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Characterization of the CysB protein of *Klebsiella aerogenes*: direct evidence that *N*-acetylserine rather than *O*-acetylserine serves as the inducer of the cysteine regulon

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The *cysB* gene of *Klebsiella aerogenes* has been cloned, sequenced and shown to complement the cysteine auxotrophic phenotype of *Escherichia coli cysB* mutants. The *K. aerogenes cysB* gene is predicted to encode a protein of 324 amino acid residues that shares approx. 95% sequence similarity with the *Salmonella typhimurium* and *E. coli* CysB proteins. Gel-retardation assays demonstrate that the purified protein binds to DNA fragments containing either the *K. aerogenes cysB* promoter or the *S. typhimurium cysJIH* promoter. Acetylserine enhances CysB binding to the *cysJIH* promoter fragment while diminishing its binding to the *cysB* promoter fragment. Fluorescence-emission-spectroscopy measurements suggest strongly that *N*-acetylserine binds to CysB apoprotein but that *O*-acetylserine does not, and support the notion that *N*-acetylserine is the physiological inducer of cysteine biosynthesis.

INTRODUCTION

Cysteine biosynthesis in *Salmonella typhimurium* and *Escherichia coli* involves the uptake and reduction of inorganic sulphate to sulphide, the synthesis of *O*-acetylserine from serine and acetyl-coenzyme A catalysed by serine transacetylase, and the reaction of *O*-acetylserine with sulphide in a reaction catalysed by *O*-acetylserine (thiol) lyase (reviewed in Kredich, 1987). This process involves the products of some 16 or so genes, dispersed among at least nine chromosomal loci (Kredich, 1992; Neuwald et al., 1992). Expression of a number of these genes, collectively referred to as the cysteine regulon, is co-ordinated. In both species, the cysteine regulon is derepressed in response to sulphur limitation. Derepression is dependent on the activity of a tetrameric regulatory protein, encoded by the *cysB* locus, the activity of which is thought to be responsive to fluctuations in the intracellular concentration of acetylserine (Bielinska and Hulanicka, 1986; Kredich, 1987; Miller and Kredich, 1987; Ostrowski and Kredich, 1989; Monroe et al., 1990). [*O*-Acetylserine undergoes a spontaneous re-arrangement to *N*-acetylserine on time scales of minutes to hours in aqueous solution around neutral pH, so that solutions of *O*-acetylserine inevitably contain varying amounts of *N*-acetylserine depending on the age and the pH of the solution. Unless otherwise stated, acetylserine refers therefore to mixtures of the L isomers of O- and N-acetylated species.] *cysE* mutant strains, which lack serine transacetylase, cannot be derepressed by sulphur limitation in the absence of an external source of acetylserine. Studies *in vivo* and *in vitro* of the *cysJIH* promoters of *S. typhimurium* and *E. coli* show that transcription is dependent on CysB protein and either *O*-acetylserine or its derivative *N*-acetylserine. Similarly, CysB protein and acetylserine are required *in vitro* for transcription initiation from the *cysP* promoter of *S. typhimurium* (Hryniewicz and Kredich, 1991).

In *cysB–lac* fusion strains of *E. coli*, in which expression of the *lac* genes is controlled by the *cysB* promoter, introduction of $cysB^+$ alleles causes a 10-fold reduction in β-galactosidase protein and *lac* mRNA levels, indicating that the *cysB* gene is subject to negative autoregulation (Jagura-Burdzy and Hulanicka, 1981; Bielinska and Hulanicka, 1986). *S. typhimurium* CysB protein has been shown to inhibit transcription initiation at its own promoter in *in vitro* 'run-off' assays. This inhibition is partially reversed by *N*-acetylserine, which blocks the binding of CysB protein in the *cysB* promoter (Ostrowski and Kredich, 1991).

The nucleotide sequences of the *cysB* genes of *S. typhimurium* and *E. coli* have been determined; they encode proteins of 324 amino acid residues that are 95% identical at the amino acid sequence level (Ostrowski et al., 1987; Tei et al., 1990). They share a putative 'helix-turn-helix' (residues 19–38), which is characteristic of that found in a number of DNA-binding proteins (Henikoff et al., 1988; Brennan and Matthews, 1989; Harrison, 1991). A putative N-terminal helix-turn-helix motif is a property of the family of prokaryotic regulatory proteins collectively known as the 'LysR' family, of which CysB is a member, that share extensive amino acid similarity over a 286 residue sequence (Henikoff et al., 1988). Most of the LysR-family proteins activate gene expression at one or more loci, while negatively regulating the expression of their own genes. The activities controlled by the LysR proteins include amino acid biosynthesis, CO_2 fixation, ion transport, antibiotic resistance, virulence (*Vibrio cholerae*), the initiation of nodulation and chromosomal replication (Henikoff et al., 1988 and references therein; Lindquist et al., 1989; Goldberg et al., 1991; Thöny et al., 1991; Viale et al., 1991; Rahiv-Manor et al., 1992). The capacity of a number of these proteins to respond to changes in cofactor concentrations by binding some promoters more tightly and simultaneously binding others less tightly, raises the interesting mechanistic question of whether they possess more than one DNA-binding site, or more

Abbreviations used: CBS, CysB-binding site; DTT, dithiothreitol; IPTG, isopropylthiogalactoside.

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than one mode of DNA binding. With the goal of answering this question by X-ray crystallographic analysis, we have carried out a characterization of the CysB protein of *K. aerogenes*.

A further interesting question concerns the nature of the inducer of the cysteine regulon. Genetic studies and earlier biochemical work led to the suggestion that the cysteine precursor, *O*-acetylserine, serves this function (Jones-Mortimer et al., 1968; Kredich, 1971). However, *O*-acetylserine is susceptible to a non-enzymic intramolecular acetyl migration reaction, which proceeds at an appreciable rate at neutral pH yielding *N*-acetylserine. It has since been shown that *N*-acetylserine is more potent than *O*-acetylserine in stimulating transcription from the *cysJIH* promoter *in vitro* (Ostrowski and Kredich, 1989). Therefore, it is possible that *N*-acetylserine is the actual internal inducer of the cysteine regulon and that the inducer function attributed to *O*-acetylserine is merely the result of the spontaneous formation of the N-acetylated species in the course of these experiments. To address this question, we have used fluorescence emission spectroscopy to monitor directly the interaction of CysB protein with acetylated serines.

MATERIALS AND METHODS

Chemicals

All reagents used were of analytical reagent grade and were purchased from commercial suppliers. *N*-Acetyl-L-serine was prepared from *O*-acetyl-L-serine according to procedures described by Narita (1958). *O*-Acetylserine (0.5 g) was dissolved in 3 ml of water and NaOH was added in aliquots until the pH remained unchanged at 8. The mix was then passed down a Dowex-50W cation-exchange column. The sample was then frozen and lyophilized, redissolved in ethanol and *N*-acetylserine was recrystallized by the addition of ether. The melting point of the material (128.6–129.2 °C) confirmed that the desired product was pure. Infra red absorption spectra were also recorded on a Perkin–Elmer 1720 Infrared Fourier Transform spectrometer. The conversion of *O*-acetylserine into *N*-acetylserine is accompanied by the loss of absorbance bands at 1980 cm^{-1} and 1750 cm^{-1}, associated with the ester carbon–oxygen bond in *O*-acetylserine, and the appearance of a band at 1560 cm^{-1}, signalling the presence of the secondary amide carbon–nitrogen bond in *N*-acetylserine.

Bacterial strains and plasmids

K. aerogenes NCTC 418 (ATCC 15380) was obtained from Dr. J. T. Smith (University of London, London, U.K.). *E. coli* strains DM700 (F^-, Δ(*cysB-topA*)217, *acrA11*) and AB1369 (F^-, *thi-1*, *argE3*, *cysB38*, *proA2*, *his-4*, *galK2*, *lacY1*, *mtl-1*, *xyl-5*, *tsx-5*, *tsx-29*, *supE44*) were obtained from Dr. J. C. Wang (Harvard University, MA, U.S.A.). *E. coli* strain JM101 (*supE*, Δ(*lac-proAB*), F′(*proAB*$^+$, *lac*I^{q}ZΔM15, *traD36*) was obtained from Amersham International. Plasmid pKK223-3 was obtained from Pharmacia. Plasmid pRSM15 containing the *S. typhimurium cysJIH* promoter (Ostrowski and Kredich, 1989) was obtained from Dr. N. M. Kredich (Duke University Medical Centre, NC, U.S.A.).

DNA manipulations

Standard molecular biological procedures were used throughout the course of these studies (Sambrook et al., 1989). Genomic DNA was prepared from *K. aerogenes* NCTC 418 essentially by the method of Marmur (1961). A library of partial *Sau3*AI restriction fragments (9–23 kb) of *K. aerogenes* genomic DNA was constructed in the λEMBL3 vector, using a 'Gigapack' packaging extract and *E. coli* VCS257 obtained from Stratagene. DNA sequencing was performed by the dideoxynucleotide-chain-termination method using a T7 DNA polymerase-based kit supplied by Pharmacia. Oligonucleotides used for DNA sequencing and amplification by PCR were synthesized on an Applied Biosystems model 392 synthesizer.

The following oligonucleotides were used as PCR primers; CB1, 5′-GGGGAATTCAGGAGTCAGCCATGAAACTA-3′; CB2, 5′-AAGATTACTGCAGTGAATTTAGGCC-3′; CB3, 5′-CGGCAGAGAGAGTGCATTTCTCTGC-3′; CB4, 5′-CTGTTGTAGTTTCATGGCTGATATC-3′; CB5, 5′-CCGCCTTGTGACGGGCA-3′; and CB6, 5′-TGGAGCCGGTGTCGTCA-3′. For overproduction of CysB protein, the *K. aerogenes cysB* coding sequence was amplified from pKA29 (Lynch, 1989) using oligonucleotides CB1 and CB2 as primers in PCR reactions. CB1 directs the introduction of an *Eco*RI site 17 bp upstream of the ATG codon and a consensus Shine–Dalgarno sequence at an optimal location. CB2 introduces a *Pst*I site at a position 176 bp downstream of the coding sequence. The PCR product generated from pKA29 using CB1 and CB2 (a 1182 bp fragment) was digested with *Eco*R1 and *Pst*1, ligated to similarly cut pKK223-3 DNA and the ligation mixture was used to transform *E. coli* JM101 (Brosius and Holy, 1984). Cells harbouring plasmid pKKCysB, in which transcription of the *cysB* gene is under the regulation of the P_{tac} promoter, were identified among ampicillin-resistant colonies.

Two DNA fragments were prepared by PCR amplification and employed as ligands in binding assays of *K. aerogenes* CysB protein. A 314 bp fragment that includes the *K. aerogenes cysB* promoter and flanking sequence (bases −299 to +15 in Figure 1) was prepared by PCR amplification using pKA30 DNA as a template and the oligonucleotides CB3 and CB4 as primers. Similarly, a 364 bp fragment encompassing the *S. typhimurium cysJIH* promoter (Ostrowski and Kredich, 1989) was generated by PCR amplification from plasmid pRSM15 template using the oligonucleotides CB5 and CB6 which are, respectively, complementary to nucleotides −175 to −159 and +189 to +173 of the *S. typhimurium cysJIH* promoter (Ostrowski and Kredich, 1989).

Purification of *K. aerogenes* CysB protein from *E. coli*

E. coli JM101, harbouring the plasmid pKKCysB, were grown to an A_{600} value of 1 in 5 l of Luria–Bertani medium (Sambrook et al., 1989) containing 100 mg/l ampicillin with vigorous aeration at 37 °C. Expression of recombinant CysB protein was induced by the addition of isopropylthiogalactoside to a final concentration of 1 mM and the cells were shaken for a further 5 h before harvesting by centrifugation and freezing at −70 °C. Recombinant *K. aerogenes* CysB protein was purified from clarified cell lysates using streptomycin sulphate and $(NH_4)_2SO_4$ precipitation steps essentially as described by Miller and Kredich (1987) for the purification of the *S. typhimurium* CysB protein. The CysB fraction, in buffer A (50 mM Tris/HCl buffer, pH 7.5/1 mM ETDA) plus 1 M $(NH_4)_2SO_4$, was loaded on to a phenyl-Sepharose column (52 ml). The column was developed with a 700 ml descending $(NH_4)_2SO_4$ (1 M to 0 M) gradient in buffer A. CysB eluted towards the end of the gradient [50 mM $(NH_4)_2SO_4$]. Finally, the protein, in 20 mM Tris/HCl buffer (pH 7.5), was loaded on to a Pharmacia Mono Q column (1 ml), and eluted with a 40 ml NaCl (0–1 M) gradient. CysB protein elutes at

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K.a  GCGACCGGCTGGTCAGCTTTCTTTATCGACGGTAAGTGGACGGAAGCCAAAAAGTAATGGCGTTCGTTCGGGCAGGAAA (-325)
K.a  A  T  G  W  S  A  F  F  I  D  G  K  W  T  E  A  K  K  *

     AGCGCGCTTTTCCTGCTCAGGCGACCGGCAGAGAGAGTGCATTTCTCTGCCGTGTCGTCTTCCCCTCTTTGCGTTAATTTC (-244)
     CTCCCCTTATAACCACCTATATTATTTTTTCGTTAGCGTCTATACAGTGTTGATATAAATGATATAGTGGTTATAGTTAAT (-163)
     GCCTTTTTTATTATTAAAACGTCTTATACATCATCGCTTTCAGGATGCATTGCGGCGCCACTATGGCCAACTGCCGGGAGC (-82)
     CCAGGGCCTTCATCGGCGACAAGTTGCCTGAACGTAAAGTGTATTAAGCGGCTTCATTTTACGCGCAAACGGATATCAGCC

K.a  ATGAAACTACAACAGCTTCGCTACATCGTTGAGGTGGTTAACCATAACCTGAATGTTTCATCCACCGCCGAAGGGCTCTAT (81)
K.a  M  K  L  Q  Q  L  R  Y  I  V  E  V  V  N  H  N  L  N  V  S  S  T  A  E  G  L  Y

K.a  ACCTCCCAGCCGGGTATCAGTAAGCAGGTCCGCATGCTGGAGGATGAGCTGGGCATTCAGATCTTTGCCCGCAGCGGTAAA (162)
K.a  T  S  Q  P  G  I  S  K  Q  V  R  M  L  E  D  E  L  G  I  Q  I  F  A  R  S  G  K
E.c                                                                   S

K.a  CACTTAACCCAGGTGACGCCGGCCGGGCAGGAGATCATCCGCATCGCCCGCGAAGTGCTGTCGAAAGTCGATGCCATCAAA (243)
K.a  H  L  T  Q  V  T  P  A  G  Q  E  I  I  R  I  A  R  E  V  L  S  K  V  D  A  I  K

K.a  TCTGTCGCCGGAGAGCATACCTGGCCTGACAAAGGGTCTCTTTACGTCGCCACCACCCATACCCAGGCGCGCTATGCGTTA (324)
K.a  S  V  A  G  E  H  T  W  P  D  K  G  S  L  Y  V  A  T  T  H  T  Q  A  R  Y  A  L
S.t                                                 I
E.c                                                 I

K.a  CCGGGAGTGATCAAGGGGTTTATTGAACGCTACCCGCGGGTGTCGCTGCATATGCATCAGGGCTCGCCGACTCAGATTGCG (405)
K.a  P  G  V  I  K  G  F  I  E  R  Y  P  R  V  S  L  H  M  H  Q  G  S  P  T  Q  I  A
E.c     N

K.a  GAGGCTGTGTCGAAAGGCAATGCCGATTTCGCCATCGCCACCGAGGCGCTACATCTGTATGACGATCTGGTGATGCTGCCA (486)
K.a  E  A  V  S  K  G  N  A  D  F  A  I  A  T  E  A  L  H  L  Y  D  D  L  V  M  L  P
E.c  D                                                           E

K.a  TGCTATCACTGGAACCGGTCGATTGTAGTGACCCCCGAGCATCCTCTGGCCACCAAGGCTTCGGTGTCCATAGAGGAACTG (567)
K.a  C  Y  H  W  N  R  S  I  V  V  T  P  E  H  P  L  A  T  K  G  S  V  S  I  E  E  L
S.t                                      D              A  T  S        T        A
E.c                    A                 D              G     K  A  I  T

K.a  GCCCAGTATCCATTGGTAACCTATACCTTTGGTTTTACCGGCCGCTCCGAACTGGATACCGCGTTTAACCGTGCGGGCCTG (648)
K.a  A  Q  Y  P  L  V  T  Y  T  F  G  F  T  G  R  S  E  L  D  T  A  F  N  R  A  G  L

K.a  ACGCCGCGCATTGTTTTTACCGCCACCGATGCCGACGTCATCAAAACCTATGTTCGCCTGGGGCTGGGGGTAGGGGTAATC (729)
K.a  T  P  R  I  V  F  T  A  T  D  A  D  V  I  K  T  Y  V  R  L  G  L  G  V  G  V  I

K.a  GCCAGCATGGCGGTGGATCCGGTCTCTGACCCGGATCTGGTGAAGCTGGATGCGAACGGTATTTTCAGCCACAGCACCACT (810)
K.a  A  S  M  A  V  D  P  V  S  D  P  D  L  V  K  L  D  A  N  G  I  F  S  H  S  T  T
S.t                       L  A                 R  I        H  D
E.c                       L  A                 R  I        H  D

K.a  AAGATAGGTTTCCGCCGTAGCACCTTCTTACGAAGCTACATGTATGATTTTATTCAACGTTTTGCTCCCCATTTGACCAGG (891)
K.a  K  I  G  F  R  R  S  T  F  L  R  S  Y  M  Y  D  F  I  Q  R  F  A  P  H  L  T  R

K.a  GACGTGGTAGATACCGCGGTGGCTTTACGCTCGAATGAAGATATTGAAGCGATGTTCAAAGATATAAAACTTCCGGAAAAA (972)
K.a  D  V  V  D  T  A  V  A  L  R  S  N  E  D  I  E  A  M  F  K  D  I  K  L  P  E  K
S.t                                         E                 Q
E.c              A                          E        V        Q

K.a  TAACCCTGAAAATAATAATGGCACTCCCTGCGAGGAGTGCCATTAACTATGATGGACTGTATTTAACAACTTAAACCTCGA (1053)
     ACCAATCACAAAATTAATAATTTTTGGCAACACGTTTTTCATTATGCCCGCCGCGATCAAATAACCGGTTAAATATATTCC (1134)
     ATCAGGCCTAAATTCACTGGATTAATCTTTTATCCATAATTAAGATTTATCTGTACCTGATGATTTGAGCAATTGGTGGTG (1215)
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Figure 1 DNA sequence of the *topI-cysB* region of *Klebsiella aerogenes* NCTC 418

The 1537 nucleotide sequence shown includes the 3′ end of the *topI* gene (−403 to −347), the *topI-cysB* intergenic region (−346 to −1), the *cysB* coding sequence (1 to 972), and sequence downstream of the *cysB* gene (976 to 1215). The predicted amino acid sequence of the *K. aerogenes* CysB protein is shown below the DNA sequence; in addition the *E. coli* (E.c) and *S. typhimurium* (S.t) CysB protein sequences are shown at positions where there is a divergence from the *K. aerogenes* (K.a.) CysB protein sequence. Two sequences which form perfect inverted repeats are indicated (by opposed arrows) downstream of the *topI* gene, together with a single such sequence located downstream of the *cysB* gene sequence.

0.25 M NaCl and appears as a single band on both Coomassie Blue-stained SDS/polyacrylamide and isoelectric focusing gels. Protein concentrations were determined using the Coomassie Blue Protein Assay reagent kit (Pierce) with BSA employed as a standard.

Gel-retardation assays

DNA binding by purified CysB protein was assayed by gel-mobility shift (Fried and Crothers, 1981; Garner and Revzin, 1981) under similar conditions to those described by Ostrowski and Kredich (1991). Binding assays were carried out in 20 μl reaction volumes containing end 5′^{32}P-labelled DNA fragments (approx. 20 ng) and various amounts of CysB protein (0–8 μg/ml) in a binding buffer consisting of 40 mM Tris/HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.1 mg/ml BSA; where indicated, *N*-acetylserine was included in the binding reaction buffer at a final concentration of 8–16 mM. The reactions were incubated at 25 °C for 5 min. Gel loading buffer (3 μl) (80 mM Tris/HCl, pH 8.0, 200 mM KCl, 2 mM DTT, 50% (v/v) glycerol, 0.2 mg/ml BSA and 0.1% Bromophenol Blue) was added and the samples loaded on to 5% (w/v)

acrylamide (82:1, acrylamide/bis-acrylamide) gels (0.1 cm × 6 cm) which had been pre-run at 3 V/cm for 30 min. Electrophoresis was performed at 10 V/cm for 1 h in 10 mM Tris/HCl, pH 8/1 mM EDTA. Radiolabelled DNA bands were visualized by autoradiography.

Fluorescence emission spectroscopy

Fluorescence emission spectra were measured in a Perkin–Elmer LS-5B luminescence spectrometer attached to a 3700 Data Station. The excitation wavelength used was 280 nm with a bandpass of 10 nm. CysB protein was diluted to a concentration of 3 μg/ml (21 nM tetramers) in a quartz cuvette in either Tris (50 mM), bis-tris (50 mM) or citrate (50 mM) buffers ranging from pH 8.0 to pH 5.0, in the presence and absence of *O*-acetylserine or *N*-acetylserine at 25 °C. Fluorescence emission was monitored over the wavelength range 300–400 nm, employing a slit width of 2.5 nm and a scan speed of 240 nm/min. In the titration experiments, 1–30 μl aliquots of ligand were added with a Hamilton syringe to 3 ml of diluted CysB protein and the value of the relative fluorescence was plotted at 340 nm. The fractional saturation (v) was calculated as $(F_i - F_0/F_{\text{max.}} - F_0)$, where F_i represents the relative fluorescence after the ith addition of ligand and F_0 and $F_{\text{max.}}$ are the values of the relative fluorescence emission at 0 and 100 mM *N*-acetylserine respectively.

RESULTS AND DISCUSSION

Cloning and sequencing of the *cysB* gene of *K. aerogenes* NCTC 418

In a previous study, a *K. aerogenes* genomic library was screened by plaque hybridization, for sequences homologous to the *E. coli topA* gene (Lynch, 1989). A number of 'positive' clones were obtained which allowed a full characterization of the *K. aerogenes topI* gene encoding the DNA topoisomerase I of this species (Lynch, 1989). Two pBluescript-KS⁻ plasmid derivatives, pKA29 and pKA30, were subsequently constructed which harbour a 3.5 kb *Pst*I genomic DNA fragment cloned in opposite orientation with respect to the *lac* promoter of the vector. Sequencing of pKA29 and pKA30 suggested that the inserted fragment in these clones consists of 1.7 kb of coding sequence at the 3′-end of the *topI* gene followed by 1.8 kb of downstream sequence (Lynch, 1989). In *E. coli* and *S. typhimurium* the genes encoding the DNA topoisomerase I and CysB proteins are tightly linked, with 213 and 413 bp respectively separating the coding sequences (Ostrowski et al., 1987); should this genetic organization and tight linkage be conserved in *K. aerogenes*, it was expected that pKA29 and pKA30 would contain the *K. aerogenes cysB* gene. Two different *E. coli cysB* mutant strains (DM700 and AB1369) were therefore transformed with pKA29 and pKA30 and ampicillin-resistant transformants of each were screened for growth on suitably supplemented, cysteine-deficient M9 minimal medium containing 50 mg/l ampicillin. Both plasmids complemented the cysteine auxotrophic phenotypes of *E. coli* DM700 and AB1369, suggesting that they encode a CysB-like protein. It is clear from these results and previous observations that the *cysB* gene product is functionally interchangeable among *E. coli*, *S. typhimurium* and *K. aerogenes* and therefore that the essential transcriptional regulatory components of the cysteine regulon are conserved among the three species.

The nucleotide sequence of 1.6 kb of genomic DNA immediately downstream of the *topI* gene has been determined. The sequence, shown in Figure 1, contains an open reading frame which is predicted to encode a protein with high sequence similarity to the CysB proteins of *E. coli* and *S. typhimurium*. This 972 nucleotide open reading frame has coding sequence for a protein of 324 residues which differs from the *S. typhimurium* and *E. coli* CysB proteins in only 15 and 21 positions respectively; most of the amino acid replacements are conservative. This degree of sequence identity among homologous proteins from the three species is lower than that observed for the catabolite activator protein (Osuna and Bender, 1991), but significantly higher than that observed between the β-galactosidase genes of *K. aerogenes* and *E. coli* (Buvinger and Riley, 1985).

The *topI-cysB* intergenic distance in *K. aerogenes* is 346 nucleotides which is intermediate between those previously observed for *E. coli* and *S. typhimurium* (Ostrowski et al., 1987). Two sequences which form perfect inverted repeats are located immediately downstream of the *topI* gene and may be involved in termination of the *topI* mRNA transcript; inverted repeat sequences resembling ρ-independent transcription terminators are located downstream of the *E. coli* and *S. typhimurium topA* genes (Ostrowski et al., 1987, Tse-Dinh and Wang, 1986). An inverted repeat sequence located 12 bp downstream of the gene may be involved in transcription termination of the *cysB* mRNA.

The transcriptional start site for the *K. aerogenes cysB* mRNA has not been determined though a comparison of the sequence of the *topI-cysB* intergenic region with those of *S. typhimurium* and *E. coli* reveals a number of sequence elements likely to be important for transcriptional regulation (Figure 2a). The −35 and −10 promoter elements for the major *cysB* mRNA start site of *S. typhimurium* are indicated (Ostrowski and Kredich, 1991);

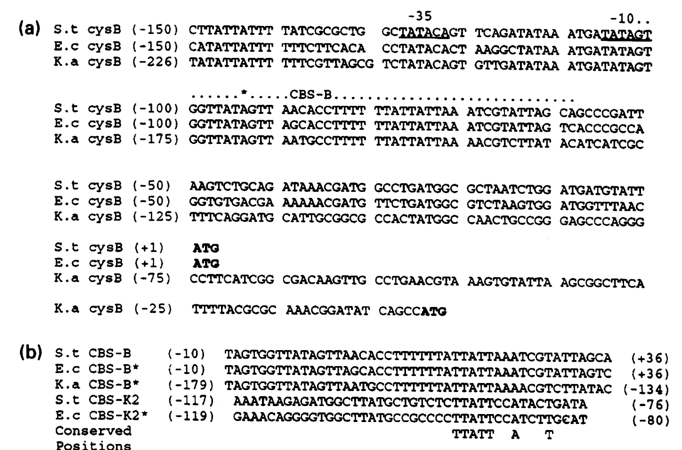


Figure 2 A comparison of the DNA sequences located upstream of the initiation codons of the *cysB* genes of *S. typhimurium* (S.t), *E. coli* (E.c) and *K. aerogenes* (K.a)

(**a**) The numbering is relative to the ATG initiation codon, shown in bold type. The −35 and −10 'consensus' promoter elements corresponding to the major *in vivo* transcriptional start-point (denoted by an asterisk) of the *S. typhimurium cysB* mRNA (Ostrowski and Kredich, 1991) are underlined. The minimal binding site for the *S. typhimurium* CysB protein determined by DNAase I protection studies (nucleotides −10 to +36) is indicated above the *S. typhimurium* sequence by a dotted line. (**b**) An alignment of DNA sequences to which the binding of CysB protein has been demonstrated to be, or is expected (denoted by asterisks) to be, inhibited by acetylserine. The CBS-K2 site is located upstream of the CBS-K1 site in the *cysK* promoter region of *S. typhimurium* and *E. coli* (Monroe et al., 1990). Acetylserine inhibits CysB binding to the former while enhancing the binding to the latter. The numbering system is relative to the major transcriptional start points *in vivo* for each gene except for the *K. aerogenes* sequence where the numbering system is the same as that used in Figure 1. The conserved positions are shown on the bottom line.

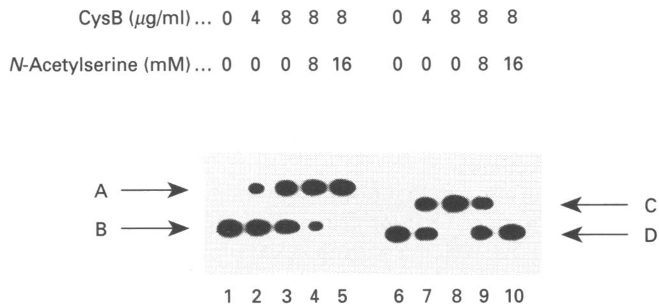


Figure 3 Binding of *K. aerogenes* CysB protein to DNA monitored by gel-mobility-shift assays

Lanes 1–5 the *S. typhimurium cysJIH* promoter fragment (20 ng/lane) and lanes 6–10 the *K. aerogenes cysB* promoter fragment (20 ng/lane). The DNA fragments (in binding buffer) were incubated in the presence of the indicated concentrations of CysB protein and acetylserine. Arrows B and D indicate the bands associated with the free *cysJIH* and *cysB* promoter fragments respectively, while arrows A and C indicate the bands associated with the corresponding fragments in complex with CysB protein. Acetylserine enhances the CysB-dependent gel mobility shift of the *S. typhimurium cysJIH* promoter fragment and reduces the CysB-dependent gel mobility shift of the *K. aerogenes cysB* promoter fragment under the assay conditions employed.

these elements are perfectly conserved in the DNA sequences located upstream of the *E. coli* and *K. aerogenes cysB* genes. If, as is likely, these elements constitute the *cysB* promoter in *K. aerogenes*, then the 5′ untranslated portion of the mRNA is predicted to be 169 nucleotides, which is significantly longer than the 94 nucleotides observed in *S. typhimurium* and *E. coli.*

Also indicated in Figure 2(a) is the CBS-B site which represents the binding site for the CysB protein (CBS) as determined by DNAaseI footprinting of the *Salmonella cysB* promoter region (Ostrowski and Kredich, 1991). This site extends from position -10 to $+36$. An alignment of this sequence with other sequences to which CysB binding is inhibited by acetylserine, is shown in Figure 2(b). There are only seven positions at which identical bases occur in all five sequences.

DNA binding

To characterize the *K. aerogenes* CysB protein *in vitro*, the plasmid pKKCysB was constructed to direct overproduction of recombinant protein in *E. coli.* In this plasmid, the *K. aerogenes cysB* gene is under the transcriptional regulation of the P_{tac} promoter. CysB protein was purified to apparent homogeneity from IPTG-induced *E. coli* JM101 cells harbouring pKKCysB as described in the Materials and methods section. The yield of purified protein was estimated to be 5–10 mg/l of induced cells.

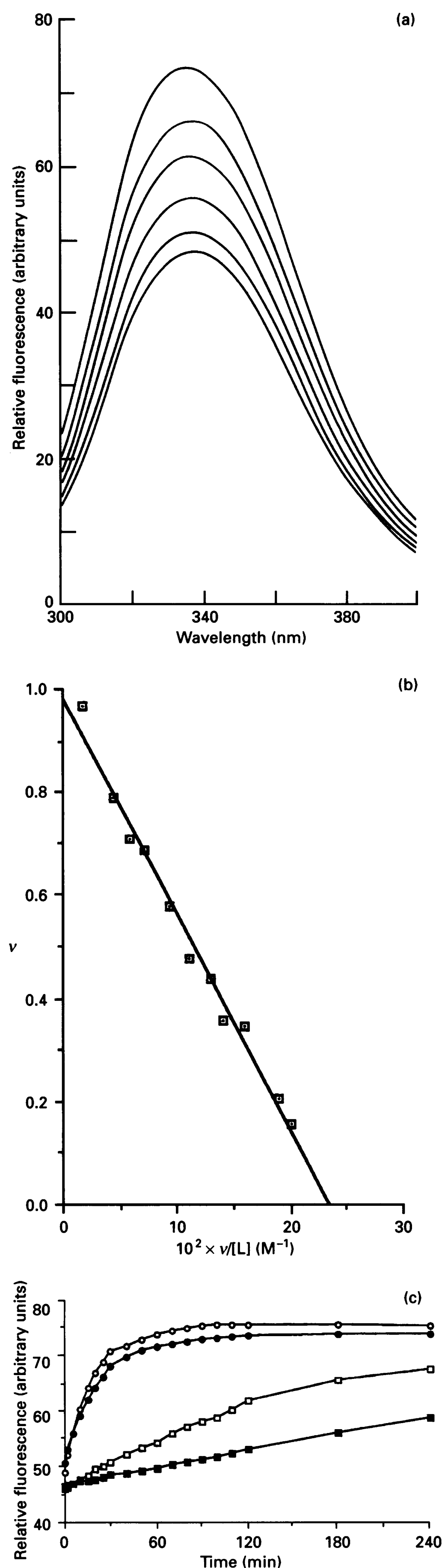
The binding of the protein to specific fragments of DNA was studied using gel-mobility-shift assays. First, a 314 bp PCR-amplified DNA fragment spanning the *K. aerogenes cysB* promoter (bases -299 to $+15$ in Figure 1) was incubated with CysB protein as described in the Materials and methods section. Figure 3 shows that CysB causes the electrophoretic migration of this DNA fragment to be retarded, presumably due to protein–DNA complex formation. The observed retardation of the DNA is substantially reversed by the inclusion of *N*-acetylserine in the incubation mix, suggesting that the cofactor lowers the affinity of CysB for its own promoter. From similar assays, in which the concentration of CysB protein present in the incubation mix was varied, we have estimated the value of the dissociation constant of the CysB–*cysB* DNA complex to be 10 nM (results not shown); a similar value (7 nM) was calculated for the complex of *Salmonella* CysB protein and a cognate *cysB* promoter fragment (Ostrowski and Kredich, 1991). Our estimate is based on the assumption that the protein has lost no activity during purification and storage and that it functions as a tetramer (Miller and Kredich, 1987). When 16 mM *N*-acetylserine is included in the incubation mix, the apparent K_d increases at least 25-fold.

A 364 bp fragment encompassing the *S. typhimurium cysJIH* promoter was amplified by PCR from pRSM15 (Ostrowski and Kredich, 1989) and used as the DNA ligand in similar experiments. As is shown in Figure 3, preincubation of this fragment with purified *K. aerogenes* CysB protein resulted in the appearance of a new electrophoretic band consistent with the formation of the more slowly migrating protein–DNA complex. In the presence of *N*-acetylserine the faster-migrating DNA band disappeared and all of the DNA was retarded, implying that *N*-acetylserine increases the affinity of CysB for the *cysJIH* DNA, as was previously observed for binding of the *S. typhimurium* CysB protein to a DNA fragment containing the same promoter (Ostrowski and Kredich, 1989). Titration of the fraction of the DNA that is shifted in the gels, as a function of the CysB concentration, allowed a value of 50 nM to be estimated for the dissociation constant of the complex, which dropped to less than 5 nM in the presence of 16 mM *N*-acetylserine (results not shown).

Thus the inducer, *N*-acetylserine, has opposing effects on DNA binding by *K. aerogenes* CysB protein, that are dependent on the nature of the sequence presented. The affinity of CysB for the *cysJIH* promoter fragment is increased *in vitro* by *N*-acetylserine, consistent with the role of the cofactor as a co-activator in the protein's *in vivo* action as a positive regulator at this promoter. In the case of the *cysB* promoter fragment, the inhibition of CysB binding by *N*-acetylserine observed *in vitro* supports the idea that the cofactor acts as an inducer of *cysB* expression by blocking the action of CysB protein as a repressor at this promoter. These findings are in agreement with observations of Kredich and co-workers (Ostrowski and Kredich, 1989, 1991). Similar-length DNA fragments, which lack CysB regulatory sequences, are retarded only at significantly higher concentrations of CysB protein, and this retardation is insensitive to *N*-acetylserine (results not shown).

Cofactor binding

The binding of acetylserine to CysB protein has proved intractable to direct assay by equilibrium dialysis, because of the limited solubility of the protein (Kredich, 1992). Instead, cofactor binding has previously been inferred only indirectly through its effects on the interaction between CysB protein and promoter sequences, in the type of experiment described above. There is a second complication in experiments assaying cofactor binding, which arises because *O*-acetylserine, the precursor of cysteine *in vivo*, is unstable *in vitro* with respect to an acetyl migration reaction which produces *N*-acetylserine. This rearrangement occurs spontaneously in aqueous solutions at room temperature with a half-time that decreases markedly with pH; it has been measured at 70 and 3 min at pH 7.5 and 8.5 respectively at 29 °C (Flavin and Slaughter, 1965). As a consequence, experiments utilizing *O*-acetylserine will contain varying amounts of *N*-acetylserine depending on the age of the solution and its pH. This reaction may well have a physiological significance, because it has been shown that *N*-acetylserine acts as an inhibitor of CysB binding to the *cysB* promoter and is 10–30 times more potent than *O*-acetylserine as an inducer of *cysJIH* transcription *in vitro* (Ostrowski and Kredich, 1989, 1991).



Fluorescence emission spectroscopy has proved to be a valuable and sensitive approach to monitoring protein–ligand interactions. For CysB and *O*-acetylserine binding, it offers the advantage that measurements can be made after short incubation times, before significant concentrations of *N*-acetylserine are allowed to accumulate.

The bottom curve in Figure 4(a) shows a fluorescence emission spectrum of purified CysB protein in Tris/HCl buffer (pH 7.5) in the region of 300–400 nm with the excitation wavelength set at 280 nm. In the initial experiments purified CysB protein was incubated with increasing concentrations of *N*-acetylserine. Fluorescence emission spectra were recorded, typically 0.5–5 min after mixing, and these are presented in Figure 4(a). There is a large (up to 60 %) enhancement of the protein fluorescence across the emission spectrum as the protein is titrated with *N*-acetylserine. Under these conditions, it is expected that the principal fluorophores will be the indole side chains of tryptophan residues, of which CysB protein has only two, so that the magnitude of the fluorescence increase is not surprising. There was no detectable change in the protein fluorescence upon addition of serine, cysteine or *N*-acetylcysteine at concentrations up to 100 mM, nor were any of these species able to block or reverse the enhancement of the fluorescence emission brought about by *N*-acetylserine. Solutions of free acetylserine in buffer exhibited no measurable fluorescence under these conditions.

These observations are consistent with the interpretation that the change in protein fluorescence is the result of specific binding of *N*-acetylserine to CysB protein, with the ligand either directly or indirectly altering the environment of either one or both tryptophan residues. In this context, it is interesting to note that the mutant *E. coli* strain *cysB484*, a cysteine auxotroph that cannot be de-repressed for the cysteine regulon, carries a Trp-186 → Arg mutation in CysB (Kredich, 1992; Colyer and Kredich, unpublished work); this tryptophan is conserved in the *K. aerogenes* CysB protein (Figure 1). From a Scatchard plot of the titration data, the dissociation constant of the CysB–*N*-acetylserine complex and the stoichiometry of ligand binding were estimated to be 4.1 ± 0.6 mM and one per monomer respectively (Figure 4b).

In a second experiment, the fluorescence emission spectrum of a solution containing CysB protein and freshly prepared *O*-acetylserine was recorded at pH 8.0. For *O*-acetylserine we also observed an enhancement of the protein fluorescence emission; however, in this case the enhancement was time-dependent as shown in Figure 4(c) (open circles). The protein fluorescence emission increases with time, over a period of 2 h. In contrast, when *N*-acetylserine was added to CysB protein, the increase in the intrinsic protein fluorescence was immediate and invariant with time. In a further experiment, CysB protein was mixed with

Figure 4 Effect of acetylserine on the fluorescence emission spectrum of purified CysB protein

(**a**) The relative fluorescence emission of CysB protein at a tetramer concentration of 21 nM (3 μg/ml) was measured over the wavelength range of 300–400 nm. From bottom to top, fluorescence of the protein in Tris/HCl buffer, pH 7.5, 25 °C at 0, 1, 2.5, 4.5, 12 and 60 mM *N*-acetylserine. The excitation wavelength was 280 nm. (**b**) Scatchard plot of the fractional saturation *v* versus *v*/[L] where L is *N*-acetylserine. *v* was calculated as described in the Materials and methods section. These results suggest a stoichiometry of one *N*-acetylserine residue binding per CysB monomer with a K_d value of 4.1 ± 0.6 mM. (**c**) Time course of the fluorescence emission at 340 nm after the addition of freshly prepared *O*-acetylserine (30 mM) to CysB protein at pH 8.0 (○), pH 7.0 (●), pH 6.0 (□) and pH 5.0 (■). Fresh *O*-acetylserine stock solutions were made up from the solid before each time course and were immediately added to the incubation mix. It was established at the end of each time course (i) that the pH had not dropped by more than 0.05 pH units, and (ii) that addition of *N*-acetylserine to 60 mM brought about an immediate increase in the relative fluorescence to a value of 75–77.

freshly prepared solutions of *O*-acetylserine at a series of pH values in the range of 5 to 8 and fluorescence emission spectra were recorded at time intervals up to 4 h. As shown in Figure 4(c), the rate of appearance of the fluorescence enhancement decreased as the pH was lowered. This behaviour was not observed when this experiment was repeated with *N*-acetylserine which produced an immediate and time-invariant enhancement of the fluorescence at each of the pHs tested (results not shown). Furthermore, the fluorescence change induced by *N*-acetylserine was not inhibited by *O*-acetylserine regardless of the order in which these reagents were added to the protein sample.

These results are consistent with the interpretation that CysB protein has a much higher affinity for *N*-acetylserine than it does for *O*-acetylserine, and that the fluorescence enhancement following the addition of *O*-acetylserine is due to the binding of *N*-acetylserine formed in the transacylation reaction. Indeed, the time course of the fluorescence enhancement correlates well with the time course of the conversion of the *O*-acetylserine into *N*-acetylserine monitored by infra red spectroscopy (results not shown). As the pH is lowered, the rate of the acetyl migration reaction slows down and this is reflected in the lower rate of appearance of the fluorescence change. Although these fluorescence experiments collectively provide evidence that *N*-acetylserine is the strongly preferred ligand of CysB, it is possible that *O*-acetylserine is binding to the protein in an alternative mode or at a second site and that this binding does not cause a change in the intrinsic protein fluorescence. *O*-Acetylserine and *N*-acetylserine are, however, structurally dissimilar, both stereochemically and in their net charge at neutral pH, so that it seems unlikely that these two species could be accommodated at the same site in the protein.

In addition to elevated concentrations of acetylserine, derepression of the cysteine regulon in *S. typhimurium* and *E. coli* also requires sulphur limitation. It has been reported that sulphide and thiosulphate are able to inhibit competitively acetylserine-stimulated promoter binding and transcription initiation mediated by CysB (Ostrowski and Kredich, 1990; Hryniewicz and Kredich, 1991). Neither sulphide nor thiosulphate caused any changes in the CysB protein fluorescence emission spectrum at concentrations in the range of 0–50 mM, nor did addition of these species serve to reverse or diminish the fluorescence enhancement caused by *N*-acetylserine (results not shown).

The fluorescence data presented here provide the first direct measurements of cofactor binding to CysB protein. Moreover, the observations imply that the effects that acetylserine exerts on the binding of CysB protein to promoter DNA fragments in the gel-retardation assays are caused exclusively by the N-acetylated species. This conclusion is supported, and given a functional significance, by the greater potency of *N*-acetylserine relative to *O*-acetylserine in *in vitro* transcription assays of *S. typhimurium* CysB protein, and suggests strongly that *N*-acetylserine may be the intracellular inducer of the cysteine regulon (Ostrowski and Kredich, 1989).

Concluding remarks

CysB protein from *K. aerogenes* is closely similar in its primary structure and solution behaviour to its well-characterized homologue from *S. typhimurium*. The fluorescence measurements reported here demonstrate *N*-acetylserine binding to the apoprotein and provide strong evidence that *N*-acetylserine, rather than *O*-acetylserine, is the physiological inducer of cysteine biosynthesis. As *N*-acetylserine is likely to be the predominant intracellular form of free acetylserine, it presumably represents a more suitable species for the bacterial cell to use to signal a readiness to synthesize cysteine.

The different locations of the CysB-binding site relative to the transcription start sites for *cysJIH* (−76 to −35) and *cysB* (−10 to +36) account for the observation that CysB binding activates transcription at the former promoter while inhibiting it at the latter. However, the mechanism by which *N*-acetylserine enhances the binding of CysB protein to the *cysJIH* promoter and simultaneously inhibits its binding to the *cysB* promoter has not been resolved and remains an open and intriguing question. Overexpression of CysB protein is a first step towards the goal of the studies initiated here, which is the crystallization and determination of the X-ray crystal structure of CysB. We have recently obtained small crystals of intact CysB protein, which are, as yet, unsuitable for X-ray analysis (R. Tyrrell, unpublished work). Encouragingly, we have better quality crystals of a 233 residue N-terminal chymotryptic fragment of the protein which possesses DNA- and cofactor-binding properties (Tyrrell et al., 1994). These crystals diffract to 2.1 Å spacing, and work is in progress to determine their structure. There is currently no three-dimensional structural information on any member of the LysR family proteins, lending these studies a wider significance.

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