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Site-directed mutagenesis of the ferric uptake regulation gene of *Escherichia coli*

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The 12 histidine and four cysteine residues of the Fur repressor of *Escherichia coli* were changed, respectively, to leucine and serine by site-directed mutagenesis of the *fur* gene. The affects of these mutations were measured *in vivo* by ligation of the mutated genes to a wild-type *fur* promoter followed by measurement of the ability of these plasmids to regulate expression of a *lacZ* fusion in the aerobactin operon. *In vitro* affects were assayed by insertion of the mutated genes in the expression vector pMON2064 attended by isolation of the altered Fur proteins and appraisal of their capacity to bind to operator DNA. The results suggest that cysteine residues at positions 92 and 95 are important for the activity of the Fur protein.

Keywords: iron, mutagenesis, repressor

Introduction

The ferric uptake regulation (Fur) protein is the major repressor of various iron uptake systems in *Escherichia coli*. These include the ferrous uptake system and high affinity uptake systems, which employ virtually ferric-specific chelators called siderophores such as aerobactin and enterobactin (Hantke 1987a, Bagg & Neilands 1987b). Fur is also involved in the repression of transcription of several bacterial toxins, and in the regulation of a wide variety of genes coding for functions, such as superoxide dismutase and succinate utilization, which seem to be loosely related to respiration by a mechanism which is unclear at this time (Bagg & Neilands 1987b, Neilands 1990). Several other bacteria, such as species of *Vibrio*, *Yersinia* and *Pseudomonas*, also appear to employ analogs of the Fur protein to perform iron-mediated repression of gene expression (Staggs & Perry 1991, 1992, Litwin *et al.* 1992).

A C-terminal, 76 amino acid tryptic core of Fur, as well as the intact protein, binds two metal ions per monomer, although we cannot discount the possibility that the N-terminal domain contributes ligands to the binding sites (Coy & Neilands 1991). Biochemical analysis indicates that activation occurs by two metal ions per dimer binding to the C-terminal domain, which induces a conformational change in the N-terminal domain and allows operator binding (Coy 1993). This suggests that only one of the two metal binding sites per monomer is actually involved in activation.

Iron chemistry in aqueous solutions is dominated by the divalent ferrous and trivalent ferric oxidation states (Crichton 1991). Based on the low solubility of ferric iron, as well as the kinetic and thermodynamic properties of ferrous iron complexes, Williams proposed that the divalent form of iron would be involved in regulation of gene expression (Williams 1982). This hypothesis was confirmed by the finding that metals like Mn(II) and Co(II), which should remain divalent at neutral pH in their uncomplexed forms, are able to repress transcription of Fur-regulated genes *in vivo* (Bagg & Neilands 1987a). Furthermore, all the first row divalent transition ions, including ferrous, but not ferric iron are able to activate this repressor (as assessed by DNase I footprinting analysis) *in vitro* (de Lorenzo *et al.* 1987). Using protease sensitivity as a measure of activation, one finds that Ga(III) (an analog for ferric iron) neither activates Fur or inhibits activation by other divalent first row transition metals, indicating that the activating binding site is specific for ferrous iron (Coy 1993).

Since the activating metal binding site appears to be specific for the ferrous form of iron, predictions can be made regarding the possible ligands involved. Ferrous iron is a relatively soft metal ion, while ferric iron is relatively hard. Based on the rule that hard acids are specific for hard bases and soft acids are specific for soft bases (Glusker 1991), one would predict that the binding site employs soft bases as ligands. Of the ligands proteins use

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to bind metal ions, relatively soft bases such as histidine and cysteine would be better suited to select ferrous over ferric iron rather than harder ligands such as carboxylate, carbonyl or tyrosine ligands. The primary structure of Fur suggests that both of the former residues might be involved in metal binding. The repressor protein has 12 histidine residues, a relatively high number for a 147 amino acid protein. Of the four cysteine residues in Fur, two are found in a CysXYCysGly motif that is commonly used as a ligand by iron–sulfur proteins (Bagg & Neilands 1987b). Based on these facts, we explored the role of these potential ligands in Fur by using site-directed mutagenesis to change each of the histidines to leucine (which occupies roughly the same volume as histidine) and each of the cysteines to serine.

Materials and methods

Molecular genetics

Standard DNA manipulations and cloning procedures were performed as described in Sambrook *et al.* (1989) and Perbal (1988). For site-specific mutagenesis, the *fur* gene from pMON2064 was isolated by digestion with *Eco*RI and *Nco*I (see Figure 1) and cloned into pTZ18U (Mead *et al.* 1986). Mutagenesis was performed using the

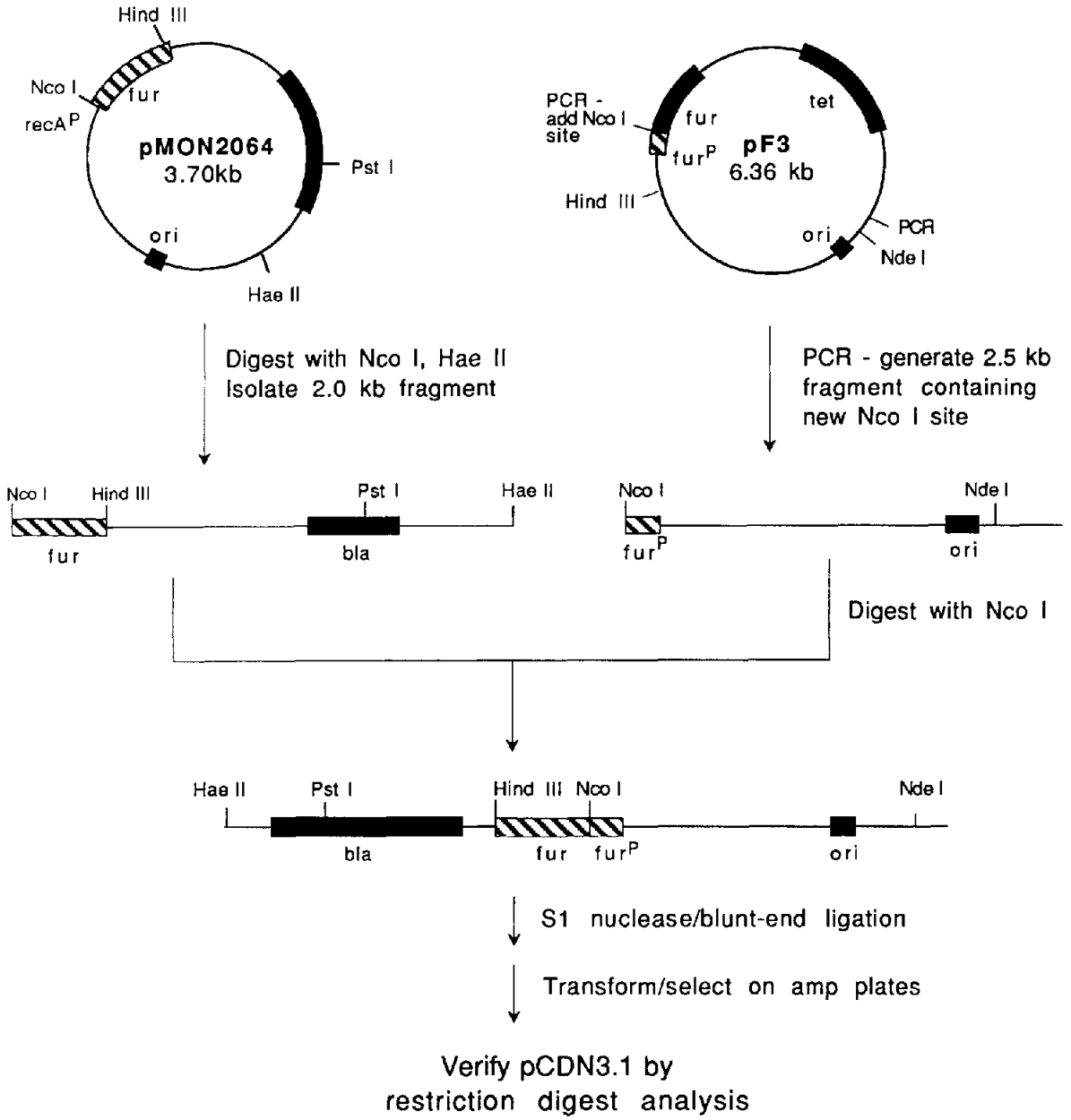

Figure 1. Construction of pCDN3.1. bla, ampicillin b-lactamase; fur^P, *fur* promoter; ori, origin of replication, recA^P, *recA* promoter, tet, tetracycline resistance.

method of Kunkel *et al.* (1987), as modified by McClary *et al.* (1989). Oligonucleotides were obtained from the DNA Synthesis Facility of the Department of Molecular and Cell Biology, UC Berkeley. Mutations were detected by sequencing (Sanger *et al.* 1977) and the entire mutant *fur* genes were then sequenced in order to confirm the absence of second-site mutations. Mutant genes were than cloned back in to the expression vector pMON2064 from which wild-type *fur* and been excised.

Plasmid pCDN3.1 was constructed in order to readily insert mutant *fur* genes under the control of the *fur* promoter (see Figure 1). pMON2064 (Wee *et al.* 1988) (which contains *fur* under control of the *recA* promoter) was digested with *Nco*I and *Hae*II, and the resulting 2.0 kb band, which contains the *fur* gene, was isolated from an agarose gel. The polymerase chain reaction was used to amplify a 2.5 kb fragment from plasmid pF3 (Bagg & Neilands 1987b), which contained the *fur* promoter, flanked by a 3′ *Nde*I site and a 5′ *Nco*I site. The two fragments were ligated, treated with S1 nuclease and the recombinant plasmid was recircularized by blunt end ligation. After transformation of JM109, the desired clone, which contains the *fur* gene under control of the *fur* promoter, was identified by restriction analysis, and named pCDN3.1. Mutant *fur* genes could be inserted by isolation from the pMON2064 constructs and ligation into isolated pCDN3.1 vector band which can be liberated by digestion with *Eco*RI and *Nco*I.

In vitro *characterization of mutant proteins*

Protein was isolated by the method of Wee *et al.* (1988) and stored at 4 °C as an 80% $(NH_4)_2SO_4$ precipitate. Protein concentration was determined by the method of Bradford (1976) and purity assessed by SDS–PAGE (Laemmli 1970). DNA gel shift assays and DNase I footprinting were performed essentially as described in de Lorenzo *et al.* (1988), using the aerobactin operator-containing fragment from p6g and the *fur* operator-containing fragment from p100a (Coy & Neilands 1991), respectively, as probes. Metal binding by equilibrium dialysis, cadmium-induced charge-transfer spectra and protease sensitivity were measured as described previously (Coy & Neilands 1991).

In vivo *characterization of mutants*

In vivo activity of the *fur* mutants was assessed by measuring their ability to repress transcription of an aerobactin operon–*lacZ* fusion. Derivatives of *fur* in pCDN3.1 were used to transform BN4023, a *fur* mutant that carries an *iucC::lacZ* fusion. Cells were grown overnight in M9 (Miller 1972) plus 0.04% casamino acids in the presence of either 100 μM $FeCl_3$ or 200 μM bipyridyl and assayed for β-galactosidase activity as described previously (Miller 1972). Sensitivity to chelators was measured in a similar manner, except that cells were grown in LB containing varying bipyridyl concentrations for 6–8 h. Manganese resistance was assayed as described previously (Hantke 1987b).

Results

We were successfully able to purify all the mutant proteins on zinc-iminodiacetic acid agarose, suggesting that they had a general structure which is similar to intact Fur and that no single histidine or cysteine residue is necessary for binding to this metal ion affinity resin. Table 1 summarizes the activities of the mutants determined both *in vitro* and *in vivo*. There is good agreement between regulation of the aerobactin operon and manganese sensitivity for the various mutants, as would be expected since it has been previously hypothesized that manganese resistance arises by escape from manganese-induced iron starvation caused by the repressor activity of the Fur protein (Hantke 1987b). Manganese-induced inhibition of growth was relieved when bacteria were grown anaerobically using nitrate as a terminal electron acceptor, but this could be due to either a reduced need for iron and use of ferrous iron or to oxygen toxicity. Surprisingly, relatively few potential ligands were found to be necessary for activity. We will consider below the properties of mutants which behaved differently from wild-type Fur.

H32L was unique among the mutants in that there was substantial disagreement between the results obtained *in vivo* and *in vitro*. *In vivo*, this protein is inactive based on both tests used, while *in vitro* the protein proved indistinguishable from wild-type. In the gel shift assay, saturating concentrations of manganese are used, which could

Table 1.

Strain	β-Galactisidase units			Mn(II) resistance[a]	$R_{1/2}^{rel}$[b]
	+iron	+bipyridyl	bipyridyl/iron		
Wild-type	66	1502	22.8	–	1
H31L	104	1594	15.3	–	1
H32L	354	1696	4.8	+	1
H70L	58	1687	29.1	–	1
H85L	64	1251	19.5		1
H86L	68	1343	19.8	–	1
H87L	60	1566	26.1	–	1
H89L	101	1458	14.4	–	1
C92S	796	2254	2.8	+	58
C95S	306	2217	7.2	+	116
H117L	59	1426	24.2	–	1 (14)
H124L	58	1416	24.4	–	1
H131L	79	1329	16.8	+/–	1
C132S	50	1132	22.6	–	1
C137S	75	1171	15.6	–	1
H142L	80	1343	16.8	–	1
H144L	39	1277	32.8	–	1
fur	1727	2692	1.6	+	/

[a] + indicates growth in the presence of 10 mM $MnCl_2$, – indicates no detectable growth in the presence of manganese. +/– indicates barely detectable growth.

[b] We define $R_{1/2}^{rel}$ as the ratio of the concentration of mutant repressor required to bind 50% of the DNA in the gel shift assay and the same value for the wild-type protein. When 1 is reported, the value was visually estimated. Other values were obtained by Hill plot analysis of quantitations performed by densitometry, as described previously (Coy 1993).

potentially mask differences that appear because of the possibly lower metal ion concentrations found in the cell. Since H32L binds DNA with the same affinity as wild-type protein, it was relatively straight forward to perform a Mn(II) titration with the DNase I footprinting assay as was described previously (Coy & Neilands 1991). This experiment revealed that H32L has a metal ion sensitivity which is indistinguishable from wild-type Fur. This result is supported by the finding that equilibrium dialysis shows that H32L binds cadmium ions with a stoichiometry which is the same as that of wild-type Fur. Finally, H32L has cadmium-induced charge transfer spectra which are virtually indistinguishable from the wild-type protein.

Mutant H117L also seems to behave in a manner which is distinguishable from wild-type Fur. At least one preparation of the protein was considerably less active than wild-type in the gel shift assay, as indicated by the bracketed $R_{1/2}^{rel}$ value shown in Table 1. However, other preparations behaved the same as wild-type Fur. Using the footprinting Mn(II) titration, this mutant seemed to bind to metal ions in the activating site with identical affinity as wild-type Fur. Furthermore, there was no readily apparent difference when this protein was tested for metal ion binding by equilibrium dialysis. Finally, as shown in Table 1, H117L seemed to be able to repress expression of the aerobactin operon normally. These data suggest that this protein might be susceptible to denaturization *in vitro*. The instability of the repressor is confirmed by the susceptibility of its cysteine thiol groups to oxidation. If wild-type Fur is dialyzed against buffer without dithiothreitol (DTT), then one can measure 3.5–4 thiol groups per monomer using dithiodinitrobenzioc acid, as described previously (del Cardayre & Neilands 1991). However, H117L loses two of its four thiol groups when treated in the same manner.

Cysteines 92 and 95 both appear to be essential for activity both *in vivo* and *in vitro*. As is shown in Table 1, both proteins are seriously impaired in their ability to carry out repression *in vivo* and to bind to DNA in the gel shift assay, although the effect of changing cysteine 92 seems to be in both cases somewhat more severe than the effect of changing cysteine 95. The double mutant C92S,C95S has properties which are similar to C92S. It is interesting to compare the effects of these mutations on the *in vivo* and *in vitro* activities of the proteins. *In vivo*, the switch from cysteine to serine seems to depress the activity of the proteins from 4- to 10-fold, based on the ratio of activities in the presence of bipyridyl and iron. *In vitro*, the mutations seem to lower activity of the proteins about 100-fold, based on the $R_{1/2}^{rel}$ values. This suggests that the structure of these proteins is destabilized such that there is a loss in activity during the purification process.

That these proteins have a destabilized structure is confirmed by the fact that purified protein preparations derived from these mutants exhibit two bands of molecular weights of 17.2 and 15.5 kDa in SDS–PAGE. This differs from wild-type Fur and most of the other mutants, which exhibit a single band of 17.2 kDa apparent molecular weight. The only exceptions are C132S and C135S, which exhibit much smaller amounts of the 15.5 kDa band. The amount of 15.5 kDa band is variable with respect to preparation, with some samples having virtually undetectable amounts while in others it comprises about 50% of the total protein present and seems to increase if the protein is stored at 4 °C. This suggests that the 15.5 kDa band is a degradation product of intact Fur. This hypothesis was proven by mapping the location of degradation to the C-terminus of Fur with CNBr using the method described previously (Coy & Neilands 1991). The instability of these mutants is confirmed in proteolysis experiments, which indicate that they are about 10-fold more sensitive to trypsin digestion than wild-type Fur. Both mutants are also susceptible to cysteine oxidation since, after dialysis in buffer which lacks DTT, one can only detect about 0.1 thiol group per monomer, indicating that the three remaining cysteines had been nearly completely oxidized.

Using equilibrium dialysis, one can see that these mutations also affect the ability of Fur to bind to metal ions. With Mn(II), both C92S and C95S bind 0.8 metal ions per monomer while intact Fur binds 1.5 metal ions per monomer in this experiment. This would suggest that the mutations knock out metal binding by one of the two sites per monomer. Less convincing results were obtained with Cd(II) though, since while C92S was only able to bind one metal ion, C95S binds about 1.6 metals per monomer. One danger in the interpretation of these experiments is that it is possible that one is detecting the effects of protein inactivation *in vitro*, as was outlined above. To obtain results about the affinity of this type of mutant for metal ions *in vivo*, BN4023 harboring pCDN3.1 with either wild-type or C95S Fur were grown on Luria broth (an iron-rich medium) in the presence of varying concentrations of the chelator bipyridyl and the activity of the *iucC::lacZ* fusion assayed as shown in Figure 2. C95S was selected because it has a less severe phenotype than C92S, thus improving the repression signal. As can be seen, C95S

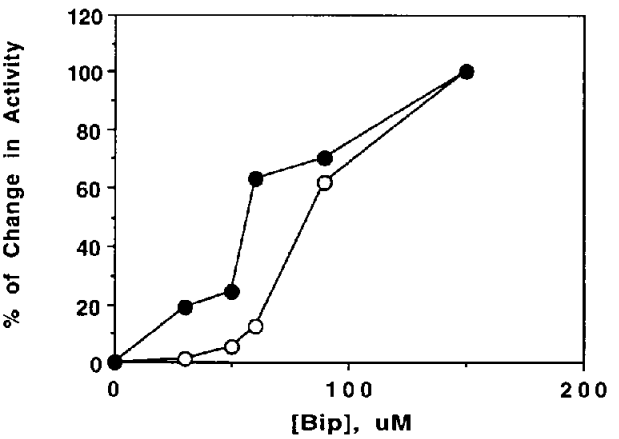

Figure 2. *In vivo* bipyridyl sensitivity of wild-type and C95S Fur proteins. To normalize the data and allow easy comparison, β-galactosidase activity for wild-type Fur (○) and C95S are reported as the percentage of the total change in activity observed between 0 and 150 μM bipyridyl.

is considerably more sensitive to bipyridyl than wild-type Fur, suggesting that its activating metal ion binding site has lower affinity for iron than wild-type Fur. C95S is also less sensitive to iron concentrations than wild-type Fur, as demonstrated by performing the same experiment in M9 (a low iron medium) supplemented with varying concentrations of iron. For example, at 1 μM ferric ammonium citrate, the *iucC::lacZ* fusion is repressed to 95% of the total observed with wild-type Fur while it is repressed only 80% of the total with C95S. Both data indicate that the activating site of C95S binds ferrous iron less tightly than that of wild-type Fur.

Discussion

Although both histidine and cysteine are likely potential ligands for a ferrous-specific protein like Fur, two factors make the former amino acid a particularly attractive candidate. Nitrogen ligands tend to bind metal ions weaker than thiol-containing ligands (Hay 1984) and would thus be more appropriate for a metal activated protein like Fur which should and does bind to ferrous iron with relatively low affinity. Furthermore, there are 12 histidines in Fur, with 10 of them located in the C-terminal 76 amino acid domain which is necessary and sufficient for metal binding. Proteins typically contain only three percent histidine. It is thus surprising that this study has generated no convincing evidence to indicate that any of the histidines are involved in metal binding. The results with H32L are intriguing since it seems to be active *in vitro* and inactive *in vivo*, but metal titrations indicate that this residue is probably not involved in metal binding. It is possible that this mutation somehow allows the protein to be inactivated *in vivo*, although it is curious that there is no evidence for destabilization of the structure in the purified protein. In this regard it is worth noting that mutagenic analysis of the lambda repressor indicates that some mutations which destabilize the protein *in vivo* do not lead to structural thermodynamic instability (Parsell *et al.* 1990). Perhaps H32L renders the protein susceptible to either binding to heat shock proteins or to digestion by specific intracellular proteases (SDS–PAGE analysis suggests that the former is more likely, data not shown) and this system is either overloaded upon induction or somehow inactivated by nalidixic acid, leading to normal yields when the protein is purified. The fact that H117L is sometimes isolated in an inactive form also indicates that it is a potential ligand, although both metal titrations and the *in vivo* activity of the protein argue against this, and oxidative susceptibility indicate that it might simply slightly perturb the structure of Fur. It is perhaps significant that it is the histidine closest to the two essential cysteines of Fur, which might render this position less tolerant to amino acid changes.

What explanation can we offer to account for the lack of apparent implication of histidines in metal binding? One explanation is that we have simply missed the involvement of histidine. Some changes in ligands in proteins can result in a less than order of magnitude decrease in metal ion binding affinity. Furthermore, site directed mutagenesis experiments with ferredoxin indicate that upon changing one of the metal binding cysteines in the protein to alanine, a nearby cysteine was recruited to take its place, resulting in an active protein (Martin *et al.* 1990). Given the high density of potential ligands in the C-terminal domain of Fur, such an occurrence in this study cannot be excluded. Finally, it is possible that the histidine residues in Fur play a role which is unrelated to binding of the activating metal ion, such as acting as sensors for pH changes in the cell (Saito *et al.* 1991), or rendering the repressor responsive to regulation by metal catalyzed oxidation (Stadtman 1992).

Cysteines 92 and 95 are the best potential candidates for ligands of the activating metal ion binding site which have been revealed in this study. Both are clearly impaired in function both *in vivo* and *in vitro*. Equilibrium dialysis provides evidence which suggests that these mutants are impaired in metal binding, but interpretation of these experiments is difficult because of the possibility of denaturization of the proteins *in vitro* and because there are two metal binding sites per Fur monomer, only one of which is believed to be involved in activation. More conclusively the *in vivo* metal ion titration indicates that the C95S mutation seems to reduce the affinity of Fur for activating metal ions. Unfortunately, both mutations also destabilize the structure of Fur as measured by a variety of techniques, which means that, considering these data alone, we cannot exclude the possibility that the effect on metal binding is an indirect one (i.e. by destabilizing structure) rather than a direct one resulting from alteration of the ligands. But it makes sense that since metal binding seems to drive conformational changes in Fur, it might not be possible to alter the ligands without simultaneously altering the general structure.

Cadmium-induced charge-transfer spectra have previously been obtained which suggest that cysteine residues are involved in Cd(II) binding to the activating site of Fur (Coy & Neilands 1991). This makes both the clear effect that altering cysteines 92 and 95 and the lack of effect of altering 132 and 137 very interesting. Combined, the data suggest rather strongly that cysteines 92 and 95 are involved in activating metal binding. The finding that only the two cysteines (which are probably in a single binding site since they are in the CysXYCysGly motif) seem to be involved in metal binding is also in agreement with biochemical data which suggests that only one of the two binding sites per Fur monomer is involved in activation (Coy 1993).

However, spectroscopic evidence also indicates that thiolates are probably not involved in the binding of activators such as copper or iron, despite the fact that competition experiments indicate that these metal ions bind in the same site as Cd(II) (Coy 1993). This suggests that Fur must somehow modulate the reactivity of the thiol groups such that they do not interact with these metals in the manner characteristic of other proteins. This could be achieved through suppression of deprotonization of the cysteine ligands, by either having the metal binding site in

a hydrophobic pocket or by having a negative charge which inhibits formation of negatively-charged thiolate anions. In this regard it is worth considering the particular sequence motif in which the necessary cysteines are found, CysXYCysGly. The homology with other metal binding sites bolsters the hypothesis that these residues are involved in the activating binding site of Fur. This motif is found in numerous iron–sulfur proteins such as rubredoxin, as well as in the HIV *gag* protein and methionyl-tRNA synthetase, where it apparently participates in zinc binding (Fourmy *et al.* 1993). Structural analysis of these proteins suggest that the terminal glycine in this motif is necessary to allow the amide proton of the next amino acid to interact with the S^- in the second cysteine residue in the motif, thus stabilizing the negative charge and altering the redox potential of the iron–sulfur protein (Adman *et al.* 1975). Although the alteration of reactivity of the cysteines in the Fur protein is clearly different (in fact, possibly of the opposite nature), it is conceivable that this terminal glycine is also used in Fur to somehow perform this alteration.

Finally, it is interesting to note that an important regulator of mammalian iron metabolism, the iron-responsive element binding protein, has recently been found to be identical to cytosolic aconitase and apparently uses an [4Fe–4S] iron–sulfur cluster as a metal sensor binding site (Rouault *et al.* 1992). It thus seems that altered iron–sulfur centers are employed in regulation of iron metabolism from bacteria to man.

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