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Negative Cooperativity of Uric Acid Binding to the Transcriptional Regulator HucR from *Deinococcus radiodurans*

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Members of the MarR family of winged helix transcriptional regulators have been shown to regulate multidrug and oxidative stress response, pathogenesis, and catabolism of aromatic compounds. Many respond to anionic lipophilic compounds in their capacity to bind DNA, and the co-crystal structure of MarR bound to salicylate revealed two ligand-binding pockets, SAL-A and SAL-B. The MarR homolog, HucR, from *Deinococcus radiodurans* has been shown to repress expression of a predicted uricase, and DNA-binding by HucR is antagonized by uric acid, the substrate of uricase. We provide a biochemical investigation of DNA-binding and uric acid-binding by HucR. Equilibrium analytical ultracentrifugation indicates that HucR exists as a dimer. Intrinsic fluorescence spectra suggest that the association of the HucR dimer with its cognate DNA involves conformational flexibility in the globular interior and/or dimerization domain of the protein, and near-UV circular dichroism spectra indicate a concomitant change in the helical twist of the DNA duplex. DNA-binding affinity, measured by electrophoretic mobility-shift assays, for HucR mutants bearing single amino acid substitutions suggests the importance of the β -hairpin “wing” in DNA binding. Analysis of intrinsic fluorescence spectra demonstrates that uric acid induces conformational changes in HucR and binds with an apparent $K_d = 11.6 (\pm 3.7) \mu\text{M}$ and a Hill coefficient of 0.7 ± 0.1 , indicating negative cooperativity. Fluorescence and DNA-binding properties of the HucR variants indicate that SAL-A is a low-affinity, uric acid-binding site and that negative cooperativity exists between homologous, high-affinity sites. The conservation of residues comprising site SAL-A suggests that it is a low-affinity, ligand-binding site in MarR homologs. Mechanistic considerations suggest that HucR is regulated by uric acid to maintain optimal cellular levels of this scavenger of free radicals in response to oxidative stress and DNA damage.

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Keywords: winged helix-turn-helix; MarR; ligand; oxidative stress; intrinsic fluorescence

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Introduction

The identification of the MarR (multiple antibiotic resistance regulator) family of transcriptional regulators was initiated by genetic experiments that identified strains of *Escherichia coli* K-12 with enhanced resistance to structurally diverse antibiotics, oxidative

stress agents, and organic solvents.^{1–5} The locus responsible for the resistance phenotype was identified as the *marRAB* operon.^{6,7} Transcriptional repression was shown to be conferred by the product of the first gene of this operon, MarR, which binds with high affinity ($K_d \sim 1 \text{ nM}$) as a homodimer to two sites in the *marRAB* promoter/operator region.^{4,8} MarR-mediated repression was shown to be relieved, both *in vitro* and *in vivo*, by structurally diverse phenolic compounds, including some of the antimicrobial agents to which the operon confers resistance.^{4,9–11} Structural determination identified MarR as a winged helix DNA-binding protein.¹²

Abbreviations used: HucR, hypothetical uricase regulator; CD, circular dichroism; EMSA, electrophoretic mobility-shift assay; BSA, bovine serum albumin.

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Numerous prokaryotic MarR homologs with physiological regulatory roles in antibiotic and oxidative stress response (MarR, MexR, EmrR, PecS, and HucR), the production of virulence factors (PecS and ExpG), and the catabolism of aromatic compounds (HpaR, HpcR, CinR, BadR, CbaR, HucR, and HcaR) have since been identified.^{9,13–19} The DNA-binding capacities of a number of these MarR family members have been shown to be altered by anionic lipophilic compounds, often by the suggested target of the regulated genes.

The structure of the MarR dimer bound to the effector salicylate has been resolved at 2.3 Å, revealing two ligand-binding sites, designated as SAL-A and SAL-B, on either side of the proposed DNA recognition helix.¹² The proximity of the ligand-binding sites in MarR to residues shown to be critical for MarR binding activity²⁰ suggested a potential mechanism of regulation. In addition, the crystal structure of an MarR family member, MexR from *Pseudomonas aeruginosa*, has been resolved at 2.1 Å.¹⁸ Several conformational states of the MexR dimer were observed in the asymmetric unit, suggesting that the mechanism of ligand-induced effects on the DNA-binding activity of this protein involves alterations to the relative orientations of the DNA binding domains from each half of the dimer. However, without combined structural data for each protein in its DNA-bound, ligand-bound, and apo conformations, the DNA-binding and ligand-response mechanisms of these MarR homologs remain unknown.

We previously reported the biochemical characterization of HucR, a novel MarR homolog from *Deinococcus radiodurans*.¹⁷ HucR binds as a homodimer with very high affinity ($K_d = 0.3$ nM) to a single 21 bp site containing *E. coli* σ^{70} -like promoter elements driving its own expression and that of an adjacent, predicted uricase (*dr1160*) (shown to be a functional enzyme *in vitro*; our unpublished results). The affinity of HucR for its cognate binding sequence is antagonized by certain anionic lipophilic compounds, most notably uric acid. Analyses *in vivo* indicate that uric acid upregulates transcription of *hucR* and *dr1160* in *D. radiodurans* and suggest that uricase activity in *D. radiodurans* total protein extracts is increased in cells grown in the presence of uric acid.

In this study, we provide the first biochemical investigation into the mechanisms of DNA interaction and ligand response for a member of the MarR family. Spectroscopic evidence reveals that the mechanism by which HucR binds its sequence-specific, DNA-binding site involves conformational flexibility in the protein and in the DNA helix. We show that HucR possesses at least two uric acid-binding sites, with dissimilar ligand-binding affinities. Moreover, our data suggest that the highly conserved ligand-binding site corresponding to SAL-A in MarR is a weak ligand-binding site in HucR, indicating that antagonistic effect of uric acid on HucR-DNA interaction is not mediated primarily by the SAL-A site.

Results

Complex formation is accompanied by conformational changes in both HucR and *hucO*

Previous footprinting analyses of the binding interaction between HucR and its sequence-specific double-stranded DNA-binding site suggested that the process involved deformation of the DNA.¹⁷ To assess the role of conformational changes in the DNA double helix in the HucR binding mechanism, we used circular dichroism (CD) spectroscopy to analyze HucR-mediated changes in the secondary structure of a 31 bp DNA fragment (*hucO*-31) containing the 21 bp HucR binding site. Protein contributions to the CD signal in the near UV are minimal; above 240 nm, ellipticity is dominated by contributions from the purine and pyrimidine bases of the DNA duplex. The CD spectrum of *hucO*-31 is characteristic of B-form DNA (Figure 1(a)). Under stoichiometric binding conditions with a molar ratio of HucR dimer:DNA of 1:1 yielding 100% saturation of *hucO*-31, HucR induced a significant

Figure 1. Binding-induced conformational changes in DNA and protein. (a) Near-UV CD spectra of 2 μ M *hucO* in the absence of protein (black, filled circles) or in complex with 4 μ M HucR (red, open triangles). Data were corrected for protein and solvent contributions to the signal. HucR–*hucO*-31 complex formation was verified by an electrophoretic mobility-shift assay (data not shown). (b) DNA-induced changes in the intrinsic fluorescence spectrum of HucR. The intrinsic fluorescence spectrum of 1.52 μ M HucR was measured in the absence or in the presence of increasing concentrations of *hucO*-31: 0:1 molar ratio of *hucO*-31 to HucR (black, filled circle); 0.25:1 (red, filled square); 0.5:1 (green, diamond); 0.75:1 (blue, triangle); 1:1 (orange, inverted triangle).

increase in the magnitude of the positive CD band at 275 nm. Changes in the ellipticity band at 275 nm have been correlated with changes in the DNA winding angle.^{21–24} The HucR-mediated effect on the *hucO*-31 CD band at 275 nm is consistent with an increase in the number of base-pairs per turn of the DNA, or a decrease in the helical twist of the DNA duplex.

Secondary structure prediction algorithms suggest that the HucR monomer is composed of seven α -helices and two β -strands,^{25,26} with helices 4 and 5 comprising the two α -helices of the helix-turn-helix motif. HucR contains two tryptophan residues, located at positions 20 and 72 in the primary amino acid sequence, and two tyrosine residues, at positions 62 and 79. Secondary structure prediction places W20 in helix 1, Y62 in helix 2, and W72 and Y79 in helix 3. Structural modeling of HucR, and structural analysis of the homologs MexR and MarR, places helix 3 in the globular interior of the monomer and helices 1 and 2 at the dimer interface (Figure 2). None of these residues is expected to be in the wing or DNA recognition helix and hence changes in the intrinsic fluorescence spectrum of HucR would indicate altered environments of the fluorophores in the dimerization domains and hydrophobic cores of the homodimer. Using an excitation wavelength of 280 nm, the

Figure 2. Model of HucR monomer. The known structure of the closely related MarR family member, MexR, was used as a template to model HucR *via* SWISS-MODEL. The predicted DNA recognition helix is colored green. The β -strands and loop comprising with “wing” motif are colored blue and yellow, respectively. Side-chains of S104 and R118 are shown in red, ball and stick representation. The backbone position of P89, part of the MarR SAL-A pocket, is colored magenta. W20 and W72 are indicated in orange. The N terminus is labeled N. Residues 178–181 of HucR are not shown.

measured emission signal is dominated by the tryptophan residues, which have a much higher quantum yield relative to tyrosine. The intrinsic fluorescence of HucR was measured as a function of *hucO*-31 concentration to monitor protein conformational changes upon complex formation (Figure 1(b)). The intrinsic fluorescence spectrum of HucR is characterized by an emission maximum at 338 nm and a “shoulder” at 328 nm. Titration of HucR with *hucO* resulted in a significant quenching of the entire fluorescence spectrum, with no change in the wavelength of maximal fluorescence or in the relative intensity of the shoulder. This quenching effect was saturated at an *hucO* to HucR monomer molar ratio of 0.5:1, consistent with previous work demonstrating that HucR binds its cognate DNA site as a homodimer. This observation, an electrophoretic mobility-shift assay (EMSA) showing complete saturation of DNA at a 1:1 stoichiometry of HucR dimer to DNA, and footprinting analyses revealing a pseudopalindromic binding sequence,¹⁷ indicate that the protein is largely active.

The equilibrium sedimentation profile of HucR was attained to determine the oligomeric state of HucR in the absence of DNA (Figure 3). Data were best fit to a model describing a single, non-associating protein species, yielding a molecular mass average of $43,016 \pm 4374$ for HucR. As the molecular mass of an HucR monomer calculated from its primary amino acid sequence is 19.7 kDa, the equilibrium sedimentation data suggest that HucR exists predominantly as a homodimer in the absence of DNA. Although we cannot rigorously rule out other association models, the reported

Figure 3. Equilibrium sedimentation profile of HucR. The absorbance of 40 μ M HucR (monomer equivalents) is shown as a function of the radial cell position (lower panel). Data were fit to a model describing a single, non-associating, species. Residuals to the fit were distributed randomly (upper panel).

association model is consistent with crystallographic and biochemical data for other MarR homologs.^{12,18,19,27} Also, this supports the accuracy of the K_d value of 0.3 nM reported for the HucR dimer–*hucO* association, as the binding mechanism is likely uncomplicated by monomer–dimer equilibrium.

Uric acid–HucR interactions

Uric acid was shown to be an efficient negative effector of the capacity of HucR to bind its cognate DNA, with a concentration of 0.26 mM being required to reduce HucR–DNA complex formation to 50%, compared to 5.2 mM for salicylic acid, and 46 mM for acetylsalicylic acid.¹⁷ To monitor binding of HucR to its ligands, we measured the intrinsic fluorescence of HucR as a function of ligand concentration. Absorption limitations at the excitation wavelength of 280 nm determined the maximum concentration of ligand for which fluorescence intensities could be fully corrected for inner filter effects. At concentrations up to 50 μ M, uric acid induced a strong quenching effect on the emission spectra of HucR. The quenching of the fluorescence emission maximum at 338 nm as a function of the concentration of uric acid was fit to the Hill equation, yielding an apparent dissociation constant for HucR–uric acid interaction of $11.6 (\pm 3.7) \mu$ M (Figure 4(a)). The fit provided a Hill coefficient (n_H) of 0.7 ± 0.1 , suggesting negative cooperativity. Analysis of the data using equations for non-equivalent, non-interacting sites resulted in fits with large error, suggesting that measurements of fluorescence quenching at concentrations of uric acid below 50 μ M predominantly monitors binding at higher-affinity binding site(s), and that the observed negative cooperativity is either between equivalent sites in a protein homodimer, or between the higher-affinity site and one, or more, lower-affinity ligand-binding sites within the same monomer. Confirmation of negative cooperativity is provided by transformation of the data (Figure 4(b)).²⁸ A Scatchard plot of uric acid-induced quenching of HucR fluorescence at 338 nm reveals a clear deviation from linearity, and the direction of the curvature is indicative of negative cooperativity. The double-reciprocal plot is hyperbolic, also indicating negative cooperativity (Figure 4(b)).^{28,29} Separate titrations of HucR with salicylic acid and acetylsalicylic acid up to final concentrations of 100 μ M and 120 μ M, respectively, resulted in essentially no effect on the intrinsic fluorescence of HucR (Figure 4(a)), consistent with previous data showing little effect on DNA–HucR complex formation at these compound concentrations.¹⁷

To monitor the effects of higher concentrations of ligand on HucR fluorescence, the emission spectra were corrected for inner filter effects of ligand absorbance at emission wavelengths, and absorbance at the excitation wavelength was compensated for by normalizing the emission spectrum to the maximal fluorescence intensity. In this way,

Figure 4. Binding isotherms for HucR–ligand interactions, at low concentrations of ligand. Quenching of HucR fluorescence at 338 nm is plotted against concentration of ligand: uric acid (filled circles, continuous line); salicylic acid (filled squares, broken line); acetylsalicylic acid (filled triangles, dotted line). Data were collected up to 100 μ M salicylic acid and 120 μ M acetylsalicylic acid, demonstrating no fluorescence quenching.

alterations to the HucR emission spectrum could be observed, in addition to the fluorescence quenching observed at low concentrations of uric acid. Titration of HucR with uric acid resulted in a gradual red-shift in the wavelength of maximal fluorescence, from 338 nm for unbound HucR to 341 nm at 200 μ M uric acid (Figure 5). This red shift saturated at 200 μ M uric acid, as the fluorescence maximum remained at 341 nm at concentrations of uric acid up to 1 mM. The fluorescence shoulder, characteristic of the HucR spectrum, is sensitive to the concentration of uric acid. The shoulder intensity, relative to the fluorescence maximum,

Figure 5. HucR fluorescence spectra, normalized to the fluorescence intensity maximum. Representative spectra from one of two separate experiments, showing HucR in the absence of uric acid (black, filled circle) or in the presence of 30 μ M uric acid (red, filled square), 200 μ M uric acid (blue, filled diamond), and 1 mM uric acid (green, filled triangle).

underwent a gradual quenching with increasing concentration of uric acid. These effects on the HucR emission spectrum were not induced by the low-affinity ligands, salicylic acid and acetylsalicylic acid (Supplementary Data, Figure S1). In comparison to the red shift observed with uric acid, separate titrations up to a concentration of 1 mM for each compound did not shift the wavelength of maximum fluorescence from 338 nm. In addition, neither salicylic acid nor acetylsalicylic acid induced quenching of the fluorescence shoulder. From these data, we conclude that three characteristic alterations to the fluorescence spectrum of HucR are indicative of uric acid binding: (1) quenching, observable at low concentrations of ligand; (2) a red shift in the wavelength of maximum fluorescence intensity; and (3) quenching of the intensity of the shoulder at an emission wavelength 10 nm below the wavelength of maximum intensity.

HucR mutants

The observation of negative cooperativity in uric acid binding to HucR (Figure 4) is consistent with three possible models: (1) there are at least two separate uric acid-binding sites per HucR monomer and the negative cooperativity exists between these sites; (2) there is only one uric acid-binding site per HucR monomer and the negative cooperativity is between homologous sites in a homodimeric assembly; or (3) there are multiple uric acid-binding sites per monomer and the negative cooperativity is between homologous sites in a homodimer. The co-crystal structure of MarR with salicylate reveals two binding pockets for salicylate within each half of a homodimer, designated as sites SAL-A and SAL-B.¹² However, the physiological relevance of these binding pockets remains to be determined, as the protein crystals were saturated at the exceedingly high concentration of salicylate of 250 mM.

Primary sequence alignment of HucR with its most closely related homologs from the MarR family revealed a high degree of conservation of residues that form the ligand-binding pockets of MarR.^{12,17} Most striking in the alignment is the conservation of residues that form ligand-binding pocket SAL-A in MarR. The guanidinium group of R118 (numbering according to HucR primary sequence) forms a hydrogen bond with the carboxylate group of salicylate at this site (~ 3 Å); R118 is strictly conserved in the alignment.¹⁷ The threonine residue that hydrogen bonds to the hydroxyl group of salicylate in site SAL-A (~ 3 Å) is strictly conserved amongst the HucR homologs in the alignment, but is replaced by a homologous serine in HucR (S104). The hydrogen bonding residues in site SAL-B are less well conserved in the alignment, but HucR retains R109, which coordinates with the carboxylate group of salicylate.

To determine whether the mechanism of ligand recognition suggested by the MarR co-crystal structure is retained by HucR, we generated two HucR mutants, HucR-R118A and HucR-S104A, each containing a single amino acid substitution in ligand-binding site SAL-A. Secondary structure prediction, sequence alignment against MarR, and structural modeling of HucR (Figure 2) positions R118 in the β -hairpin motif that forms the characteristic “wing” structure of winged helix proteins and positions S104 in the fifth α -helix of HucR that forms the DNA recognition helix of the winged helix motif. Therefore, changes in both the ligand-binding and DNA-binding properties of HucR were expected from each mutation.

DNA-binding properties of HucR mutants

We measured the affinities of HucR-R118A and HucR-S104A for the HucR binding site, *hucO*. EMSA revealed an apparent K_d of $0.1(\pm 0.0)$ nM ($\Delta G_{\text{assoc}} = -13.4$ kcal/mol) for the interaction of HucR-S104A with *hucO*, compared to the apparent K_d of $0.3(\pm 0.0)$ nM previously measured for wild-type HucR (Figure 6). The affinity of HucR-R118A for *hucO* was reduced approximately sixfold, revealing an apparent K_d of $1.9(\pm 0.1)$ nM ($\Delta G_{\text{assoc}} = -11.8$ kcal/mol). The complex of each HucR mutant with *hucO* dissociates during electrophoresis, as reflected in a fractional saturation less than 100% before correction for dissociation during electrophoresis. In addition, whereas only a single protein–DNA complex is observed in each HucR and HucR-R118A titration, a second complex may be distinguished at high concentrations of HucR-S104A. EMSA measurements of DNA binding for the HucR variants under stoichiometric conditions ($[DNA] \gg K_d$) demonstrated that HucR,¹⁷ HucR-S104A, and HucR-R118A bind *hucO* at molar ratios of 1.9:1, 1.8:1, and 1.9:1, respectively (data not shown), consistent with fluorescence data (Figure 1(b)) and observations of a pseudopalindromic binding sequence,¹⁷ indicating

Figure 6. Affinity measurements of HucR mutants for the HucR binding sequence (*hucO*). (a)–(c) EMSAs in which 0.1 nM *hucO* was titrated with protein. Uncomplexed *hucO* and protein–DNA complexes are identified by arrows. (a) Wild-type HucR: 0, 0.05 nM, 0.125 nM, 0.25 nM, 0.5 nM, 1.0 nM, 2.5 nM, 5.0 nM, 15.0 nM. (b) HucR-S104A; protein concentrations as for (a). (c) HucR-R118A: 0, 0.25 nM, 0.5 nM, 2.5 nM, 5.0 nM, 10.0 nM, 15.0 nM, 20.0 nM. (d) Binding isotherms for HucR-S104A (continuous line, filled circles; binding to 0.01 nM *hucO*) and HucR-R118A (broken line, filled squares). Data were collected in triplicate. Fractional saturation is reported as the mean $\pm$ S.D.

that HucR binds DNA as a dimer, suggesting that the mutant proteins also approach 100% activity.

The protein–DNA complex instability observed for both HucR-S104A and HucR-R118A suggested the possibility that the DNA-binding specificities of the HucR mutants were reduced, relative to the wild-type. A measure of the sequence specificity in binding by HucR-S104A and HucR-R118A was obtained by a direct comparison of the abilities of unlabeled *hucO* and pGEM5 to compete for protein binding with 0.1 nM 32 P-labeled *hucO* (Figure 7). Reduced fractional saturation on the gel implies competition in solution. Each HucR mutant is highly sequence-specific in its DNA-binding capacity, as reported for the wild-type protein. Titration of unlabeled *hucO* to a concentration of 5 nM elicited a clear reduction in labeled complex

Figure 7. Competition assays. Binding reactions involved 0.1 nM 32 P-labeled *hucO* and titration of up to 15 nM unlabeled *hucO* or pGEM5, followed by the addition of protein. Complexes were resolved by EMSAs. The first lane in each gel contains only labeled DNA. Concentrations of unlabeled competitor DNA are indicated above the corresponding lanes. Complexed and uncomplexed *hucO* are identified by arrows. (a) HucR-S104A, (b) HucR-R118A.

formation with HucR-S104A and HucR-R118A. However, when using pGEM5 as a competitor, only a slight reduction in the labeled *hucO*–protein complex is observed at a concentration of 15 nM pGEM5, for each mutant. Since pGEM5 contributes a greater than 12-fold molar excess of base-pairs compared to *hucO*, the competition assays demonstrate the retention of a definite sequence-specific binding for both HucR mutants.

Consistent with the apparent DNA binding constants for the HucR variants (Figure 6), the DNA-responsive intrinsic fluorescence spectra of the proteins suggest altered mechanisms of DNA association (Figure 8). A comparison of the intrinsic fluorescence spectra of HucR, HucR-S104A, and HucR-R118A reveals significant differences. Whereas the measured fluorescence maximum is at 338 nm for each protein, the peak intensity is 86.2 for HucR, 73.2 for HucR-S104A, and 111.6 for HucR-R118A, suggesting that the environments of either one, or both, of the tryptophan residues differ in these HucR variants, and that each mutation causes a global conformational change that propagates to the globular interior and to the dimerization domain; while a conformational change would be needed to alter the environment of the tryptophan(s), such changes may be modest. When complexed with a stoichiometric quantity of *hucO*, such that all protein is expected to be in complex with DNA, fluorescence quenching is observed for each protein, indicating altered environments for one or both tryptophan residues. However, the degree of quenching induced by complex formation differs significantly between the proteins. HucR interaction with its cognate DNA-binding site leads to a quenching of intrinsic fluorescence by 10.0%, compared to 5.4% for HucR-S104A, and 22.2% for HucR-R118A. From these two observations, we conclude that protein conformational changes upon DNA binding by HucR, HucR-S104A, and HucR-R118A also differ.

Figure 8. Fluorescence spectra of free and DNA-bound HucR variants. The intrinsic fluorescence spectrum is shown for 1.52 μ M each HucR variant before (continuous line, filled circles) or after (broken line, open triangles) incubation with 0.76 μ M *hucO*-31. (a) Wild-type HucR, (b) HucR-S104A, and (c) HucR-R118A.

Uric acid binding by HucR mutants

As both R118 and S104 mediate hydrogen bond contacts with a ligand in the crystal structure of MarR, and as both of these residues are highly conserved in the MarR family, we analyzed the roles of each of these side-chains in uric acid recognition by HucR. For each HucR mutant, increasing concentrations of uric acid reduced formation of the protein-*hucO* complex (Figure 9(a) and (b)). However, in contrast to the case of wild-type HucR, where DNA complex formation was abolished in the presence of approximately 1 mM uric acid, we observed faint smears extending above the band of free *hucO* at concentrations of uric acid up to 10 mM. This effect was shown not to be a result of uric acid or its solvent affecting the DNA migration (data not shown). Additional controls confirmed that the uric acid solvent did not affect formation of the protein-*hucO* complex (data not shown). We therefore interpreted the smearing above the band of free DNA to be residual DNA complex formation with HucR-S104A and HucR-R118A. At concentrations of uric acid below 1 mM, a steep decrease in

Figure 9. DNA-binding antagonism by uric acid. Protein-*hucO* association was challenged with increasing concentrations of uric acid, and complexes resolved by EMSA. Reactions contained 0.1 nM 32 P-labeled *hucO*. Lane 1 contains free DNA. For protein-containing reactions, uric acid concentrations are 0, 0.1 mM, 0.5 mM, 1, 5 mM, and 10 mM. (a) HucR-S104A. (b) HucR-R118A. (c) Normalized protein-*hucO* complex formation as a function of uric acid concentration for wild-type HucR (continuous line, filled circles), HucR-R118A (broken line, filled squares), and HucR-S104A (dotted line, filled triangles). Experiments were performed in triplicate and data are reported as mean $\pm$ S.E.

normalized DNA complex formation is seen for all three variants (Figure 9(c)). At concentrations of uric acid above 1 mM, the antagonistic effect of uric acid approaches saturation; for wild-type HucR, fractional complex saturation approaches zero while fractional saturation for the HucR-mutants near saturation at $\sim$ 30%. At 10 mM uric acid, approximately 20% of the HucR-S104A complex remains and approximately 10% of the HucR-R118A complex remains.

In addition to affects on DNA affinity, the S104A and R118A amino acid substitutions in HucR, therefore, each appear to reduce the response of the protein to high concentrations of uric acid. The data are consistent with there being at least two separate uric acid-binding sites in HucR with dissimilar ligand affinities. Our data suggest that the mutations at HucR positions 104 and 118, corresponding to ligand-binding pocket SAL-A in MarR, each affect a lower-affinity uric acid-binding site.

The interaction of uric acid with HucR-S104A and HucR-R118A was measured by intrinsic fluorescence (Figure 10(a)). For HucR-S104A, the

Figure 10. Fluorescence detection of uric acid binding to HucR mutants. (a) Binding isotherms for protein–uric acid interactions. Quenching of protein fluorescence at 338 nm is plotted against concentration of uric acid for wild-type HucR (continuous line, filled circles), HucR-R118A (broken line, filled squares), and HucR-S104A (dotted line, filled triangles). (b) and (c) Fluorescence spectra, normalized to the fluorescence intensity maximum, for protein in the absence (black, filled circles) or presence of 30 μM (red, filled squares), 200 μM (blue, filled diamonds), and 1 mM (green, filled triangles) uric acid. (b) HucR-S104A. (c) HucR-R118A.

apparent K_d for interaction with uric acid was $7.4 (\pm 0.5) \mu\text{M}$, compared to $9.7 (\pm 3.3) \mu\text{M}$ for HucR-R118A, and $11.6 (\pm 3.7) \mu\text{M}$ for HucR. Interestingly, the negative cooperativity in uric acid binding by HucR was reduced, or lost, through the S104A and R118A mutations. Whereas HucR displayed an n_H of 0.7 ± 0.1 , fits for HucR-S104A and HucR-R118A revealed n_H values of 1.1 ± 0.1 and 0.9 ± 0.2 , respectively. Scatchard representations of uric acid-induced quenching of mutant fluorescence were linear. As for the wild-type protein, fits of the data for the mutants to an equation for non-equivalent, independent sites resulted in large error. As measurements of fluorescence quenching at low concentrations of uric acid predominantly monitors binding at a high-affinity site(s), the

unchanged, or small increase in, affinity for uric acid observed for both HucR mutants is consistent with each mutation residing in a low-affinity uric acid-binding site.

We then compared the effects of uric acid on the normalized fluorescence spectra of HucR, HucR-S104A, and HucR-R118A. The uric acid-induced red shift in the wavelength of maximal fluorescence intensity for HucR was observed in both HucR mutants (Figure 10(b) and (c)). For each protein, the wavelength of maximal intensity shifted from 338 nm, for the apo protein, to 341 nm at 200 μM uric acid and remained at 341 nm at a ligand concentration of 1 mM. The reduction of the shoulder fluorescence intensity relative to the peak intensity observed in HucR was retained in both HucR mutants, even at low concentrations of uric acid. We therefore concluded that these effects on the fluorescence spectra were induced by ligand binding at a site that was unaltered by the separate mutations at S104 and R118. Moreover, as the red-shift and shoulder-quenching effects were observed at concentrations of ligand below 50 μM , we conclude that these effects correspond to binding at a high-affinity site(s) and that the S104A and R118A mutations perturb a low-affinity ligand binding site(s).

Discussion

HucR–hucO association; conformational changes

The conformational changes observed in HucR and the DNA upon binding indicate that the interaction is not static. Rather, a DNA distortion is observed that may indicate a decrease in the duplex winding angle (Figure 1(a)). This is consistent with the mechanisms of site-specific DNA binding by winged helix proteins from the MerR family, which have been shown to decrease the helical twist of the DNA duplex, and bend the double helix, upon formation of complex.^{30,31} As the center of each inverted repeat in the HucR binding site is separated by one full turn of double helix, it is tempting to speculate that unwinding of the double helix is required to accommodate insertion of the recognition helices of an HucR homodimer into consecutive, major grooves. In the absence of a crystal structure of a DNA-bound MarR homolog, this is the first piece of evidence suggesting that alteration of the helical twist of the DNA duplex is required to accommodate binding by these proteins.

Quenching of the intrinsic fluorescence of HucR upon DNA complex formation suggests conformational changes in the homodimer that extend beyond the helix-turn-helix and wing motifs that are predicted to make all direct contacts with DNA; the two HucR tryptophan residues are expected to be in the globular interior of the protein and at the dimer interface (Figures 1(b) and 2). Quenching of

the intrinsic fluorescence of the indole ring of tryptophan has been associated with conformational shifts to three altered environments: (1) increased solvent exposure; (2) closer contact with amide bonds in the peptide backbone; or (3) closer contact with polar side-chains.^{32,33} Additional work will be required to elucidate the nature of the conformational changes induced in HucR and its cognate DNA-binding site upon formation of the complex.

HucR DNA-binding mechanism

Given the predicted locations of S104 and R118 in the DNA recognition helix and wing of HucR, respectively, it is not surprising that substitutions at these positions affect HucR–*hucO* association (Figure 6). The protein–DNA complex stability for each HucR-mutant, relative to that of wild-type protein, is reduced on the gel; we cannot say if this relative instability is present in solution. However, an increased k_{off} would explain the increased K_d observed for HucR-R118A. Also consistent with the altered apparent K_d values for each mutant are the intrinsic fluorescence data, suggesting altered DNA-binding mechanisms (Figure 8).

The effects of the individual mutations on DNA binding support the prediction that HucR mediates binding *via* a winged helix fold. In addition, the decreased affinity for the HucR binding site, elicited by the R118A mutation, suggests strongly that the β -hairpin wing, and not just the recognition helix, makes direct contacts with the DNA duplex. Moreover, the high degree of amino acid conservation in the wing region of MarR homologs suggests its importance in directing association with DNA.¹⁷

HucR–uric acid association and negative cooperativity

The apparent $K_d \sim 12 \mu\text{M}$ for binding of uric acid to HucR indicates an affinity significantly higher than has been observed for MarR, whose affinity for the phenolic compounds salicylate, plumbagin, 2,4-dinitrophenol, and menadione was 500 μM , 250 μM , 250 μM , and 800 μM , respectively,^{8,11,34} but similar to the drug-binding affinities of the MarR homolog, EmrR, which binds phenolic ligands with a K_d of 2.0–15.0 μM .²⁷

Our data suggest that the HucR monomer contains at least two separate uric acid-binding sites with dissimilar affinities, and that the HucR site corresponding to the MarR ligand-binding pocket SAL-A binds uric acid with low affinity. The significant conservation of residues comprising SAL-A suggests that SAL-A is a low-affinity site in MarR, as well. These conclusions are based upon the following observations. (1) Uric acid-induced quenching of HucR intrinsic fluorescence reveals negative cooperativity, indicating that at least two uric acid-binding sites are involved (Figure 4). Uric acid quenching predominantly monitors binding at a high-affinity site(s) as indicated by the poor fit of the data to equations for multiple, non-equivalent,

non-interacting sites, suggesting that negative cooperativity is between homologous sites in each monomer. (2) The S104A and R118A mutations each result in little to no change in uric acid-binding affinity, suggesting that each residue resides in a low-affinity, ligand-binding site (Figure 10). Each mutation removes the cooperativity of uric acid binding, consistent with each mutation residing in the same binding site. (3) The alterations to the normalized fluorescence spectra observed at concentrations of uric acid up to 1 mM were nearly identical for all three HucR variants, indicating that these effects were induced by ligand binding at a site unaltered by the separate S104A and R118A mutations (Figures 5 and 10(b) and (c)). Moreover, as these alterations to the emission spectra occurred at concentrations of uric acid below 50 μM , we conclude that these effects correspond to binding at high-affinity site(s). (4) Increasing concentrations of uric acid up to 1 mM result in a similar, steep antagonism of HucR–*hucO* complex formation for HucR, HucR-S104A, and HucR-R118A (Figure 9). However, unlike HucR, residual protein–DNA complex is observed for both HucR mutants up to 10 mM uric acid. These observations are consistent with each mutation residing in a low-affinity, ligand-binding site.

The loss of uric acid binding cooperativity induced by each mutation, combined with the differences in the intrinsic fluorescence spectra of the HucR variants (Figure 8), suggests the possibility that each mutation propagated a conformational change in HucR that disrupted the cooperativity between the high-affinity site(s) in each half of the homodimer. Alternatively, uric acid binding at the low-affinity site (containing HucR residues S104 and R118) might stabilize the recognition helix in a position such that ligand binding at the high-affinity site might reduce the affinity of the corresponding site in the adjacent monomer. This latter model is consistent with the expected juxtaposition of the SAL-B ligand-binding sites in the cleft between the HucR subunits of the homodimer. The data do not rule out the possibility that the negative cooperativity in HucR is between the low-affinity site comprised of residues S104 and R118, and a separate high-affinity site(s). However, ligand binding in the low-affinity, SAL-A site is unlikely to be involved in negative cooperativity, based on our observation that negative cooperativity is seen at concentrations of uric acid below 50 μM , where site SAL-A is probably not filled.

It has been proposed that the physiological benefit of negative cooperativity is that it increases the range of concentration over which the protein can respond.^{32,35} In addition, negative cooperativity can increase the protein's sensitivity to low concentrations of ligand. Given the tendency of uric acid to precipitate and the consequential deleterious effects to the cell, it would likely be advantageous for *D. radiodurans* to upregulate uricase in response to low concentrations of uric acid. However, given the ability of uric acid to scavenge

damaging reactive oxygen species,^{36,37} it seems beneficial to the cell for the uric acid-modulated response of uricase expression to be attenuated, such that an optimal concentration of uric acid is maintained below a critical threshold where precipitation would occur. Negative cooperativity could extend the range of uric acid concentration over which HucR can respond, by effectively increasing the concentration of uric acid at which all HucR-mediated uricase repression is relieved, thus allowing a basal level of uric acid to remain in the cell. Simultaneously, negative cooperativity would effectively decrease *hucR* transcription at low concentrations of uric acid, likely to maintain an optimal level of uricase in the cell.

In a similar light, uric acid-binding site SAL-A in HucR may be physiologically relevant. As suggested by Figure 9, uric acid binding in site SAL-A (in addition to binding at high-affinity site(s)) would reduce DNA-binding affinity relative to that of HucR with only the high-affinity site(s) filled. Such a system may serve to provide a constitutive level of uricase repression at concentrations of uric acid below a dangerous threshold; only if the cellular concentration of uric acid becomes high enough, would site SAL-A be filled, resulting in maximal uricase expression.

Possible basis for changes in the fluorescence spectrum of HucR on ligand or DNA binding

W20 of HucR is predicted to reside in an α -terminal α -helix that, on the basis of the MarR and MexR crystal structures, likely forms the hydrophobic dimer interface.^{12,18} On the basis of the MarR-salicylate co-crystal structure, movement of the DNA-binding domains to accommodate DNA binding would require significant distortion of MarR α -helix 1 and the C-terminal α -helices.^{8,12} The multiple structural views of the MexR homodimer indicate significant flexibility in the dimerization domain, as observed in a 17° deviation in the helix orientation of α -helix 1, and a 4.9 Å shift in its helix midpoint position.¹⁸ Moreover, as dimerization of both MexR and MarR is mediated primarily by van der Waals contacts involving their respective N and C-terminal helices, flexibility in this domain is expected, as such hydrophobic interactions do not require specific molecular orientations. Except for the wing itself, the MexR winged helix DNA-binding domain appears to be considerably less flexible; rather, each domain moves as a rigid body relative to the homologous domain in the dimer.¹⁸ HucR α -helix 3, which contains W72, corresponds to the first helix in this motif. Though we do not know the magnitude of the conformational changes that alter the environments of the HucR fluorophores, the alterations of the HucR fluorescence spectrum observed upon DNA binding and ligand binding are consistent with conformational changes in the dimerization domain containing HucR tryptophan 20 and/or

rigid movements of HucR helix 3 in the winged-helix DNA binding domain.

Proposed mechanism for uric acid-mediated allosteric control of HucR–DNA complex formation

Though there is considerable variability in the mechanisms of allosteric control of the DNA-binding properties of homodimeric winged helix proteins, the effector binding domains are generally distal to the winged helix domains, and ligand-induced conformational changes are propagated to the winged helix motif.³⁸ Indeed, all characterized prokaryotic multidrug-binding transcriptional regulators other than MarR incorporate a spatial separation of ligand-binding and DNA-binding domains.^{34,39} Even though the MarR family is proposed to share a common evolutionary history with the GntR, MerR, and Crp families,⁴⁰ MarR proteins appear to have an interesting structural organization, such that the ligand-binding and winged helix DNA-binding domains overlap almost completely.

The MexR structure reveals an “open” dimer conformation in which basic charge repulsions between the two DNA-binding domains maintain the dimer in a conformation that can be docked onto the known MexR DNA-binding site.¹⁸ Comparison to another MexR dimer suggested a mechanism of allosteric control in which the binding of a potential effector between the two DNA-binding domains neutralizes protein charges and mediates hydrophobic bonds with protein side-chains, resulting in a reduction in the relative distance between the DNA-binding domains to become incompatible with DNA binding.

HucR is strongly electropositive in its proposed DNA-binding region, and uric acid could serve to neutralize charge repulsions that maintain the DNA-binding lobes of the HucR dimer in a DNA-binding conformation. The hydrophobic rings of uric acid could potentiate van der Waals interactions with hydrophobic HucR side-chains, further distorting the relative conformations of the subunits. It is likely that the uric acid-binding site mediating such conformational events corresponds to ligand-binding site SAL-B in MarR. Ligand-binding site SAL-A is located between the DNA recognition helix and the wing region, whereas site SAL-B is located on the other side of the DNA recognition helix; the two SAL-B sites in the homodimer would be positioned in the channel between the DNA-binding lobes (6 Å wide in the MarR structure). Binding of uric acid at such a position could potentially disrupt the relative orientation of the HucR DNA-binding domains. Such a perturbation of the spatial separation of the recognition helices in the homodimer would be dependent upon the flexibility of the dimerization domain suggested by the fluorescence data. Support for our observation that site SAL-A in HucR is a low-affinity, ligand-binding site and our

hypothesis that site SAL-B mediates allosteric events comes from the fact that the residues that comprise ligand-binding site SAL-A, but not SAL-B, are highly conserved in MexR, and MexR does not respond to salicylic acid as an effector.¹⁸ This suggests that SAL-A is a low-affinity site in MarR.

The significant changes in the fluorescence spectrum of HucR that are induced upon binding uric acid and DNA likely correspond to alterations in the environments of both tryptophan residues (though we do not know to what extent each contributes). According to this hypothesis, uric acid would distort the relative positions of the DNA-binding domains in the homodimer, thus altering the environment of tryptophan 72. Such movements of the winged-helix domains would require flexibility of the N-terminal α -helices of each monomer, altering the environment of HucR tryptophan 20. Similarly linked movements would be expected for the HucR dimer to adopt a DNA-binding conformation.

Materials and Methods

Circular dichroism (CD) spectroscopy

Complementary synthetic oligonucleotides were gel-purified and annealed to generate a 31 bp fragment (*hucO-31*) containing the HucR-binding site, spanning the sequence -23 to +8 relative to the putative transcriptional start site of *dr1160*. CD spectroscopy was performed on an AVIV 202 CD spectrophotometer using a quartz cuvette with a 1 cm pathlength. DNA was diluted to a final concentration of 2 μ M in CD buffer (20 mM potassium phosphate (pH 7.0), 50 mM KCl) and titrated with concentrated HucR to a final concentration of 4 μ M monomer. After each titration, samples were equilibrated at 25 °C for 15 minutes before data were collected. Ellipticity measurements were collected at 25 °C from 340 nm to 190 nm in steps of 1 nm. All data were corrected for buffer contributions to the signal, and samples containing HucR were corrected for additional ellipticity contributions from the protein. Experiments were performed in duplicate (with representative data reported from one experiment).

Intrinsic fluorescence measurements

Fluorescence emission spectra from 280 nm to 440 nm were recorded on a Jasco FP-6300 spectrofluorimeter with an excitation wavelength of 280 nm at 25 °C using a 0.5 cm pathlength cuvette. All experiments were performed with 0.03 mg/ml of protein (1.52 μ M wild-type or mutant HucR) in 40 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.1% (w/v) BRIJ 58, 100 mM NaCl, and 10 mM MgCl₂, unless stated otherwise. Parallel absorbance spectra were recorded for each sample from 190–450 nm. For measurements of protein-*hucO-31* complexes, HucR (wild-type or mutant) was titrated with concentrated *hucO-31*, mixed, and equilibrated for 15 minutes before scanning. For the protein-ligand binding assays, stock solutions of uric acid, salicylic acid, and acetylsalicylic acid were prepared as described.¹⁷ Serial ligand dilutions were prepared such that the addition of 0.5 μ l to the protein sample would

attain the desired concentration of ligand. Samples were mixed and incubated for 60 seconds before each scan.

The corrected protein fluorescence intensity at each wavelength ($F_{\text{corr}}(\lambda)$) was obtained from the observed fluorescence by first correcting for dilution from titration and background fluorescence to obtain $F_c(\lambda)$. Inner filter effects were then resolved by the following correction factor:

$$F_{\text{corr}}(\lambda) = F_c(\lambda) \times 10^{(A_{\text{ex}}/2 + A_{\text{em}}/2)}$$

where A_{ex} and A_{em} are the absorbance at the excitation and emission wavelengths, respectively.⁴¹ $F_{\text{corr}}(\lambda)$ is only reported for samples where both A_{ex} and A_{em} are less than 0.2. For ligand-binding assays where A_{ex} exceeded 0.2, normalized fluorescence intensities were obtained by partial correction of the inner filter effect ($F_{\text{pcorr}}(\lambda)$):

$$F_{\text{pcorr}}(\lambda) = F_c(\lambda) \times 10^{(A_{\text{em}}/2)}$$

before being normalized to the maximal F_{pcorr} . For fluorescence measurements involving acetylsalicylic acid, $F_c(\lambda)$ was calculated by incorporating an additional, experimentally determined factor to correct for quenching resulting from the solvent, ethanol.

Percentage quenching (%Q) induced by DNA binding was calculated by:

$$\%Q = 100(1 - (F_{\text{corr}}(338 \text{ nm})\{\text{Protein} + \text{DNA}\} / F_{\text{corr}}(338 \text{ nm})\{\text{Protein}\}))$$

For ligand binding at low concentrations of ligand, quenching at 338 nm (Q_{338}) was calculated by:

$$Q_{338} = 1 - (F_{\text{corr}}[X] / F_{\text{corr}}[0])$$

where $F_{\text{corr}}[X]$ and $F_{\text{corr}}[0]$ are the corrected fluorescence intensities at 338 nm for ligand concentrations $X \mu$ M and 0 μ M, respectively. As a control, no uric acid-induced quenching of lysozyme fluorescence was observed up to 50 μ M uric acid. Uric acid-binding isotherms were generated by non-linear fits to the Hill equation:

$$Q_{338} = \{n(1/K_d)^{n_H}(U)^{n_H}\} / (1 + (1/K_d)^{n_H}(U)^{n_H})$$

where n represents the quenching plateau, U is the concentration of uric acid, K_d is the observed dissociation constant, and n_H is the Hill coefficient. Data are reported as the mean $\pm$ S.D. for two separate experiments.

Sedimentation equilibrium

A 150 μ l HucR sample at $A_{280}=0.65$ was dialyzed overnight at 4 °C against 350 ml of AU buffer (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂). The reference and solution sectors of an analytical cell with a double-sector centerpiece were loaded with 125 μ l of AU buffer and 110 μ l of HucR sample, respectively. Equilibrium analysis was performed at 20 °C at 10,000 rpm using a Beckman Optima XL-A analytical ultracentrifuge equipped with a An-60 Ti rotor. The cell was scanned at 294 nm at five hour intervals with a step size of 0.004 cm until the system reached equilibrium. The partial specific volume of HucR at 20 °C was determined to be 0.7387 cm³/g from the primary amino acid sequence using the program SEDNTERP, and the solution density calculated to be 1.00120 g/cm³. Equilibrium sedimentation data were analyzed using Origin Equilibrium software and fit to an equation describing a single ideal protein species. Fits of the data to models describing a self-associating system resulted in molecular mass

calculations with large error and that were not multiples of the HucR molecular mass.

Cloning, overexpression, and purification of HucR mutants

Primers were designed to introduce the desired amino acid substitutions into the *hucR* coding sequence *via* whole plasmid PCR using the recombinant plasmid pSPW1, which contains the *hucR* coding region as a template.¹⁷ Arginine 118 was converted to alanine using primers R118A-FWD (5'-CTG ATC GAG GCC CGC GAG GAC-3'; nucleotide substitutions yielding R118→A in bold) and R118A-REV (5'-GCC CTT TTC GAG CAG CCG CAC-3'). Serine 104 was converted to alanine using primers S104A-FWD (5'-CCT TCG ACG GCC AAC CGG ATC-3'; nucleotide substitutions yielding S104→A in bold) and S104A-REV (5'-CCC GGA AAT GGC GGC CAG G-3'). PCR products were treated with DpnI and transformed into *E. coli* TOP10 (Invitrogen). The fidelity of the recovered plasmids, identified as pR118A and pS104A, was verified by DNA sequencing. Plasmids pR118A and pS104A were transformed individually into *E. coli* BL21(DE3)pLysS. HucR mutants were expressed, and cells lysed essentially as described for wild-type HucR, with the exception that protein expression was induced with 0.8 mM isopropyl-1-thio- β -D-galactopyranoside for 75 minutes.¹⁷ Cell lysates were precipitated on ice with ammonium sulfate to 60% saturation and the resulting pellets resuspended in 100 ml of the appropriate buffer: for HucR-R118A purification, HA buffer (20 mM Tris-HCl (pH 7.5), 50 mM KCl, 4.8% (v/v) glycerol, 1 mM Na₂EDTA, 4.3 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride) and for HucR-S104A purification, HAPT buffer (20 mM potassium phosphate (pH 6.0), 50 mM KCl, 10% (v/v) glycerol, 1 mM Na₂EDTA, 0.02% (v/v) Tween-20, 4.3 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride) and subsequently dialyzed overnight against 40 volumes of the respective buffer (this and all subsequent purification steps were performed at 4 °C). Each dialysate was subsequently centrifuged at 11,000 g for 20 minutes and the supernatants processed as described below.

For HucR-R118A purification, the supernatant was loaded onto CM-cellulose and DEAE-Sepharose columns linked in tandem and equilibrated with HA buffer (pH 7.5). The HucR-R118A-containing flow-through and wash fractions were dialyzed against HAP buffer (pH 6.0) (same as HA buffer, except that 20 mM potassium phosphate (pH 6.0) replaces Tris-HCl) and loaded onto a CM-cellulose column equilibrated with HAP buffer (pH 6.0). The HucR-R118A-containing flow-through was loaded onto a hydroxyapatite column equilibrated with HAP buffer (pH 6.0). The HucR-R118A-containing flow-through and wash fractions were loaded onto a heparin column equilibrated with HAP buffer (pH 6.0). HucR-R118A was eluted with a linear gradient of 50 mM–1 M KCl in HAP buffer (pH 6.0). Peak fractions were pooled, dialyzed against HAP buffer (pH 6.0), and loaded onto a Cibacron Blue 3GA column equilibrated with HAP buffer (pH 6.0). The column was eluted with a linear gradient of 50 mM–1 M KCl and peak fractions pooled. HucR-R118A was concentrated, and the concentration of KCl reduced to ~50 mM, using a Centrprep centrifugal filter device. The concentration of glycerol was subsequently increased to 20%.

For HucR-S104A purification, the supernatant was loaded onto a CM-cellulose column equilibrated with HAPT buffer (pH 6.0). The HucR-S104A-containing

flow-through and wash fractions were loaded onto a heparin column equilibrated with HAPT buffer (pH 6.0). The HucR-S104A-containing wash was loaded onto a Cibacron Blue 3GA column equilibrated with HAPT buffer (pH 6.0). The column was eluted with a linear gradient of 50 mM–1 M KCl and peak fractions pooled. HucR-S104A was concentrated and the concentration of KCl reduced to ~50 mM. The concentration of glycerol was subsequently increased to 20%.

Each mutant was purified to greater than 95% homogeneity, as determined by SDS-PAGE (Supplementary Data, Figure S2). HucR-R118A and HucR-S104A concentrations were determined spectrophotometrically using $\epsilon_{280} = 13,512 \text{ M}^{-1} \text{ cm}^{-1}$ and verified by SDS-PAGE using bovine serum albumin (BSA) as a standard.

Electrophoretic mobility-shift assay (EMSA)

The DNA used for protein–DNA affinity measurements was prepared by PCR amplification from the *D. radiodurans* genome, as described, and spans a 180 bp sequence containing a single HucR binding site.¹⁷ The resulting PCR product, *hucO*, was gel-purified and ³²P-labeled with phage T4 polynucleotide kinase. EMSA was performed as described for the wild-type protein.¹⁷ For HucR-R118A and HucR-S104A DNA-affinity determination, 0.1 nM or 0.01 nM ³²P-labeled *hucO*, respectively, was titrated with up to 20 nM or 15 nM protein dimer. The exponential decay constant for each complex dissociation in the gel was calculated and fractional saturation corrected, as described.⁴² Each binding isotherm was generated by a non-linear fit of three data sets to the equation:

$$\text{Fractional saturation of } hucO = (n(P)/K_d)/(1 + (P/K_d))$$

where n represents maximal fractional saturation, P is the concentration of free protein dimer, and K_d is the apparent equilibrium dissociation constant. Each K_d is reported as the mean $\pm$ S.D. Calculated values of n from the HucR-S104A and HucR-R118A binding isotherms were 0.9 ± 0.0 and 1.0 ± 0.0 , respectively. Measured complex was identical for binding incubation times of 45 seconds to 60 minutes, indicating that thermodynamic equilibrium is reached quickly (data not shown). The DNA-binding stoichiometry of HucR mutants was determined by titrating 0.1 μ M labeled *hucO* with up to 0.5 μ M protein monomer. Binding conditions for the competition assays were as described above, except that a slightly larger, gel-purified PCR product of 241 bp was used (containing a single HucR binding site). Each binding reaction contained 0.1 nM ³²P-labeled *hucO* titrated with up to 15 nM unlabeled *hucO* or pGEM5. Protein was added last to the binding reactions, at a final concentration of 2.5 nM HucR-S104A dimer or 10 nM HucR-R118A dimer. Protein–*hucO* binding conditions for HucR-S104A and HucR-R118A, analyzing uric acid as an effector, were as described for wild-type HucR, except that protein dimer concentrations were 3.75 nM and 12.5 nM for HucR-S104A and HucR-R118A, respectively. Data were fit to an equation for two single-exponential decays:

Normalized fractional complex formation

$$= (n_1 e^{-jL}) + (n_2 e^{-kL})$$

where L is the concentration of uric acid, n_1 and n_2 are fractional complex amplitudes, and j and k are decay constants. Fits to a single exponential resulted in lower R^2 values relative to fits to a double exponential (e.g. for HucR-S104A, $R^2 = 0.8390$ and 0.9984 , respectively).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2005.05.027](https://doi.org/10.1016/j.jmb.2005.05.027)

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