblad & Noyes, 1984). However, the biochemical properties of bindin have precluded the use of this approach in assessing the specific effects of chemical modification. The two readily reversible arginine reagents, cyclohexanedione and CQS, do not inactivate bindin. The effective reagents for arginine modification, phenylglyoxal and butanedione, are not always readily reversible due to the stability of the condensation adduct. Some studies have reported that nucleophilic displacement of reagent or loss of stabilizing borate ions from the inactivating adduct is accompanied by a return of biological activity (Enoch & Strittmatter, 1978; Vallejos et al., 1978). We found that the fucan binding activity of butanedione- and phenylglyoxal-treated bindin was reactivated to less than 2% of maximal values after dialysis into Tris- or arginine-containing buffers (data not shown). DEPC treatment of histidine produces both mono- and disubstituted products; the former adduct is readily reversed by treatment with neutral hydroxylamine (0.05–3 M) (Miles, 1977). However, we found that the fucan binding activity of untreated binding controls was sensitive to hydroxylamine treatment (data not shown). The reported cleavage of asparagine–glycine bonds by strong nucleophiles such as HA (Bornstein & Balian, 1977) may be the cause of this loss, but this remains to be established. Citraconic acid modification can be usually reversed by mild acid treatment (pH 4–5 for few hours at 40 °C) (Atassi & Habeeb, 1972). Citraconic anhydride treated bindin is recalcitrant to reversal of inactivation under such conditions, suggesting that lysine modification may lead to irreversible denaturation of the active structure.

Characterization of fucan binding to model basic polyamino acids provided an independent means of assessing essential residues of bindin. The three types of basic polyamino acids display different salt sensitivities for their fucan binding activities, which is most likely due to the different bonding characteristics of their nitrogenous base. A comparison of the ionic strength dependence of fucan binding for bindin and polyarginine reveals a marked resistance to disrupt by high concentrations of NaCl. These results strengthen the suggestion that arginine residues are critical parts of the active site of bindin as determined by our chemical modification study. The structural reason for the enhanced salt stability of bindin and polyarginine may be due to the formation of a resonating, cyclic bonding system between the guanido group of arginine and the sulfate ester, which is shown in Figure 7A (Ichimura et al., 1978). The coplanar, resonating bond between the two guanido nitrogens and two sulfate oxygens has hybrid characteristics between the hydrogen bond and the ionic bond which may account for its enhanced salt stability. A cyclic, bidentate bond between carboxylate, phosphate, and sulfate ionic substituents of the ligand and arginine residues in several proteins has been postulated in other studies (Ichimura et al., 1982; Hart et al., 1987). An interesting feature of this type of noncovalent bond is that its formation is sensitive to the orientation of the sulfate and guanido moieties. The ring-shaped bond can only form if the orientations of the guanido and sulfate moieties are coplanar and complementary. If the pair is not in the correct orientation, a longer range, nondirectional salt bridge would be expected to form as shown in Figure 7B. The spatial orientation of critical arginine residues of bindin relative to the sulfate esters on the polysaccharide may be involved in determining the observed polysaccharide binding specificity.

Registry No. Arg, 74-79-3; His, 71-00-1; Lys, 56-87-1; poly(Arg), 25212-18-4; poly(Arg), SRU, 24937-47-1; poly(Lys), 25104-18-1; poly(Lys), SRU, 38000-06-5; poly(His), 26062-48-6; poly(His), SRU, 26854-81-9; fucoidan, 9072-19-9.

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An NH_4^+-Dependent Protein Synthesis Cell-Free System for Halobacteria†

J. L. Sanz, I. Marín, M. A. Balboa, D. Ureña, and R. Amils*
Centro de Biología Molecular CSIC-UAM, Cantoblanco, Madrid 28049, Spain
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ABSTRACT: An efficient poly(U)-dependent polyphenylalanine system for extreme halophilic archaebacteria is described. The system was tested with eight different species including the six genera described to date: *Halobacterium*, *Haloferax*, *Haloarcula*, *Halococcus*, *Natronobacterium*, and *Natronococcus*. In all of them the optimal ionic conditions are similar. The system is extremely dependent on NH_4^+, with the optimal concentration being 1.5 M $(NH_4)_2SO_4$, and has virtually no dependence on K^+. The internal concentration of NH_4^+ and K^+ of three halobacteria with different degrees of halophilia was measured. A close correlation between the internal concentration of K^+ and the external concentration of cations, as well as a relatively low concentration of NH_4^+, was obtained in all cases. The possible importance of all the variables of the system is discussed.

Halobacteria are a large group of archaebacterial microorganisms that concentrate high levels of K^+ within the cell to overcome the extreme external osmotic pressure. They colonize very diverse habitats such as solar salterns, desertic terminal lakes like the Great Salt Lake or the Dead Sea, soda lakes with very alkaline pHs like the Wadi-Natrum and the Magadi, or man-made substrates like salt fish, sausage, and tanned leather.

All the reported cell-free protein synthesis systems for this type of archaebacteria have been performed with phylogenetically and taxonomically similar halobacteria, belonging to the *Halobacterium* genus (Bayley & Griffiths, 1968; Kessel & Klink, 1981; Saruyama & Nierhaus, 1985). Recently, Torreblanca et al. (1986) proposed a new taxonomic classification for the nonalkalophilic halobacteria based on numerical taxonomy and the lipid composition of the membrane. They describe two new genera, *Haloarcula* and *Haloferax*, including some of the species previously ascribed to the *Halobacterium* genus.

The differences in the ionic conditions of the habitats in which the three genera of nonalkalophilic halobacterial bacillus

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* Address correspondence to this author.

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