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Rational Design and Molecular Characterization of a Chimaeric Response Regulator Protein

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BvgA and EvgA are closely related response regulators from *Bordetella pertussis* and *Escherichia coli*. To analyze the domain borders and linker sequences of these proteins, we used limited proteolysis and matrix-assisted laser desorption/ionization-mass spectrometry analysis of the in-gel-digested proteolytic fragments. The thermolysin-sensitive linker regions were found to extend from Leu130 to Thr144 for BvgA and from Leu127 to Ser133 for EvgA. These data provided the rationale for the construction of the chimaeric protein HA. HA carries the EvgA receiver and BvgA output domains, fused in the central part of the linker sequences of the parent proteins. Thermolysin-sensitive sites of HA were found at positions identical with those in the EvgA and BvgA linker sequences, indicating intact folding of its receiver and output domains. Consistent with this, the chimaera showed virtually unchanged phosphorylation and dimerization properties.

However, BvgA and HA differed in the effect of phosphorylation on their DNA-binding activities. In the case of BvgA, phosphorylation resulted in an increased affinity and specificity in DNA binding, whereas the DNA-binding properties of HA were not affected by phosphorylation. The chimaera HA was unable to activate transcription of the BvgA-dependent *fha* promoter, either *in vivo* or *in vitro*. These results indicate that the phosphorylation-induced activation of BvgA requires specific interactions between the receiver and output domains that are disturbed in the chimaera.

Keywords: limited proteolysis; mass spectrometry; filter-binding assay; analytical ultracentrifugation; two-component signal transduction

Two-component signal transduction systems are present in eubacteria, archaea, several lower eukaryotes, and plants.[1,2] Signal transduction by these systems involves phosphotransfer reactions between a histidine residue located in the transmitter domain of a sensor protein and an aspartate residue in the receiver domain of a response regulator protein. Phosphorylation of the receiver domain leads to the activation of the effector function of the response regulator located in its output domain.[3–5]

The crystal structures of isolated domains of several response regulators and of the full-length protein NarL have been solved.[6–12] The receiver domains are about 120 to 125 amino acid residues in size and have a doubly wound α/β pattern consisting of a central five-stranded parallel β-sheet surrounded by five α-helices.[13] In the case of non-phosphorylated NarL, the three-dimensional structure revealed that the DNA-binding helix-turn-helix motif in the output domain is sterically hindered by the receiver domain. It is believed that phosphorylation of the receiver domain induces a conformational change in order to allow the output domain to interact efficiently with its DNA target sequence. A strictly conserved lysine residue

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Abbreviations used: MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; EMSA, electrophoretic mobility shift assay.

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present within loop β5-α5 of the receiver domains appears to have a crucial function in this process. This residue is located close to the phosphorylation site and phosphorylation causes its back-and-forth movement.[7,8] The phosphorylation-induced repositioning of this residue is connected with movements of the surface-exposed β4-α4 region and the loop α4-β5, which may be involved in homodimerization of some response regulators.[7,8,11,14–16]

Little is known about the mechanism by which the conformational change is transduced from the receiver to the output domain: In some cases, phosphorylation-induced dimerization of the response regulators may be involved in their activation and co-operative interactions may contribute by the induction of oligomerization of response regulators upon binding to DNA.[14,17–21]

Recently, we presented a novel strategy for the efficient identification of domain borders and linker sequences of multidomain proteins including the response regulator NtrC.[22,23] This method is based on the combination of limited proteolysis of susceptible protein surface areas using proteases with broad substrate specificities and mass spectrometric peptide mapping. In the present study, we applied this method to characterize the domain borders and linker sequences of the highly related response regulators BvgA and EvgA. In fact, their receiver and output domains belong to the same classes, the so-called C1 and the FixJ/NarL subfamilies, respectively.[3,5,24–27] BvgA is part of the BvgAS two-component phosphorelay system of *Bordetella pertussis* and is the master regulator of virulence.[28–30] EvgA is part of the EvgAS two-component phosphorelay system of *Escherichia coli*, which is involved in the regulation of drug efflux systems.[27,31] The identification of the domain borders of the two response regulators provided the structural information required for the construction of the chimaeric protein HA, consisting of the defined EvgA receiver and the BvgA output domains. The biophysical and biochemical properties of this chimaera were characterized and their implications for the structure-function relationships of response regulators are being discussed.

Identification of domain borders and linker sequences in EvgA and BvgA

To identify their domain borders and linker sequences, the purified BvgA and EvgA proteins were subjected to limited proteolysis with thermolysin. The limited proteolysis of BvgA led to two distinct cleavage products migrating in the gel corresponding to molecular masses of about 14 kDa and 8 kDa, respectively (data not shown). Increasing the enzyme to substrate ratios from E:S of 1:1440 (w/w) to E:S of 1:360 (w/w) resulted in strong reduction of the 23 kDa band and in increasing intensities of the bands migrating at 14 kDa and 8 kDa. From this observation, we conclude that the 14 kDa band very likely corresponds to the receiver domain of BvgA, whereas the 8 kDa corresponds to the output domain of BvgA, indicating that thermolysin cleaved preferentially in the linker regions separating the two domains. The decreased intensity of the 8 kDa band at E:S of 1:180 indicates that the output domain is proteolytically less stable than the receiver domain. EvgA behaved very similarly (data not shown).

Precise molecular masses of the thermolytic fragments of EvgA and BvgA, respectively, were determined by matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) analysis from the fragment mixtures (Table 1). The result confirmed that limited proteolytic cleavage produced domain fragments. MALDI-MS analyses further revealed that thermolysin digestion produced heterogenic product mixtures due to the presence of several cleavage sites in close proximity. In the resulting mixtures, ion signals with differences in molecular masses were found that represented domain fragments of different lengths. The MALDI mass spectrum of the fragment mixtures from the response regulators obtained after two hours with E:S of 1:360 showed the presence of singly and multiply charged ions for BvgA and EvgA fragments, respectively, in the mass range between 7000 and 16,000 Da. In addition, the spectra revealed that the 14 kDa products consisted of up to at least four products in the case of EvgA, and at least two products in the case of BvgA. For EvgA, the major product showed an ion signal at m/z 14,212 corresponding to the N-terminal receiver domain (residues 1-130). The major product of BvgA with an ion signal at m/z 14,122 represented the N-terminal receiver domain (residues 1-129). Similarly, with EvgA, an ion signal at m/z 8256 was observed for the C-terminal domain (residues 134-204) and with BvgA a signal at m/z 8845 was indicative for its C terminus (residues 130-209). Hence, we conclude that thermolysin cleaved EvgA and BvgA initially at susceptible peptide bonds located in the linker regions, leaving the tightly folded domains mostly undigested.

According to our previously published strategy, an internal sequence verification was performed by further in-gel-digestion of thermolytic fragments with trypsin after separation by SDS-PAGE as described.[22,23,32] The doubly digested products were analysed by mass spectrometric peptide mapping. The peptides obtained from the bands migrating with molecular masses of about 23 kDa could be assigned as the entire response regulators BvgA and EvgA, respectively, whereas the tryptic peptides obtained from the 14 kDa bands were found to be derived exclusively from the receiver domains (data not shown). After tryptic digestion of the 8 kDa bands, only peptides derived from the respective BvgA or EvgA output domains were identified (data not shown). The sites of the soluble response regulators accessible to thermolytic cleavage are therefore concentrated on a small sequence stretch of the proteins. The thermolytic cleavage sites were found in a position very similar to that of the sequence stretch that resembles the

Table 1. Mass spectrometric molecular mass determinations of the response regulators BvgA, EvgA and HA and corresponding domain fragments obtained by limited thermolysin proteolysis

Protein	Position	$[M + H]^+_{(calcd)}$	$[M + H]^+_{(obsd)}$
EvgA	1-204	22,691.3	22,691
$Receiver_E$	1-126	13,680.8	13,681
	1-129	14,069.3	14,064
	1-130	14,211.5	14,212
	1-133	14,454.7	14,457
$Output_E$	134-204	8255.5	8256
BvgA	1-209	22,951.9	22,949
$Receiver_B$	1-129	14,126.6	14,122
	1-144	15,750.3	15,744
$Output_B$	130-207	8661.0	8660
	130-209	8845.3	8845
	145-207	7037.3	7037
	145-209	7221.5	7222
HA	1-204	22,289.8	22,292
$Receiver_E$	1-126	13,680.8	13,681
	1-129	14,064.3	14,064
	1-139	15,087.3	15,087
$Output_B$	130-204	8244.5	8246
	140-204	7221.5	7222

MALDI-MS analyses were carried out using a Bruker Reflex III time-of-flight mass spectrometer (Bruker-Daltonik, Bremen, Germany). Fragment mixtures obtained from limited proteolysis experiments were analysed in linear mode with 25 kV acceleration voltage. A portion (0.3 µl) of sample solution was mixed with 0.3 µl of a saturated solution of sinapinic acid in 60 % (w/v) CH_3CN, 0.1 % (w/v) TFA in water directly on the target. Analyses of peptide mixtures generated from in-gel proteolytic digestions were performed in reflector mode without further purification. Acceleration voltages were set to 20 kV (ion source), and 23 kV (reflector), respectively. A portion (0.3 µl) of sample solution was mixed with 0.3 µl of a saturated solution of a-cyano-4-hydroxycinnamic acid (CHCA) in 30 % CH_3CN, 0.1 % TFA in water directly on the target. The air-dried samples were subsequently recristallized from 0.4 µl of 60 % CH_3CN, 0.1 % TFA in water.

linker region proposed for the NarL protein (Figure 1).[6]

Biochemical properties of truncated protein fragments derived from limited proteolysis

After proteolysis of BvgA or EvgA with thermolysin, the histidine kinases EvgS and BvgS were added to the peptide mixtures in the presence of [γ-^{33}P]ATP. Analysis by SDS-PAGE revealed the radioactively labelled bands corresponding to the intact histidine kinases and response regulators. Moreover, labelling of several bands could be observed that migrated at about 14 kDa and corresponded to the truncated receiver domains (data not shown). This demonstrates that the isolated receiver domains of EvgA and BvgA are active enzymes with the ability to transfer the phosphoryl group from the autophosphorylated histidine kinases to the aspartate residues.

Using the thermolytic peptide mixture of BvgA and a DNA fragment containing the BvgA-binding site of the *fha* promoter (see below), we performed electrophoretic mobility shift assays (EMSA). We found that an additional complex was present that migrated faster than the complexes formed with native BvgA (data not shown). This additional complex very likely consists of the DNA fragment and the peptide comprising the output domain of BvgA, suggesting that the isolated output domain

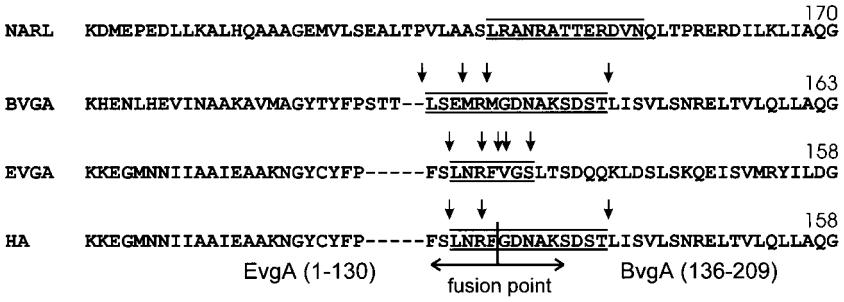

Figure 1. Sequence alignment of linker regions from BvgA, EvgA, HA and the related NarL protein. The entire sequences of the proteins were aligned using the ClustalW WWW service at the European Bioinformatics Institute. Lines above and below the sequences indicate linker sequences. Arrows depict cleavage sites of limited proteolysis with thermolysin (↓). The fusion point in the chimaeric protein HA is depicted and numbers in parentheses refer to amino acid positions as in intact parent proteins.

Table 2. Structural and functional properties of EvgA, BvgA and the chimaeric response regulator HA

	EvgA	HA	BvgA
Molecular mass (kDa):			
Calculated (monomer)	22.7	22.3	23.0
Measured by AUC[a]	43.1±3.1	44.4±4.6	45.1±3.1
Association state	Dimer	Dimer	Dimer
Phosphorylation by	EvgS	EvgS	BvgS
Phosphorylation of limited proteolyis products	Yes	Yes	Yes
Half life of phosphoproteins (at 30 °C (min))[b]	21.2	15.5	17.0
Activation of *fha* promoter *in vivo*[c]	No	No	Yes
Activation of *fha* promoter *in vitro*[d]	No	HA: no	BvgA: no
		HA-P: no	BvgA-P: yes
Binding at the *fha* promoter[e]:			
Half maximal binding at the *fha* promoter (nM)		HA: 156, HA-P: 170	BvgA: 74, BvgA-P: 3.0
Binding specificity (as compared to BvgA)		HA: −55-fold, HA-P: −55-fold	BvgA: 1, BvgA-P: 14-fold
Co-operative/linear binding		HA: linear, HA-P: linear	BvgA: co-operative, BvgA-P: co-operative

[a] Analytical ultracentrifugation was carried out as described.[21,33]
[b] The half-life of the phosphorylated proteins was determined using acetyl-^{32}P as described.[19]
[c] The *in vivo* activation of the *fha* promoter by the various response regulators was determined as described in Results.
[d] Single-round run-off transcription assays using purified RNA polymerase (either a wild-type *B. pertussis* RNP or a hybrid *E. coli* RNP containing the σ^{72} factor of *B. pertussis*) were performed as recently described.[36,36]
[e] Details for *in vitro* binding experiments of response regulators at the *fha* promoter are given in Figure 2.

retained DNA-binding activity. This conclusion is supported by data obtained with the purified BvgA output domain N-terminally fused to maltose-binding protein or to a His tag, which also demonstrated significant binding of the isolated output domain to *fha* promoter sequences (A.B. and R.G., unpublished results).[24]

Construction of a chimaeric response regulator and its structural characterization by limited proteolysis and mass spectrometry

After the definition of the linker sequences that connect the receiver with the output domains in BvgA and EvgA, respectively, we selected a suitable fusion point on a rational basis for the creation of the chimaeric protein HA. The HA protein consists of amino acid residues 1-130 derived from EvgA and of amino acid residues 136-209 derived from BvgA (Figure 1). As described above, the purified protein HA was subjected to limited proteolysis by thermolysin and mass spectrometric characterization of the derived peptides. The results obtained with HA were very similar to those obtained with the parent proteins. For example, limited proteolysis of HA resulted in the appearance of 14 kDa and 8 kDa bands in varying amounts depending on the enzyme to substrate ratio. As for the parent proteins BvgA and EvgA, these bands correspond to the receiver and output domains, respectively (data not shown).

Following the experimental strategy described for the parent proteins, the HA receiver domain was determined to comprise amino acid residues 1-126, and the output domain was determined to start with Leu140 (Figure 1). The analysis of the protein chimaera by limited proteolysis provided strong indications that the receiver and output domains are folded properly, because, similar to the parent proteins, thermolysin cleavage occured only within the predicted hybrid linker sequence spanning the amino acid residues 127-139 (Figure 1). It is noteworthy that cleavage within the HA linker occured at positions identical with those in the corresponding linkers of the two parent proteins (Figure 1). In contrast, limited thermolysin cleavage of another chimaeric protein derived from EvgA and BvgA fused within the receiver domain resulted in additional cleavage sites outside of the linker, probably due to altered folding of the hybrid receiver domain (M.B., A.B., R.G. & M.O.G., unpublished results). Moreover, the spectroscopic analysis based on circular dichroism of BvgA, EvgA and HA provided further evidence for intact folding of the receiver and output domains of the chimaeric protein HA (data not shown).

Analysis of the biochemical and biophysical properties of the chimaeric protein HA

Phosphorylation of the chimaeric HA protein was analysed *in vitro* by addition of purified BvgS or EvgS and of [γ-^{33}P]ATP. Confirming our previous results, the HA protein carrying the EvgA receiver domain could be phosphorylated by EvgS

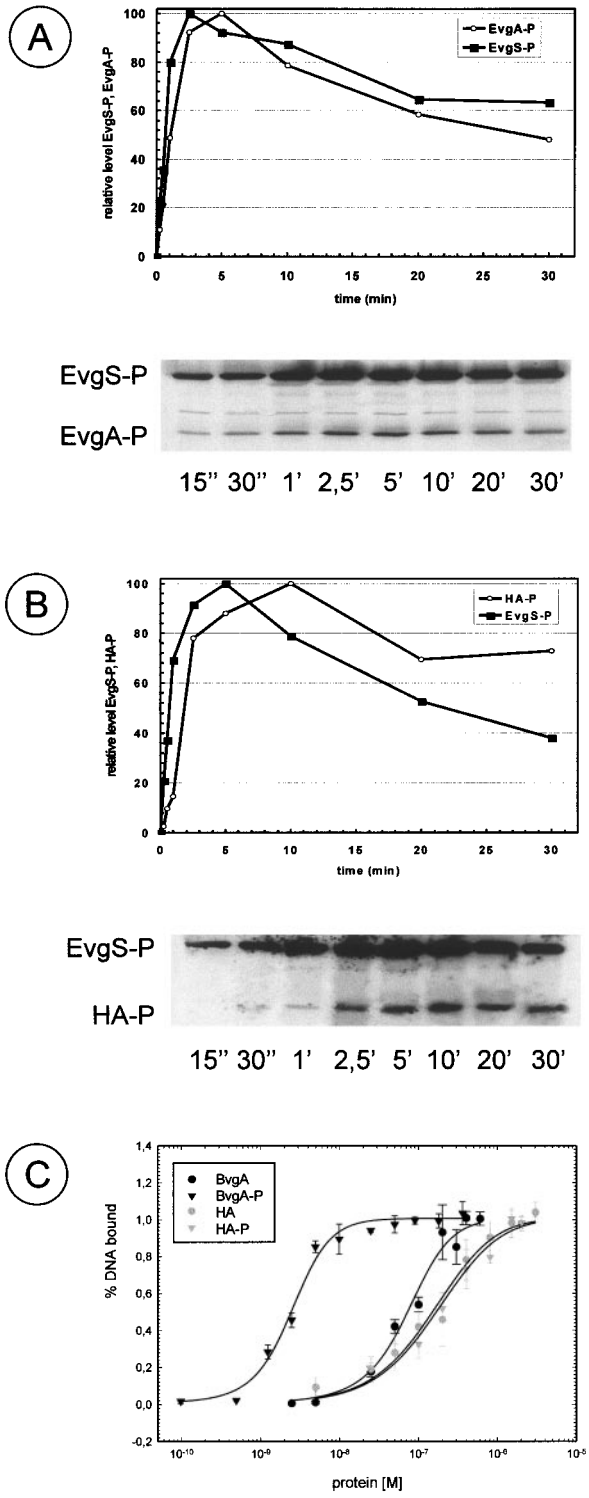

Figure 2. Biochemical properties of the chimaeric HA protein. (a) Time-course of relative auto- and transphosphorylation of the EvgS histidine kinase and of EvgA. (b) Time-course of relative auto- and transphosphorylation of the EvgS histidine kinase and of HA. The autoradigrams of the protein bands after SDS-PAGE analysis and, at the top of each gel, the results of quantification of the gel with a PhosphorImager are shown. Time is depicted on the *x*-axis (in minutes). Relative phosphorylation values are given with respect to the strongest signal on each gel taken as 100 %. The experiments were carried out as described[29] using [γ-^{33}P]ATP (specific activity 3000 Ci mmol^{-1}) and a 4:1 ratio of the response regulators and the kinases (final concentration of 1 μM). Quantitative densitometry was performed using a PhosphoImager (Molecular Dynamics Inc.) according to standard protocols. (c) DNA-binding characteristics of BvgA and HA. Binding was determined using a 77 bp DNA fragment containg the *fha* promoter from sequence position −102 to −26, which was end-labelled with [^{32}P]ATP. Various amounts of the proteins were incubated with [^{32}P]DNA (final concentration < 0.1 nM) in assay buffer (10 mM Tris (pH 7.5), 50 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 100 μg ml^{-1} BSA, 10 % (w/v) glycerol) in a total volume of 50 μl. After incubation at 4 °C or at room temperature for 25 minutes, 45 μl aliquots were taken and filtered through nitrocellulose filters (NC 45, Schleicher & Schuell). The filters were washed with 250 μl of assay buffer without BSA, dried and analyzed by liquid scintillation counting. For the competition experiments, fixed concentrations of protein (60 nM BvgA, 150 nM HA) were incubated with different amounts of non-labeled specific and non-specific DNA fragments in addition to the labeled DNA probe. The ratio of the half-maximal inhibition after addition of specific or non-specific DNA was used for the calculation of the specificity of DNA binding. Calculations of binding isotherms were done as described.[44] For the binding of ligand to two binding sites, the binding isotherm is given by $Y = x/(1 + x)$, where $x = K_1[L] + K_2[L] + K_1K_{1,2}[L]^2$ with the microscopic binding constants K_1 and K_2, and a co-operative interaction constant of $K_{1,2}$. Y is the fraction of DNA to which ligand has bound and [L] is the free ligand concentration. This equals the total ligand concentration if $[L] \gg [DNA]$. For linear binding of ligand to two binding sites, $K_{1,2} = 1$.

but not by BvgS (Table 2).[29] Interestingly, the phosphorylation kinetics of the chimaeric protein revealed a profile very similar to that observed for the intact EvgA protein, and the half-life of the phosphorylated form of the chimaeric protein resembled those of the parent proteins (Figure 2, Table 2). These data demonstrate that the presence of the heterologous BvgA output domain does not interfere with the phosphorylation characteristics of the EvgA receiver in the chimaeric protein.

Previous work showed that the BvgA and EvgA response regulators are dimers in solution in the non-phosphorylated form.[33] The apparent molecular mass of the non-phosphorylated chimaeric protein HA determined by analytical ultracentrifugation was 44.4(±4.6) kDa (Table 2). The calculated molecular mass of the HA monomer is 22.3 kDa. We conclude that in solution the HA protein forms dimers in the non-phosphorylated form. Therefore, the fusion of the two response regulators at their linker sequences did not impair protein surfaces required for homodimerization.

As the chimaeric response regulator contained the DNA-binding motif of BvgA, its transcription activation potential was investigated using the well-characterized BvgA-dependent *fha* promoter.[34] For this purpose, an *E. coli* strain with a deletion

of the chromosomal *evgA* gene but carrying an *fha*::*lacZ* fusion was transformed with plasmids encoding either BvgA or the HA protein in combination with the BvgS or the EvgS histidine kinases, respectively, under the control of the IPTG-inducible *lac* promoter. A strong induction of transcription from the *fha* promoter could be detected when expression of the BvgS and BvgA proteins was induced, whereas expression of HA together with EvgS did not cause any significant transcription from this promoter (data not shown).

To further corroborate these data, we performed *in vitro* transcription experiments using purified *E. coli* RNA polymerase and, alternatively, a reconstituted hybrid RNA polymerase containing the major σ-subunit of the RNA polymerase of *B. pertussis*.[35,36] In agreement with previous reports, transcripts derived from the BvgA-dependent *fha* and *cyaA* promoters could be observed with both RNA polymerases but only in the presence of phosphorylated BvgA. Addition of equal amounts of chimaeric HA protein did not result in the detection of specific transcripts, irrespective of the phosphorylation state of HA (data not shown). Therefore, despite the presence the DNA-binding motif of BvgA and despite its unimpaired phosphorylation and dimerization properties, HA is not able to activate transcription under the given experimental conditions.

In vitro binding of the chimaeric response regulator HA at the *fha* promoter

To investigate the reason for the failure of HA to function as a transcriptional activator, we characterized its interaction with DNA. Filter-binding assays were performed using the purified proteins and a previously described 77 bp radiolabelled DNA fragment derived from the *fha* promoter that contains an inverted repeat sequence known to be required for BvgA binding.[34,37]

Unphosphorylated BvgA bound quite strongly to this oligonucleotide with half-maximal binding at 74 nM (Figure 2(c); Table 2). The steep binding curve of BvgA indicates co-operative binding. Phosphorylation had a strong effect on the binding behaviour of BvgA, since the protein concentration required for half-maximal binding decreased from 74 nM to 3 nM for phosphorylated BvgA (Figure 2(c); Table 2). The chimaeric protein HA was also able to interact with the oligonucleotide, although it exhibited different binding characteristics compared to BvgA, such as a lower affinity to the DNA probe (Table 2). Moreover, HA did not bind in a co-operative fashion to the oligonucleotide and the binding isotherm could be fitted with a model for independent binding with the same binding constant of 2.9×10^6 M^{-1} for the two sites that we had identified in the 77 bp oligonucleotide (data not shown). Most importantly, phosphorylation of the HA protein remained without effect (Figure 2(c)). Competition experiments revealed that the specificity of BvgA for the *fha* promoter sequence increased approximately 14-fold upon phosphorylation. In contrast, the binding specificity of the chimaeric protein was much lower than that of non-phosphorylated BvgA and, once more, did not change upon phosphorylation of the protein (Table 2).

Conclusions

The position of the linker regions of the highly related response regulators BvgA and EvgA were determined and on the basis of this information the chimaeric protein HA was constructed. HA contains the EvgA receiver and the BvgA output domain fused within their linker sequences. In the case of the HA protein, the characterization of proteolytic cleavage products obtained after limited proteolysis with thermolysin and the comparison of the CD spectra of the related response regulators provided clear evidence for a proper folding of the two domains. Accordingly, the chimaera exhibits several biochemical and biophysical properties in common with its parent proteins, which include its high specificity for the EvgS histidine kinase, its phosphorylation and its dimerization properties (Table 2). However, despite the presence of these essential features, the chimaera was not able to activate transcription from BvgA-dependent promoters. Several previous attempts to construct chimaeric response regulators also did not result in functional transcription factors, although the reasons for lack of activity were not investigated in these cases.[38,39] In the case of the chimaeric HA protein, significant differences between HA and BvgA were found with regard to interaction of the proteins with DNA. In contrast to BvgA, phosphorylation of HA affected neither the affinity nor the specificity of the protein for the DNA target sequence (Figure 2; Table 2). Furthermore, HA did not bind co-operatively at its target sequences. Both structural and functional data suggest that, although the individual protein domains are apparently folded properly, their communication is perturbed and does not allow activation of the output domain after phosphorylation of the receiver. In addition, structural transitions possibly induced by binding of the wild-type protein at the target DNA and required for cooperativity may not function in the chimaeric protein. Therefore, although the BvgA and EvgA proteins show extensive sequence similarities in their receiver, and in particular in their C-terminal output domains, the interacting surfaces of these domains apparently do not fit with each other to enable a productive interaction. In this respect, it is interesting to note that in the case of FixJ, the receiver and output domains were shown to influence their respective activities strongly in a reciprocal manner.[14] Moreover, even a single amino acid substitution in the C-terminal DNA-binding domain affected the biochemical properties of the receiver domain in the OmpR response regulator.[40]

The results presented here demonstrate that, although the HA protein very likely contains intact domains, the chimaera is not a functional transcription activator. This may be due to a structural problem caused by the chimaeric linker region, or to a lack of essential co-interactions of the BvgA output domain with its cognate receiver domain. It is likely that specific interactions between the receiver and output domains of the BvgA and EvgA response regulators are required for the phosphorylation-induced activation of the output domain, suggesting a direct control of output activity by the receiver. However, the function of the linker sequences of response regulators is poorly understood.[17,41–43] Future investigations will show whether the linker sequences are involved directly in information conductance between the receiver and output domains of the BvgA and EvgA proteins.

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