DeVico, A. L., Copeland, T. D., Oroszlan, S., Gallo, R. C., & Sarngadharan, M. G. (1991) *J. Biol. Chem. 266*, 6774-6779.
Dickson, C., Eisenman, H., Fan, E., Hunter, E., & Teich, N. (1982) in *Molecular Biology of Tumor Viruses: RNA Tumor Viruses* (Weiss, R. A., Teich, N. M., Varmus, H. E., & Coffin, J. M., Eds.) pp 513-648, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Englund, P. T., Kelly, R. B., & Kornberg, A. (1989) *J. Biol. Chem. 244*, 3095-3052.
Gerard, G. F. (1981) *Biochemistry 20*, 256-265.
Gerard, G. F., & Grandgenett, D. P. (1975) *J. Virol. 15*, 785-797.
Gerard, G. F., D'Alessio, J. H., Kotewicz, M. L., & Noon, M. C. (1986) *DNA 5*, 271-279.
Grandgenett, D. P., & Rho, H. M. (1975) *J. Virol. 15*, 526-533.
Johnson, M. S., McClure, M. A., Feng, D. F., Gray, J., & Doolittle, R. F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7648-7652.
Keil, B. (1982) *Methods in protein sequence analysis* (Elzinga, M., Ed.) pp 291-304, Humana Press, Clifton, NJ.
Kotewicz, M. L., D'Alessio, J. M., Driftmier, K. M., Blodgett, K. P., & Gerard, G. F. (1985) *Gene 35*, 249-258.
Kotewicz, M. L., Sampson, C. M., D'Alessio, J. M., & Gerard, G. F. (1988) *Nucleic Acids Res. 116*, 265-277.
Lee, Y. M., & Benisek, W. F. (1976) *J. Biol. Chem. 251*, 1553-1560.
Lee, Y. M., & Benisek, W. F. (1978) *Arch. Biochem. Biophys. 188*, 31-36.
Majumdar, C., Stein, Cy A., Cohen, J. S., Broder, S., & Wilson, S. H. (1989) *Biochemistry 28*, 1340-1346.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McClarin, J. A., Frederick, C. A., Wang, B.-C., Greene, P., Boyer, H. W., Grable, J., & Rosenberg, J. M. (1986) *Science 234*, 1526-1541.
Modak, M. J. (1976) *Biochemistry 15*, 3620-3626.
Modak, M. J., & Marcus, S. L. (1977) *J. Virol. 22*, 243-246.
Mohan, P. M., Basu, A., Basu, S., Abraham, K. I., & Modak, M. J. (1988) *Biochemistry 27*, 226-233.
Nanduri, V. B., & Modak, M. J. (1990) *Biochemistry 29*, 5258-5264.
Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., & Steitz, T. A. (1985) *Nature 313*, 762-766.
Rajababu, C., & Axelrod, B. (1978) *Arch. Biochem. Biophys. 188*, 31-36.
Repaske, R., Hartley, J. W., Kavalick, M. F., O'Neill, R. R., & Austin, J. B. (1989) *J. Virol. 63*, 1460-1464.
Roth, M. J., Tanese, N., & Goff, S. P. (1985) *J. Biol. Chem. 260*, 9326-9335.
Shinnick, T. M., Lerner, R. A., & Sutcliff, J. G. (1981) *Nature 293*, 543-548.
Srivastava, A., & Modak, M. J. (1980a) *J. Biol. Chem. 255*, 917-921.
Srivastava, A., & Modak, M. J. (1980b) *J. Biol. Chem. 255*, 2000-2005.
Steczko, J., Walker, D. E., Hermodson, M., & Axelrod, B. (1979) *J. Biol. Chem. 254*, 3254-3258.
Steczko, J., Hermodson, M., Axelrod, B., & Dziembor-Kentzer, E. (1983) *J. Biol. Chem. 258*, 13248-13254.
Stone, K. L., & Williams, K. R. (1986) *J. Chromatogr. 359*, 203-212.
Tanese, N., & Goff, S. P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1777-1781.
Tanese, N., Roth, M., & Goff, S. P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4944-4948.
Thompson, G. W., Ockerman, L. T., & Schreyer, J. M. (1951) *J. Am. Chem. Soc. 73*, 1379-1381.

Structural Dynamics and Functional Domains of the Fur Protein†

Mark Coy and J. B. Neilands*
Department of Molecular and Cellular Biology, University of California, Berkeley, California 94720
Received January 25, 1991; Revised Manuscript Received May 20, 1991

ABSTRACT: Proteolytic enzymes were used to detect metal-induced conformational changes in the ferric uptake regulation (Fur) protein of *Escherichia coli* K12. Metal binding results in enhanced cleavage of the N-terminal region of Fur by trypsin and chymotrypsin. Activation of both trypsinolysis sensitivity and DNA binding have similar metal ion specificity and concentration dependencies, suggesting that the conformational change detected is required for operator DNA binding. Isolation and characterization of biochemically generated fragments of Fur as well as other data indicate that the N-terminal region is necessary for the interaction of the repressor with DNA and that a C-terminal domain is sufficient for binding to metal ions.

Iron is essential for almost all forms of life (Neilands, 1972), but it is also a potentially toxic element because it can catalyze the formation of dangerously reactive hydroxyl radicals, which can damage virtually all cellular constituents (Halliwell, 1988). These two factors make it necessary to tightly regulate the intracellular concentration of iron. In all species studied, this regulation probably occurs at the level of uptake, since no mechanism to excrete iron is known (Neilands, 1990).

In many aerobic and facultative anaerobic microorganisms, iron uptake is accomplished by synthesis of ferric specific chelators called siderophores (Neilands, 1981). Siderophores can retrieve insoluble iron from the environment or the se-

† This work was supported in part by Grants AIO4156, CRCR-1-1633, and ES07075 from the NIH, USDA, and NIEHS, respectively.
* Corresponding author.

0006-2960/91/0430-8201$02.50/0 © 1991 American Chemical Society

questered element from host tissues (Crosa, 1989). The chelated iron is taken up by a specific transport system (Neilands, 1982). The production of these uptake systems is negatively regulated by iron (Garibaldi & Neilands, 1956). Regulation of several genes involved in iron uptake in *Escherichia coli* occurs at the transcriptional level (Hantke, 1981; Bindereif & Neilands, 1985), and mutants that constituitively express these genes have been isolated. The gene that controls production of iron-uptake systems is called *fur* (ferric uptake regulation), and the *E. coli* K12 version has been mapped (Bagg & Neilands, 1985), cloned (Hantke, 1984), and sequenced (Schaffer et al., 1985). Fur acts as a classical repressor, blocking transcription in the presence of Fe(II) or other divalent first row transition elements, although ferrous ion is probably the main metal available to the repressor in the cell (Bagg & Neilands, 1987a). The use of relatively redox-inert elements such as Mn(II) greatly facilitates biochemical studies of Fur since the protein is inactivated by iron in aerobic conditions. Although the *fur* gene product is the dominant iron regulatory element in *E. coli*, there are probably Fur-independent means of regulation of gene expression (de Lorenzo et al., 1988b). The *fur* mutation is highly pleiotropic, causing, for example, defects in dicarboxylic acid transport and reductions in the level of succinate dehydrogenase (Neilands, 1990), and the *fur* gene is also involved in a complex circuit that regulates expression of superoxide dismutase (Niederhoffer et al., 1990; B. Tardat and D. Touati, unpublished results).

Our laboratory has purified the Fur protein (Wee et al., 1988) and has characterized its DNA-binding properties (de Lorenzo et al., 1987, 1988a,b). Some information about the three-dimensional structure of Fur has been obtained by NMR (Saito and Williams, unpublished data). Previous research has not examined the structure–function relationships of the Fur protein or the mechanism by which metal activates the ability of the repressor to bind with high affinity to operator DNA.

Fur is a unique and interesting protein for several reasons. It is the only repressor characterized to date that is responsive to the concentration of a nutritious metal ion. It also lacks significant sequence homology to any known DNA-binding protein (Bagg & Neilands, 1987b). Because of this, understanding the mechanism of DNA sequence recognition and ligand activation of Fur will provide valuable information about repressor action in general.

In this paper, we describe an analysis of the Fur protein using proteases as a probe of protein conformation. This study has revealed that, upon metal binding, Fur undergoes a structural transition in which the N-terminal part of the protein becomes protease sensitive. Such a change could be caused by the N-terminal region moving away from the main body of the protein or adopting a more extended structure. In the CRP and Trp repressors, changes analogous to these are observed upon ligand binding in the domain of the protein that interacts with DNA (Heyduk & Lee, 1989; Lawson et al., 1988). Evidence will then be presented that supports the following model of the functional organization and mechanism of metal ion activation of Fur: metal is bound to a C-terminal domain of the protein, and this induces a conformational change in the N-terminal region that allows it to bind to operator DNA.

Materials and Methods

Materials

CTNE,[1] TAME, and TPCK-trypsin were obtained from U. S. Biochemicals. α-Chymotrypsin (type 1–5), CNBr, Reactive Blue 2-agarose, and gel filtration molecular weight markers were obtained from Sigma Chemical Co. Centriprep 10 ultrafiltration devices were purchased from Amicon. SDS–PAGE molecular weight markers (2512–16 949 Da) were obtained from Hoefer Scientific Instruments. DEAE-Sephadex A-50 was purchased from Pharmacia. Spectra/Por 1, 3, and 6 dialysis tubes were obtained from Spectrum Medical Industries. Immobilon-P PVDF transfer membranes were purchased from Millipore. Bio-Gel P100 (fine) gel filtration media was purchased from Bio-Rad. Molecular biology enzymes were obtained from Boehringer Mannheim Biochemicals. Iminodiacetic acid agarose was obtained from Pierce Chemical Co. [α-^{32}P]dATP (6000 Ci/mmol) was purchased from Amersham. All other chemicals were of the highest quality available.

Methods

Partial Proteolysis Experiments. Fur protein was purified as described previously (Wee et al., 1988) with minor modifications. Protein concentrations were determined by UV analysis (Wee et al., 1988) or by the Bradford assay (Bradford, 1976). Molar concentrations refer to the Fur monomer. Proteolysis reactions were done in 10 mM BisTris-HCl (pH 7.0), 40 mM KCl, and 5% (w/v) glycerol (protease buffer), typically with 0.25 mg/mL Fur and either 12.5 μg/mL trypsin or 20 μg/mL chymotrypsin. Protease reactions were stopped by addition of one-half volume of 2 M urea, 3.3% SDS, 2% BME, and 100 mM Tris-HCl (pH 7.5) and boiling for 2 min (Mattick et al., 1983) and were analyzed by SDS–PAGE (Francis, 1984) or by SDS–urea gel electrophoresis (Swank, 1980). Quantitation of gels was done with a Kratos densitometer and a Fur standard curve.

Protease Activity Measurements. To measure the effect of metal or DNA on the activity of proteases, the rate of esterolysis by these enzymes was measured spectrophotometrically in the presence of the additives. Trypsin activity was measured with TAME in protease buffer, as described previously (Walsh, 1970). Chymotrypsin activity was assayed with CTNE in protease buffer, as described previously (Walsh & Wilcox, 1970). The intrinsic acceleration of proteolysis was obtained by dividing the rate of Fur trypsinolysis by the amount of acceleration observed with trypsin esterolysis in the presence of the additive.

Protein Microsequence Analysis. For sequencing, protease reactions (performed essentially as above) were stopped by adding one-half volume of 3% SDS, 33% glycerol, 0.15 M Tris-HCl (pH 6.8), and 10 mM BME and boiling the sample for 2 min. Approximately 10–25 μg of the digests was resolved on 17.5% acrylamide gels (as described above) with 0.1 mM thioglycolic acid in the cathode buffer to prevent protein derivitization (Hunkapiller et al., 1983). Proteins were transferred to Immobilon-P transfer membranes according to the method of Matsudaira (1987). Bands of interest were excised and analyzed by gas-phase sequencing at the Biomo-

[1] Abbreviations: aa, amino acid; BisTris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; BME, β-mercaptoethanol; CNBr, cyanogen bromide; CRP, catabolite repressor protein; CTNE, *N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester; dATP, 2′-deoxyadenosine 5′-triphosphate; DEAE, diethylaminoethyl; DNA, deoxyribonucleic acid; DNase I, deoxyribonuclease I; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IDA, iminodiacetic acid; LacR, *lac* repressor; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAME, *p*-tosyl-L-arginine methyl ester; TPCK, L-1-tosylamino-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; TrpR, *trp* repressor; UV, ultraviolet.

lecular Resource Center, University of California, San Francisco.

CNBr Mapping. Fur (3 mg) was digested with chymotrypsin as above in the presence or absence of 200 μM $MnCl_2$, and the reaction was stopped by the addition of 2 mM PMSF. The samples were dialyzed against 50 mM NH_4HCO_3 and 1 mM PMSF, lyophilized, dissolved in 70% formic acid, and cut with CNBr [2 times the weight of protein, as in Fontana et al. (1986)]. Samples were then lyophilized, dissolved and dialyzed in ion-exchange buffer (4 M urea, 20 mM Tris-HCl pH 8.0, 2 mM DTT, 10 mM methylamine, and 1 mM EDTA), and analyzed by SDS-urea gel electrophoresis.

Purification of FurN and FurC. All column chromatography was carried out at 4 °C. Large-scale (80–100 mg) CNBr digests of Fur were performed as above. After dialysis in ion-exchange buffer, the sample was applied to a DEAE-Sephadex column (1.7 × 20 cm), equilibrated in the same buffer. The column was washed with ion-exchange buffer (20 mL/h) to elute FurN and then with ion-exchange buffer plus 0.2 M KCl to elute FurC and intact Fur. FurN was further purified by another passage over a fresh DEAE column, followed by dialysis against 50 mM NH_4HCO_3 and by lyophilization. FurN was then dissolved in MOPS buffer [20 mM MOPS (pH 7.0), 1 mM DTT], dialyzed against the same buffer, and stored at −20 °C. The mixture of Fur and FurC was concentrated by using a Centriprep 10 spin concentrator and renatured by dialysis against MOPS buffer. Renatured FurC and Fur were then purified from denatured proteins lacking metal-binding activity by chromatography on a small Zn(II)-IDA-agarose column (1.7 × 6.8 cm) as described previously (Wee et al., 1988). After dialysis in MOPS buffer, the histidine eluate, containing renatured FurC and Fur, was applied to a Reactive Blue 2-agarose column (1.7 × 6.5 cm) equilibrated with the same buffer. FurC was eluted from the column with a MOPS buffer wash, and intact Fur was eluted with MOPS buffer plus 0.5 M KCl. FurC was concentrated and stored at 4 °C. About 5 mg of FurN and 30 mg of FurC were typically obtained from 80 mg of Fur.

Purification of Fur′ and FurT. A 15-mg sample of Fur was cut with trypsin to give a mixture of Fur′ and FurT, and digestion was terminated by adding 2 mM PMSF. The digest was desalted with a Centriprep 10 spin concentrator and applied to a Reactive Blue 2-agarose column, as described above, with 1 mM PMSF present in all buffers. The FurT fraction was recovered in the MOPS buffer wash, and Fur′ was eluted from the column with MOPS buffer plus 0.5 M KCl. Both fractions were dialyzed against MOPS buffer containing 1 mM PMSF, concentrated with a Centriprep 10 spin concentrator, and stored at −20 °C. About 4 mg of Fur′ and 2 mg of FurT was isolated by this procedure.

Gel Filtration. Gel filtration experiments were performed with a Bio-Gel P100 column (1 × 60 cm, 10 mL/h flow rate) in MOPS buffer plus 0.15 M KCl. Blue Dextran (void volume), horse heart cytochrome *c* (12.4 kDa), bovine erythrocyte carbonic anhydrase (29.0 kDa), and hen egg albumin (45.0 kDa) were used as standards.

Equilibrium Dialysis. Samples of protein (3 mL of 30 μM) in dialysis bags were equilibrated 14 h with 150-mL portions of either MOPS buffer or 20 mM MOPS and 0.15 M NaCl (pH 7.0), containing varying concentrations of $MnCl_2$ or $CdCl_2$, at 4 °C. Metal ion concentrations inside and outside the bag were determined with a Perkin-Elmer model 372 atomic absorption spectrophotometer in the flame mode.

DNA-Binding Assays. General molecular biology techniques were taken from Maniatis et al. (1982) or Perbal (1988). To facilitate DNA binding studies, the 250 bp *Eco*R1/*Dra*I *fur* gene operator-containing fragment from pVLN100 (de Lorenzo et al., 1988a) and the 250 bp *Eco*R1/*Pvu*II aerobactin operator-containing fragment from pCON6 (de Lorenzo et al., 1988b) were cloned into *Eco*R1/*Sma*I cut pTZ18R to give p100a and p6g, respectively. Electrophoretic mobility shift assays were performed as in de Lorenzo et al. (1988b), with 60 mM BisTris-borate and 100 μM $MnCl_2$ (pH 7.5) in the gel and tray buffers. Probe DNA was prepared by fill-in labeling of *Eco*R1/*Hin*dIII cut p6g or p100a with Klenow enzyme and α-[^{32}P]dATP. DNaseI footprinting was performed as in de Lorenzo et al. (1987), with the omission of EDTA from all buffers. Labeling was performed by using *Eco*R1 or *Hin*dIII cut plasmid as above; a second digestion was performed to liberate vector from insert, and the insert fragments were recovered from a 5% polyacrylamide gel.

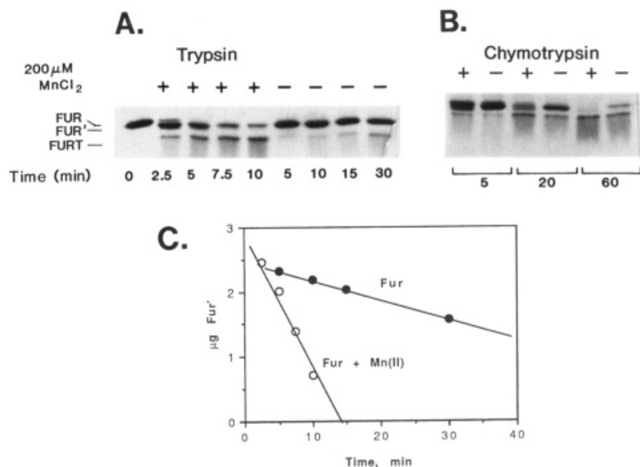

FIGURE 1: Effect of $MnCl_2$ on the kinetics of Fur proteolysis. Fur was cut with trypsin (A) or chymotrypsin (B) in the presence (+) or absence (−) of 200 μM $MnCl_2$. Aliquots were removed at various time points and proteolysis was stopped. Samples were then analyzed by 17.5% SDS-PAGE. (C) Densitometric analysis of the gel shown in panel A.

RESULTS

Metal Ion Activation of Fur Proteolysis

Figure 1 shows the effect of 200 μM $MnCl_2$ on the rate of Fur trypsinolysis. There are three distinct steps in the digestion of Fur by trypsin. The protein is quickly cut to a slightly lower molecular weight species named Fur′, which is then converted in a metal-regulated step to a tryptic core designated FurT. The latter is slowly hydrolyzed to peptides too small to be detected in this gel system. As can be seen in Figure 1, densitometric analysis of the gel reveals that Fur′ is converted to FurT 5.8 times faster in the presence of metal than in its absence. $MnCl_2$ also stimulates the activity of trypsin as measured by its effect on trypsin-catalyzed esterolysis of TAME, as shown in Table I. This acceleration of trypsinolysis is too small (1.37-fold effect for TAME) to account for the effect observed with Fur′ trypsinolysis. Furthermore, the acceleration of trypsin-catalyzed TAME esterolysis is independent of $MnCl_2$ concentration in the range tested (see Table I), while the degree of acceleration of Fur′ hydrolysis is strongly dependent on metal ion concentration, as is shown in Figure 2. Both the magnitude and concentration dependence of Mn(II) acceleration of hydrolysis differ significantly for Fur and TAME, suggesting that the increased rate of Fur trypsinolysis in the presence of metal is due to a conformational change in the Fur protein. By correcting for the increase in the rate of trypsin hydrolysis by $MnCl_2$ (as measured by the

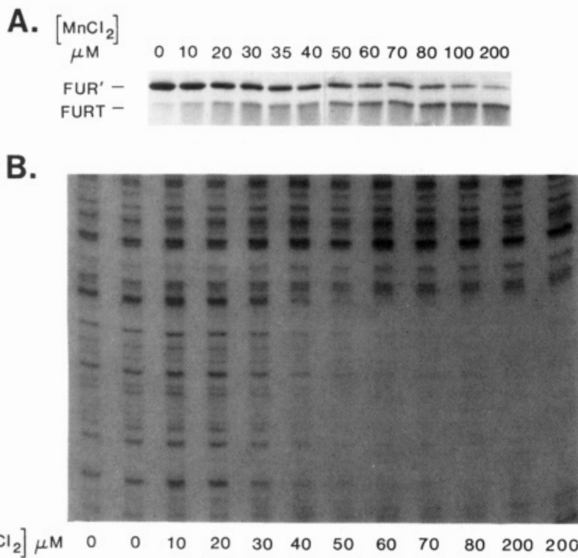

FIGURE 2: Mn(II) concentration dependence of Fur activation. (A) Metal ion dependence of Fur trypsinolysis analyzed by 17.5% SDS-PAGE. (B) DNase I footprinting analysis of the metal concentration dependence of Fur DNA binding. The *fur* operator-containing fragment of p100a was end labeled at the *Eco*R1 side and used with 47.5 nM Fur and varying metal concentrations in the binding reactions.

Table I: Effect of Various Additives on Protease Activity

additive	protease	substrate	rel act. (%)
none	trypsin	TAME	100
10 μM $MnCl_2$			136
20 μM $MnCl_2$			136
40 μM $MnCl_2$			136
50 μM $MnCl_2$			132
200 μM $MnCl_2$			144
3000 μM $MnCl_2$			139
200 μM $MnCl_2$	trypsin	Fur	580
			420[a]
10 μM $CdSO_4$	trypsin	TAME	120
25 μM $CdSO_4$			129
50 μM $CdSO_4$			130
12.5 μM $CdSO_4$	trypsin	Fur	272
			215[a]
25 μg/mL DNA	trypsin	TAME	54
75 μg/mL DNA			51
50 μg/mL DNA	trypsin	Fur	26
200 μM $MnCl_2$	chymotrypsin	CTNE	102
50 μg/mL DNA			76
100 μg/mL DNA			106

[a] Values corrected for the effect of metal on protease activity, as determined from TAME measurements.

TAME assay), the actual effect of saturating $MnCl_2$ on the rate of Fur' trypsinolysis is 4.2-fold. Figure 2 also shows the $MnCl_2$ concentration dependence of Fur DNA binding, as determined by DNase I footprinting. As can be seen by a qualitative comparison, the metal ion concentration dependence for both DNA binding and induction of sensitivity to trypsin are quite similar, indicating that the conformational change that manifests itself as increased sensitivity to trypsin might be the same metal-induced change required for DNA binding.

To identify the various species produced in Fur trypsinolysis, digested Fur was resolved by SDS-PAGE and transferred to Immobilon membranes. Bands corresponding to Fur' and FurT were excised and sequenced, giving the results shown in Figure 3. The molecular weight of each species was estimated by SDS-urea gel electrophoresis, as shown in Figure 3. Fur' is a mixture of peptides with an apparent molecular weight of 13.3 kDa, most of which have a N-terminus at A10 and a small portion of which have a N-terminus at K9. FurT has a N-terminus at S77. Since FurT is 32 amino acids shorter than FurC, but only has a slightly lower apparent molecular weight as estimated with this gel system (6.8 and 8.9 kDa for FurT and FurC, respectively), it is unlikely that very many amino acids are missing from the C-terminus of FurT. The first good candidate for tryptic cleavage in the C-terminal region of Fur would be R120, which is quite far into the protein (see Figure 3). In summary, it seems likely that tryptic

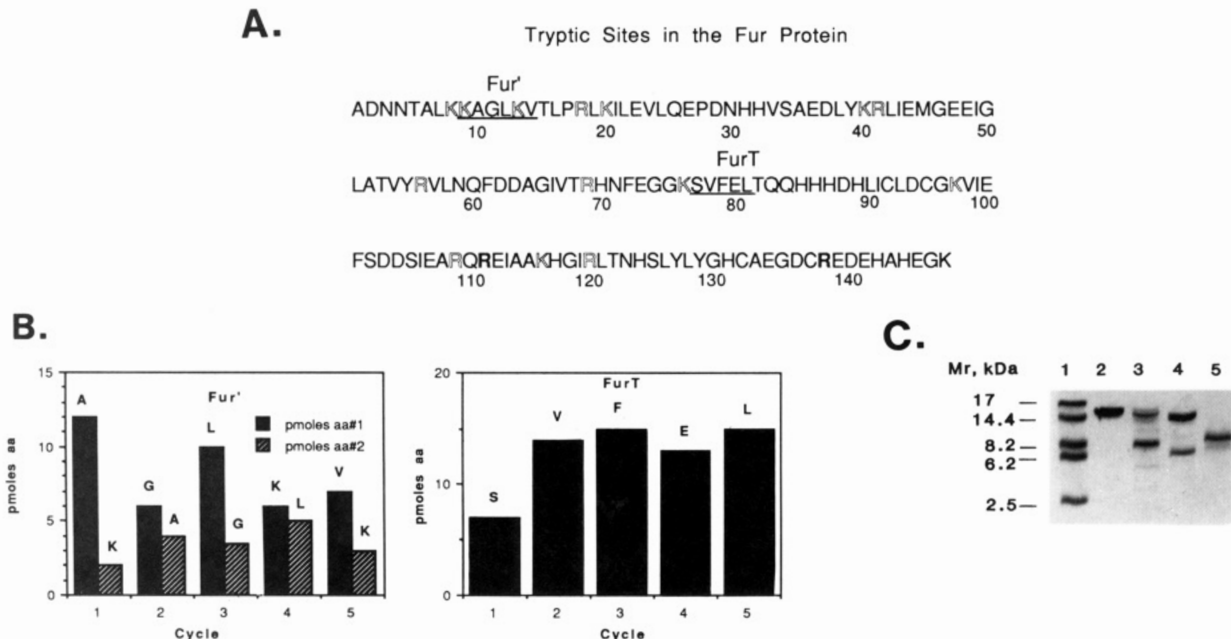

FIGURE 3: Characterization of tryptic fragments. (A) Amino acid sequence of the Fur protein, based on the sequence published by Schaffer et al. (1985). In the version used here, an A replaces T at the N-terminus (Wee et al., 1988). Shadowed letters are preferred tryptic sites. Boldface letters show presumably unreactive tryptic sites because of the presence of C-terminal acidic residues (Mihalyi, 1978). Sequences obtained for Fur' and FurT are underlined. (B) Sequence analysis data for Fur' and FurT. (C) SDS-urea gel analysis of various protease digests: (lane 1) 5 μg of molecular weight standards; (lane 2) 2 μg of Fur; (lane 3) 4 μg of Fur cut with chymotrypsin; (lane 4) 4 μg of Fur cut with trypsin; (lane 5) 2 μg of FurC cut with trypsin. Semilogarithmic plots of migration distance versus molecular weight were used to estimate the apparent molecular weights of various fragments.

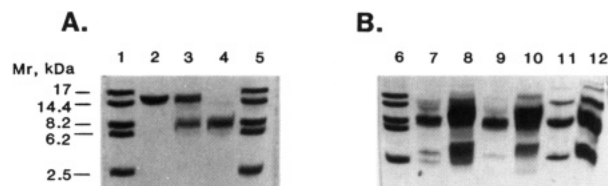

FIGURE 4: CNBr mapping of chymotryptic cleavage sites in Fur. SDS-urea gel electrophoresis was used to analyze Fur digested with chymotrypsin before (A) and after (B) cleavage with CNBr: (lanes 1, 5, and 6) molecular weight standards; (lane 2) intact Fur; (lane 3) Fur cut with chymotrypsin, no metal; (lane 4) Fur cut with chymotrypsin, 200 μM $MnCl_2$; (lanes 7 and 8) 3 and 12 μg of Fur cut with chymotrypsin (no metal) after cleavage with CNBr; (lanes 9 and 10) 3 and 12 μg of Fur cut with chymotrypsin (200 μM $MnCl_2$) after cleavage with CNBr; (lanes 11 and 12) 3 and 12 μg of Fur cut with CNBr.

cleavage of Fur occurs in the N-terminal region of the protein, and, in the presence of activating metal, Fur undergoes a conformational change that makes it more sensitive to trypsinolysis.

To determine whether the effect of metal on Fur digestion by protease is specific for trypsin, the effect of $MnCl_2$ on chymotryptic cleavage of Fur was studied. As shown in Figure 1, chymotrypsin cleaves Fur in the presence of saturating $MnCl_2$ considerably faster than in its absence. The data in Table I show that $MnCl_2$ has virtually no effect on the activity of chymotrypsin, as determined by the effect of this metal on the rate of esterolysis of CTNE, suggesting that the increase in the rate of chymotryptic digestion of Fur is due to a conformational change in the Fur protein. A CNBr mapping experiment designed to show what portion of the Fur protein is cleaved by chymotrypsin is shown in Figure 4. There is a single methionine in Fur that allows chymotryptic cleavage to be mapped to either the N- or C-terminal CNBr fragments. As can be seen in Figure 4, the amount and the size of the C-terminal fragment of Fur is virtually unchanged after chymotryptic cleavage while the amount of N-terminal fragment is decreased after proteolysis. Like tryptic cleavage, intermediates of slightly lower molecular weight than Fur (perhaps analogous to Fur′) are produced during chymotrypsinolysis. In Figure 4, such intermediates are evident in the sample cut with chymotrypsin. Following CNBr cleavage, there is a band that runs at a slightly lower molecular weight than FurN. This suggests that these intermediates have lost amino acids from their N-terminus, as has Fur′. The chymotrypsin-derived core of Fur has an apparent molecular weight of 8.1 kDa, which is quite close to the 8.9-kDa apparent molecular weight observed for FurC as determined by SDS-urea gel electrophoresis. This, along with the CNBr mapping experiment, suggests that chymotrypsin cuts Fur somewhere close to M45.

These results indicate that in the presence of activating metals Fur undergoes a conformational change that renders the N-terminal region of the protein sensitive to proteolytic cleavage. This change in conformation seems to be related to DNA binding since protease cleavage acceleration and DNA binding have a similar $MnCl_2$ concentration dependence. Furthermore, metals that activate Fur for DNA binding [Mn(II), Ni(II), and Cd(II)] also activate protease sensitivity, while Mg(II) fails to induce either change in the Fur protein (data not shown). The acceleration of Fur proteolysis in the presence of metal is analogous to the behavior of the *E. coli* CRP protein: cyclic AMP binding to the N-terminal domain of CRP induces protease sensitivity in the C-terminal DNA-binding domain (Heyduk & Lee, 1989). On the basis of the similarity of conformational changes observed between the two

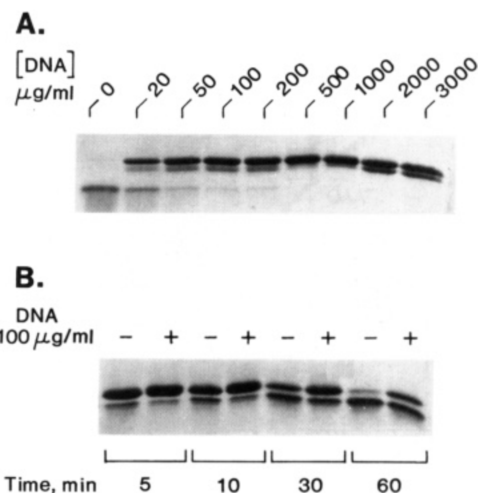

FIGURE 5: Effect of DNA on Fur proteolysis. (A) Varying concentrations of sonicated salmon sperm DNA were added to standard Fur trypsinolysis reactions (in the presence of 200 μM $MnCl_2$) and analyzed by 17.5% SDS-PAGE. (B) Fur was digested with chymotrypsin in the absence of metal with (+) or without (−) 100 μg/mL sonicated salmon sperm DNA and analyzed by 17.5% SDS-PAGE.

proteins, the metal-induced protease sensitivity of the N-terminal region of Fur suggests that this part of the protein is involved in DNA binding and the C-terminus is involved in metal binding. The following experiments were performed to assess the validity of this model.

Functional Domains of the Fur Protein

Evidence Implicating N-Terminal Involvement in DNA Binding. (A) Protease-Sensitivity Experiments. The effect of DNA on the rate of proteolysis of Fur was examined to see if DNA could block access of proteases to the N-terminal part of the protein. As shown in Figure 5, sonicated salmon sperm DNA greatly inhibits trypsinolysis of Fur in the presence of saturating $MnCl_2$. Similar results are observed in the absence of metal. Note that these experiments depend on the fact that, at high concentrations, Fur will bind to DNA independent of the sequence or presence of metal. Metal ions are only required to induce high-affinity binding to DNA (unpublished data). The results of a quantitative analysis of the effect of DNA on the rate of Fur trypsinolysis in the absence of metal (performed in the same manner as the experiments showing the quantitative effect of $MnCl_2$ on the rate of Fur trypsinolysis) are shown in Table I. DNA lowers the activity of trypsin as determined by the effect on TAME trypsinolysis, but the effect is both of lower magnitude and of different concentration dependence than is observed with Fur. The effect of DNA on chymotrypsinolysis of Fur is shown in Figure 5. As was observed with trypsin, DNA inhibits Fur proteolysis but has a negligible effect on the activity of chymotrypsin (as measured with CTNE), as shown in Table I. The inhibition of Fur proteolysis by DNA could be explained by DNA binding to the protease-sensitive N-terminal region of the protein, thus blocking access to trypsin or chymotrypsin.

(B) DNA Binding by FurN. In an effort to isolate the functional domains of Fur, the protein was cut at the single methionine residue, and the N- and C-terminal fragments (FurN and FurC respectively) were purified. Figure 6 shows the purification protocol and results. The ability of FurN to bind to DNA was tested by using the gel shift assay as shown in Figure 7. DNA binding by FurN is both weak (about 1000-fold weaker binding than intact Fur) and nonspecific (both vector and operator-containing inserts are shifted simultaneously). It appears that several FurN molecules can

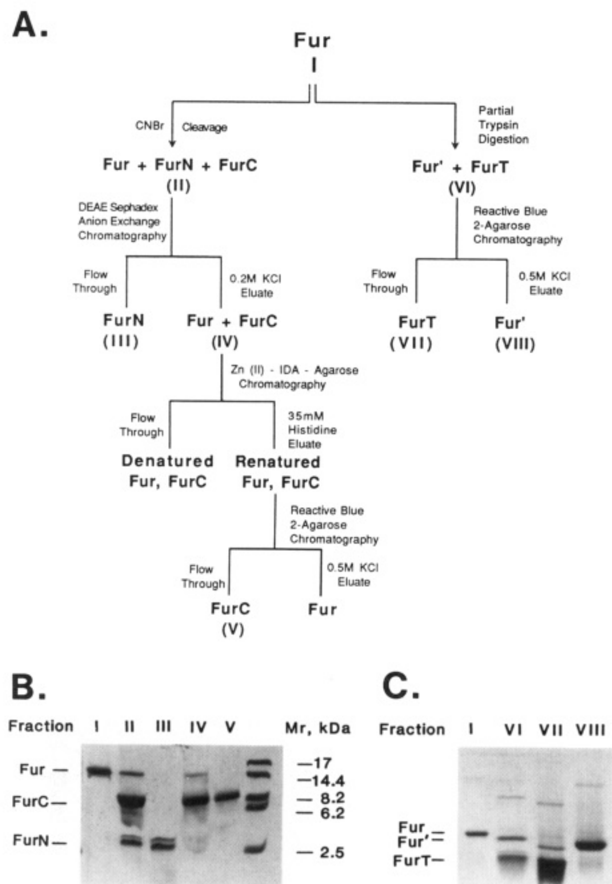

FIGURE 6: Isolation of various Fur fragments. (A) Outline of the purification procedures used in this paper. (B) SDS-urea gel electrophoresis of various fractions from the FurN and FurC purification procedure. (C) 17.5% SDS-PAGE analysis of various fractions from the Fur′ and FurT purification procedure.

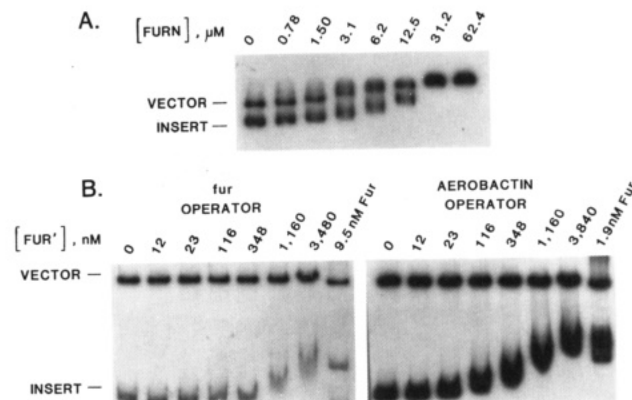

FIGURE 7: DNA binding by FurN and Fur′. (A) The electrophoretic mobility shift assay was performed as under Materials and Methods except that the final concentration of nonspecific DNA was reduced from 2.5 to 0.5 μg/mL and the gel was run for shorter periods of time than usual. $MnCl_2$ was omitted from the gel and tray buffers. The *fur* operator-containing p100a was used as a probe. FurN concentrations were estimated by using the Bradford assay, assuming a molecular weight of 5 kDa. (B) The gel shift assay was performed as under Materials and Methods, using 0.1 nM *fur* (p100a) and aerobactin (p6g) operators.

bind per DNA molecule, since the distance that the bands are shifted depends on the amount of FurN added. DNA binding is also quite sensitive to the presence of nonspecific DNA since little binding is observed when the concentration of unlabeled salmon sperm DNA in the binding buffer is increased from 0.5 μg/mL (used in the experiment shown in Figure 7) to 2.5 μg/mL [used by de Lorenzo et al. (1988a)]. The FurN–DNA complex also seems to have a quite short half-life, since running the gel for the usual time results in smears instead of distinctly shifted bands. DNA binding by FurN is also unaffected by the presence of $MnCl_2$, which was omitted in the experiment shown in Figure 7 (data not shown). FurC exhibited no DNA-binding activity in the gel shift assay under these conditions, even at a concentration of 40 μM (data not shown).

(*C*) *DNA Binding by Fur′*. Fur′, the trypsin-derived Fur fragment that lacks the first eight or nine N-terminal amino acids, was purified by the protocol given in Figure 6. The procedure exploits the ability of Fur′ to bind to Reactive Blue 2-agarose. Many proteins that interact with nucleic acids, such as micrococcal nuclease, RNase, and several restriction enzymes, bind to blue dye columns (Amicon Corp., 1980). In these experiments, proteins that bind to DNA (Fur, Fur′) adhere to the blue dye column, while those that do not bind DNA (FurC, FurT) are not retained, suggesting that it might be acting as a DNA affinity column. Figure 7 shows that Fur′ is able to bind DNA in the gel shift assay, but the specificity and the affinity of binding is lower than that seen with native Fur. Fur′ binds to the *fur* gene and aerobactin operon operators with virtually identical affinity, while intact Fur binds to the aerobactin operator about 50-fold tighter than to the *fur* operator (data not shown). There does seem to be some specificity in Fur′ DNA binding since it has higher affinity for operator-containing DNA than for nonspecific vector DNA, unlike FurN, which binds to both with about equal affinity. Fur′ does not bind DNA in the absence of metal. On the basis of the previously presented data, it seems likely that Fur′ has lost amino acids only from the N-terminus. The loss of eight or nine amino acids from the N-terminus of Fur thus results in a loss of 2–3 orders of magnitude in DNA-binding affinity and a considerable reduction in binding specificity, indicating that the N-terminal region of Fur is important for the interaction of this protein with DNA.

Evidence Implicating the C-Terminal Domain of Fur in Metal Binding. The purification of FurC, the C-terminal fragment obtained from CNBr cleavage of Fur, is shown in Figure 6. The ability of FurC to bind to Zn(II)-IDA-agarose suggests that this protein has metal-binding activity. Binding to Zn(II)-IDA-agarose is zinc dependent, and it is probably acting as a metal-binding affinity column. FurN does not bind strongly to Zn(II)-IDA-agarose, suggesting that this peptide lacks metal-binding activity.

Gel filtration analysis was used to estimate the molecular weight of FurC and to indirectly determine if it has a structure similar to the C-terminal region of intact Fur. When Fur or FurC is denatured by 4 M urea, they run in the exclusion volume of a G100 column, because random coils have a much larger effective volume than folded proteins. In gel filtration analysis, intact Fur runs as a 40.6-kDa protein, pointing to a dimer of 17-kDa monomers, which is consistent with the results obtained with HPLC by Dr. Kayoko Nakamura (Neilands & Nakamura, 1991). FurC has an apparent molecular weight of 28.9 kDa, which is about what would be predicted for a dimer of 12-kDa subunits. This suggests that FurC folds into a native-like structure and has the structural determinants required for dimerization. FurC is relatively resistant to trypsinolysis (see Figure 3C), which also indicates that it may be able to adopt a structure similar to the C-terminal region of intact Fur.

An analysis of the Mn(II) binding properties of Fur and FurC using equilibrium dialysis (shown in Figure 8) indicates that Fur and FurC bind approximately two Mn(II) per monomer. These curves show that FurC binds to manganese with

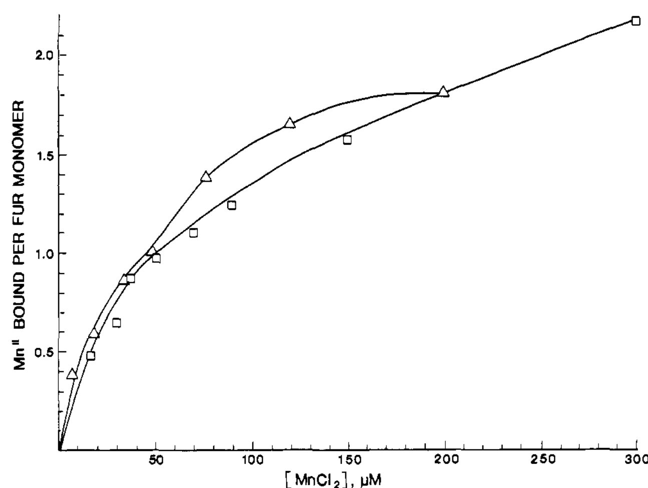

FIGURE 8: Equilibrium dialysis of Fur (Δ) and FurC (□) with $MnCl_2$. Equilibrium dialysis with 30 μM Fur or FurC was performed as under Materials and Methods, in MOPS buffer at 4 °C.

only slightly lower affinity than intact Fur (half-maximal binding is achieved at about 40 and 50 μM, respectively). This suggests that the two peptides bind to Mn(II) at the same sites and that a C-terminal domain is responsible for metal binding in intact Fur. Scatchard analysis of Fur and FurC Mn(II) binding gives consistently nonlinear plots with K_D = 38 μM and n = 2 for intact Fur and K_D = 60 μM and n = 2.1 for FurC. These data and the lack of internal homologies in the Fur amino acid sequence indicate that Fur probably has two nonidentical metal-binding sites. Equilibrium dialysis experiments have not been performed on FurT, but this protein is retained on Zn(II)-IDA-agarose, suggesting that it also has metal-binding capability.

The Interaction of Cadmium with Fur

A study of the interaction of cadmium with Fur has provided evidence that supports the model of Fur functional organization and activation outlined above.

UV Spectroscopy and Equilibrium Dialysis. As shown in Figure 9, prominent UV absorption spectra are produced when Cd(II) is added to Fur, suggesting that the metal interacts with thiolates in the protein. Similar spectra, which have been attributed to charge transfer transitions, have been observed with cadmium complexes of metallothionein. It appears that two spectrally distinct complexes are formed. When one molar equivalent of $CdSO_4$ is added to Fur, a spectrum with λ_{max} = 227 nm and $\epsilon_M = 1.2 \times 10^4$ M^{-1} cm^{-1} is observed. At higher $CdSO_4$ concentrations, a second complex is apparently formed with λ_{max} = 242 nm and $\epsilon_M = 9.5 \times 10^3$ M^{-1} cm^{-1}. Characteristics of the Cd(II)–Fur spectra are sensitive to ionic conditions of the buffer, suggesting that the metal might have access to the solvent when bound to Fur. Fur precipitates at high concentrations of cadmium in buffers with low ionic strength. Since FurC is not very soluble in buffers with high salt concentrations, extensive work has not been done with cadmium and FurC. At low salt and cadmium concentrations, Fur and FurC have quite similar Cd(II)-induced absorption spectra: both have λ_{max} = 240 nm and extinction coefficients of about 1×10^4 M^{-1} cm^{-1}. Competition between Cd(II) and Mn(II) binding to Fur cannot be detected by UV absorption, probably because Cd(II) binds to Fur much more tightly than Mn(II) (see below). Addition of $MnCl_2$ to Fur gives no detectable absorption spectra.

Equilibrium dialysis indicates that Fur has a high affinity for Cd(II) (K_D < 5 μM) and that about 2.3 Cd(II) are bound per Fur monomer, consistent with the results obtained with

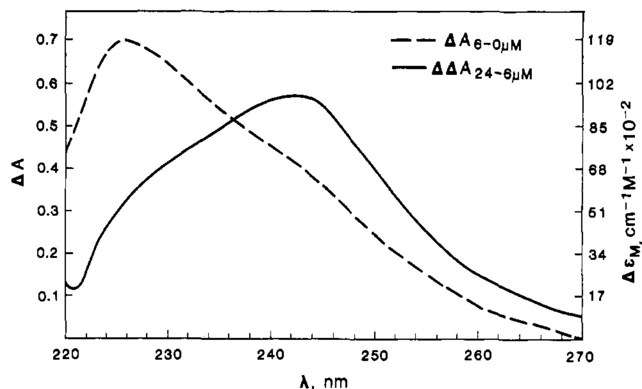

FIGURE 9: UV absorption spectra of Fur–Cd(II) complexes. $CdSO_4$ (1 mM stock) was added to Fur (6 μM) in 50 mM MOPS (pH 7.0) and 375 mM NaCl, and spectra were recorded on a Schimadzu UV160 spectrophotometer. The $\Delta A_{6-0\mu M}$ spectra (λ_{max} = 227 nm) was obtained by subtracting the Fur spectrum from the spectrum observed with Fur in the presence of 6 μM $CdSO_4$. The $\Delta A_{24-6\mu M}$ spectrum (λ_{max} = 242 nm) was obtained by subtracting the Fur–Cd(II) spectrum at 6 μM $CdSO_4$ from the spectrum observed with Fur in the presence of 24 μM $CdSO_4$.

$MnCl_2$ and the spectral evidence presented above.

Equilibrium dialysis experiments show that Cd(II) and Mn(II) can compete for binding to the Fur protein. For example, in the presence of 75 μM $MnCl_2$, Fur binds 1.1 Mn(II) per monomer, while Fur equilibrated with 75 μM $MnCl_2$ and 1.4 μM $CdCl_2$ binds only 0.4 Mn(II) per monomer. In the presence of 1.4 μM $CdCl_2$, 1.2 Cd(II) ions were bound per monomer, while 0.9 Cd(II) were bound in the presence of 75 μM $MnCl_2$. Under the right conditions, competition can be more extensive, suggesting that Cd(II) and Mn(II) bind to the same sites in the Fur protein.

Protease Studies Using Cd(II). The effect of cadmium on Fur protease sensitivity, with trypsin as a probe of protein conformation, is shown in Figure 10. The rate of Fur trypsinolysis was analyzed at various cadmium concentrations, as was done with Fur with saturating Mn(II), and is shown in Figure 10. Maximum protease sensitivity is observed in the presence of about one molar equivalent of Cd(II) per Fur monomer. Similar results have been obtained with Ni(II) as an activating metal. Equilibrium dialysis experiments indicate that both of these metals should form complexes with Fur virtually stoichiometrically under these conditions. This indicates that one metal ion per Fur monomer is required to activate protease sensitivity. As shown in Figure 10, addition of more than one equivalent of cadmium does not accelerate conversion of Fur′ to FurT but does lead to the appearance of bands with slightly lower molecular weight than Fur′. This suggests that Fur assumes a unique conformation when more than one cadmium per monomer is bound. A kinetic analysis of trypsinolysis of the Fur–Cd(II) complex (1:1 molar ratio) (summarized in Table I) indicates that cadmium accelerates the intrinsic rate of Fur′ hydrolysis 2.2-fold, which is about one-half the magnitude of the effect observed with saturating $MnCl_2$.

As shown in Figure 10, the effect of Cd(II) on Fur trypsinolysis kinetics is dominant over that of Mn(II). In the presence of both metals, Fur appears to be cut at the same rate that is observed in the presence of cadmium alone. This is what is expected, given that Cd(II) binds Fur much more tightly than Mn(II), if both metals activate Fur protease sensitivity by binding to the same site.

Footprinting Analysis of Cd(II) Activation of Fur DNA Binding. de Lorenzo et al. (1987) showed that Cd(II) is capable of inducing Fur DNA binding. A more detailed

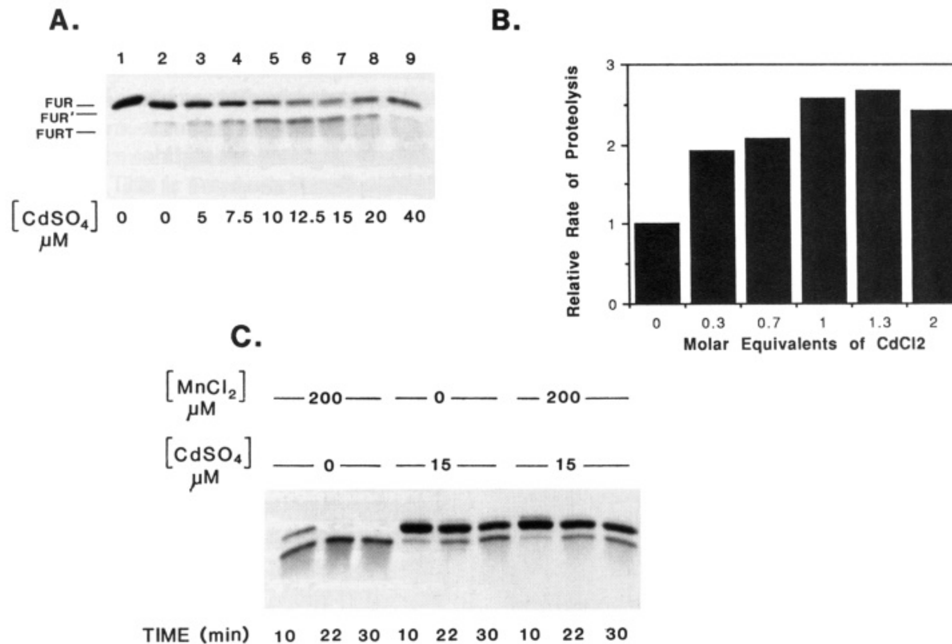

FIGURE 10: Fur trypsinolysis in the presence of Cd(II). (A) Fur (12.5 μM) was cut with trypsin in the presence of increasing concentrations of $CdSO_4$ as under Materials and Methods. Samples were resolved by 17.5% SDS–PAGE. (Lane 1) Fur that has not been cut with trypsin. (B) The effect of $CdCl_2$ on the rate of Fur trypsinolysis. Rates were determined by linear regression analysis of time course protease digestions in the presence of varying concentrations of Cd(II) that had been quantitated by densitometry, as in Figure 1B. (C) Fur trypsinolysis in the presence of Mn(II) and Cd(II). 15 μM Fur was cut with trypsin in the presence of saturating concentrations of $MnCl_2$, $CdSO_4$, or both metal ions, and analyzed by 17.5% SDS–PAGE.

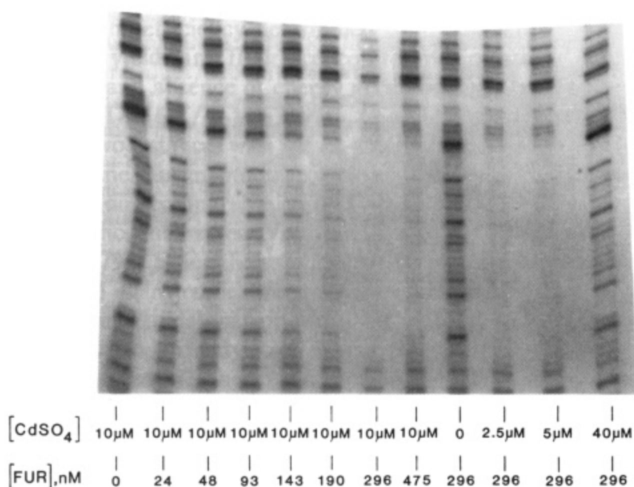

FIGURE 11: Cadmium activation of Fur DNA binding. DNase I footprinting was performed as described above with the *fur* operator probe end labeled on the *Eco*R1 side.

analysis of Fur DNA binding in the presence of cadmium, as measured by DNase I footprinting, is shown in Figure 11. This analysis shows that very low concentrations of $CdSO_4$ (<2.5 μM) fully activate the Fur protein, in contrast to Mn(II), where 50–80 μM $MnCl_2$ is required to induce maximum Fur DNA binding (see Figure 2). Fur affinity for DNA is greatly reduced at high (40 μM) concentrations of $CdSO_4$. Since high concentrations of cadmium also induce a unique conformation of Fur, as determined by protease sensitivity (Figure 11), this suggests that binding of cadmium to the second low-affinity site in the Fur protein may cause the protein to adopt a conformation that has a low affinity for DNA, although this inhibition could also be due to protein precipitation or other effects. In the presence of saturating concentrations of $CdSO_4$, relatively high concentrations of Fur (about 200 nM) are required to fully bind the *fur* operator DNA. In the presence of saturating $MnCl_2$, 70–90 nM Fur is sufficient to fully bind this DNA. This indicates that the Fur–Cd(II) complex has a lower affinity for DNA than the Fur–Mn(II) complex. The Fur–Cd(II) complex is also less sensitive to trypsinolysis than the Fur–Mn(II) complex, suggesting that the metal-induced conformational change responsible for increased protease sensitivity is the same as the metal-induced change in Fur that allows DNA binding. A comparison of the activities of Cd(II) and Mn(II) with Fur also shows that metal-binding affinity and the ability of a metal to promote DNA binding are not related. Mn(II) binds Fur more weakly than Cd(II) but it is more effective than Cd(II) in inducing DNA binding.

Discussion

Functional Organization of the Fur Protein. Many repressor proteins, such as LacR, TrpR, and CRP, have been found to have a two-domain structure, in which one domain is involved in DNA binding while the other is involved in ligand binding (Steitz, 1990; Pabo & Sauer, 1984). The results shown above indicate that a similar model might explain the functional organization of the Fur protein. The biphasic kinetics of proteolysis suggests that this repressor has two domains that differ in protease sensitivity.

FurC can refold into a protein with structural (dimerization, protease resistance) and functional (metal-binding) characteristics similar to those of intact Fur, in the absence of FurN. This indicates that at least the C-terminal region of the protein fits the criteria of a domain. Isolated ligand-binding domains have been obtained from the *lac* repressor (Platt et al., 1973) and the CRP (Eilen et al., 1978).

Work with cadmium has shown that the binding site for this metal probably involves thiolate ligands as determined by the prominent UV spectra observed, analogous to those seen with metallothionein (Vasak et al., 1981). It seems unlikely that thiolate ligands are involved in Fur binding of Mn(II), since no Mn(II)-induced spectroscopic signature has been detected. Both metals probably bind to the same pocket in Fur because each can induce both DNA binding and protease sensitivity.

Equilibrium dialysis and protease sensitivity competition experiments support the hypothesis that Cd(II) and Mn(II) bind to the same sites in the Fur protein, although it is possible that the two metals bind to Fur at different sites and induce conformational changes that prevent binding of additional metal ions. In binding Mn(II) either the cysteines of Fur may interact very weakly with the metal so deprotonation does not take place, analogous to the interaction of Mn(II) with water at neutral or acidic pH, or an intervening water molecule is perhaps bound by Mn(II) so that cysteine does not act as a direct ligand or the metal interacts with cysteines in some other manner. Alkylation studies have shown that cysteine thiol groups are essential for Fur activity (del Cardayre & Neilands, 1991). These data are consistent with our model of Fur having a C-terminal metal-binding domain since all of the cysteines are located in this region of the protein. It is interesting to note that two of the four cysteines in Fur are found in the motif CysXYCysGly, which is present in the metal-binding centers of ferredoxins and many other metal-binding proteins (del Cardayre & Neilands, 1991).

The evidence for an N-terminal DNA-binding domain is less conclusive. Although the protection by DNA of the N-terminus of Fur from protease digestion and the loss in binding affinity and specificity observed in Fur′ all indicate that the N-terminal region of is necessary for DNA binding, they do not show whether the N-terminal region of Fur is sufficient for DNA binding, since FurN does binds to DNA with low affinity and no detectable specificity. This may be because FurN does not contain all the sequence necessary to form a complete DNA-binding domain, although it is possible that the C-terminal metal-binding domain of Fur is also involved in the interaction of the protein with DNA. The exact demarcation between the N- and C-terminal domains is unclear, although the sequence of protease-derived cores of Fur suggests that the boundary lies somewhere between M45 (the approximate location of the N-terminus of chymotrypsin-derived cores) and S77 (the N-terminus of trypsin-derived cores). Molecular genetics techniques are currently being used to generate an 80 amino acid N-terminal fragment to determine if a DNA-binding domain can be isolated. Such experiments have been done with the λ*cI* and *lac* repressors (Steitz, 1990).

Metal Ion Activation of the Fur Protein. There are several possible mechanisms by which metal ions could activate the DNA-binding capability of Fur. One mechanism would involve ligand-induced dimerization of the Fur protein, as has been proposed for eukaryotic nuclear hormone receptors (Forman & Samuels, 1990). Fur probably acts as a multimer because of the dyad symmetrical nature of its operator sequence (de Lorenzo et al., 1987) and because negative complementation can be observed in fur^+ fur^- merodiploid strains (Braun et al., 1987). This mechanism seems unlikely since Nakamura has shown that Fur exists as a dimer both in the presence and absence of metal, down to concentrations of 1 μM (Neilands & Nakamura, in press). Work is currently being done in this laboratory to determine if changes in quaternary structure occur at very low protein concentrations in the presence or absence of metal.

Another model would postulate the direct involvement of protein bound metal in binding to DNA. The total absence of DNA binding by FurC and the involvement in DNA binding by the N-terminal region of Fur indicate that the activating metal ion is probably not bound by the same part of the protein that interacts with DNA. Fur-bound Fe(II) is not able to catalyze cleavage of DNA in the presence of H_2O_2, also suggesting that the metal ion is not in close proximity to the DNA (Nakamura et al., 1989). The isolation of an N-terminal fragment that binds to DNA in a manner similar to intact Fur or an X-ray structure of the ternary Fur–metal–DNA complex would be required to thoroughly test for direct metal involvement in DNA binding.

The model for the mechanism of metal ion activation of Fur DNA binding that best explains the data outlined above postulates that metal binding to Fur induces a conformational change that allows the protein to interact with DNA. The fact that Cd(II) is less effective than Mn(II) at inducing protease sensitivity and is also less effective at inducing DNA binding is the best evidence to date indicating that this conformational change is required for DNA binding. Supporting evidence is that both the conformational change that manifests itself as protease sensitivity and sequence-specific DNA binding seem to have a very similar metal ion concentration dependence (Figure 2 and unpublished data). Although Fur has two metal-binding sites per monomer, the fact that only one molar equivalent of tightly binding metals such as Cd(II) or Ni(II) is necessary to produce maximal protease sensitivity suggests that only one of these sites is actually involved in inducing this conformational change in the protein, although it is possible that activation could actually require binding of two metals to one of the monomeric subunits of a dimeric repressor.

Ligand-induced extension or loosening of the structure of DNA-binding domains has been observed in two other bacterial repressors. cAMP has been shown to induce protease sensitivity in the *E. coli* CRP, and this conformational change has been correlated with the ability of the protein to bind DNA (Heyduk & Lee, 1989). With the Trp repressor, X-ray crystallographic analysis showed that addition of tryptophan to the protein resulted in an increased mobility of the DNA-binding domain, as measured by the average isotropic temperature factor (Lawson et al., 1988). Comparison of the apo and tryptophan-bound forms of this protein in model-building experiments shows that the corepressor induces a conformational change in which the DNA-binding domain moves away from the main body of the protein and into a position that could better interact with the major groove of B-form DNA (Zhang et al., 1987). Moving the DNA-binding domain away from the main body of the protein would be expected to result in a looser or more flexible structure. In both the λcI repressor [as measured by differential scanning calorimetry (Pabo et al., 1979)] and the lac repressor [as measured by protease sensitivity (Platt et al., 1973) or by NMR (Wade-Jardetzky et al., 1978)], the DNA-binding domains have a looser structure than the rest of the protein. These examples of analogous biochemical behavior suggest that our model of Fur functional organization and mechanism of activation is valid. Fur seems to have no helix–turn–helix sequence motif as is found in the above-mentioned repressors, although CD studies have shown that it is rich in α-helices (Williams et al., 1989). This suggests that Fur solves the problem of sequence-specific DNA recognition and ligand-induced activation in a manner similar to that of the classical helix–turn–helix repressors using a very different amino acid sequence.

Added in Proof

Recently Saito et al. (1991a,b) and Saito and Williams (1991) have published NMR structures showing the folding pattern of the Fur protein.

Acknowledgments

We thank Magnus Persmark and Larry C. Ackerson for their excellent technical assistance and Dr. R. J. P. Williams for his thoughtful suggestions and constructive criticism.

Registry No. Zn, 7440-66-6; Mn, 7439-96-5; Cd, 7440-43-9.

REFERENCES

Amicon Corporation (1980) *Dye–Ligand Chromatography: Applications, Method, and Theory of Matrex Gel Media*, Scientific Systems Division, Lexington, MA.

Bagg, A., & Neilands, J. B. (1985) *J. Bacteriol. 161*, 450–453.

Bagg, A., & Neilands, J. B. (1987a) *Biochemistry 26*, 5471–5477.

Bagg, A., & Neilands, J. B. (1987b) *Microbiol. Rev. 51*, 509–518.

Bindereif, A., & Neilands, J. B. (1985) *J. Bacteriol. 162*, 1039–1046.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Braun, V., Hantke, K., Eick-Helmerich, K., Koster, W., Prebler, U., Sauer, M., Schaffer, S., Schoffler, H., Staudenmaier, H., & Zimmermann, L. (1987) in *Iron Transport in Microbes, Plants, and Animals* (Winkelmann, G., van der Helm, D., & Neilands, J. B., Eds.) pp 35–51, VCH Verlagsgesellschaft, FRG.

Crosa, J. H. (1989) *Microbiol. Rev. 53*, 517–530.

del Cardayre, S., & Neilands, J. B. (1991) in *Iron Biominerals* (Frankel, R., Ed.) Plenum Press, New York.

de Lorenzo, V., Wee, S., Herrero, M., & Neilands, J. B. (1987) *J. Bacteriol. 169*, 2624–2630.

de Lorenzo, V., Herrero, M., Giovannini, F., & Neilands, J. B. (1988a) *Eur. J. Biochem. 173*, 537–546.

de Lorenzo, V., Giovannini, F., Herrero, M., & Neilands, J. B. (1988b) *J. Mol. Biol. 203*, 875–884.

Eilen, E., Pampeno, C., & Krakow, J. S. (1978) *Biochemistry 17*, 2469–2473.

Fontana, A., & Gross, E. (1986) in *Practical Protein Chemistry—A Handbook* (Darbre, A., Ed.) John Wiley and Sons Ltd., New York.

Forman, B. M., & Samuels, H. H. (1990) *New Biol. 2*, 587–594.

Francis, R. T. (1984) *Anal. Biochem. 72*, 248–254.

Garibaldi, J. A., & Neilands, J. B. (1956) *Nature 156*, 526–527.

Halliwell, B., Ed. (1988) *Oxygen Radicals and Tissue Injury*, Federation of the American Society for Experimental Biology, Bethesda, MD.

Hantke, K. (1981) *Mol. Gen. Genet. 182*, 288–292.

Hantke, K. (1984) *Mol. Gen. Genet. 197*, 337–341.

Heyduk, T., & Lee, J. C. (1989) *Biochemistry 28*, 6914–6924.

Hunkapiller, M. W., Lujan, E., Ostrander, F., & Hood, L. E. (1983) *Methods Enzymol. 91*, 227–236.

Lawson, C. L., Zhang, R., Schevitz, R. W., Otwinowski, Z., & Joachimiak, A. (1988) *Proteins: Struct., Funct., Genet. 3*, 18–31.

Maniatis, T., Fritsch, E. F., & Sambrook, J. C. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.

Mattick, J. C., Tsukamoto, Y., Nickless, J., & Wakil, S. J. (1983) *J. Biol. Chem. 258*, 15291–15299.

Mihalyi, E. (1978) *Application of Proteolytic Enzymes to Protein Structure Studies*, CRC Press, Bethesda, MD.

Nakamura, K., de Lorenzo, V., & Neilands, J. B. (1989) in *Metal–DNA Chemistry* (Tullius, T. D., Ed.) American Chemical Society, Washington, DC.

Neilands, J. B. (1972) *Struct. Bonding (Berlin) 11*, 145–170.

Neilands, J. B. (1981) *Annu. Rev. Biochem. 50*, 715–731.

Neilands, J. B. (1982) *Annu. Rev. Microbiol. 36*, 285–309.

Neilands, J. B. (1990) in *Pseudomonas: Biotransformations, Pathogenesis, and Evolving Biotechnology*, pp 382–395, American Society for Microbiology, Washington, DC.

Neilands, J. B., & Nakamura, K. (1991) in *Microbial Iron Chelates* (Winkelmann, G., Ed.) CRC Press, New York.

Niederhoffer, E. C., Naranjo, C. M., & Fee, J. F. (1989) in *Metal Ion Homeostasis: Molecular Biology and Chemistry* (Hamer, D. H., & Winge, D. R., Eds.) pp 149–158, Alan R. Liss, Inc., New York.

O'Halloran, T. V. (1989) *Met. Ions Biol. Syst. 24*, 105–146.

Pabo, C. O., & Sauer, R. T. (1984) *Annu. Rev. Biochem. 53*, 293–321.

Pabo, C. O., Sauer, R. T., Sturtevant, J., & Patashne, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1608–1612.

Perbal, P. (1988) *A Practical Guide to Molecular Cloning*, 2nd ed., John Wiley and Sons, New York.

Platt, T., Files, J. G., & Weber, K. (1973) *J. Biol. Chem. 248*, 110–121.

Saito and Williams (1991) *Eur. J. Biochem. 197*, 43–47.

Saito et al. (1991a) *Eur. J. Biochem. 197*, 29–38.

Saito et al. (1991b) *Eur. J. Biochem. 197*, 39–42.

Schaffer, K., Hantke, S. K., & Braun, V. (1985) *Mol. Gen. Genet. 200*, 110–113.

Steitz, T. A. (1990) *Q. Rev. Biophys. 3*, 205–280.

Swank, R. T., & Munkres, K. D. (1970) *Anal. Biochem. 39*, 462–477.

Vasak, M., Kagi, J. H. R., & Hill, H. A. O. (1981) *Biochemistry 20*, 2852–2856.

Wade-Jardetzky, N., Bray, R. P., Conover, W. W., Jardetzky, O., Geisler, N., & Weber, K. J. (1979) *J. Mol. Biol. 128*, 259–264.

Walsh, K. A. (1970) *Methods Enzymol. 19*, 41.

Walsh, K. A., & Willcox, P. E. (1970) *Methods Enzymol. 19*, 31–40.

Wee, S., Neilands, J. B., Bittner, M. L., Hemming, B. C., Haymore, B. L., & Seetharam, R. (1988) *Biol. Met. 1*, 62–68.

Williams, R. J. P., Wormald, M. R., Hider, R. C., & Neilands, J. B. (1989) in *Metal Ion Homeostasis: Molecular Biology and Chemistry* (Hamer, D. H., & Winge, D. R., Eds.) pp 189–196, Alan R. Liss, Inc., New York.

Zhang, R., Joachimiak, A., Lawson, C. L., Schevitz, R. W., Otwinowski, Z., & Sigler, P. B. (1987) *Nature 327*, 591–597.