Functional Dissection of the LysR-type CysB Transcriptional Regulator

REGIONS IMPORTANT FOR DNA BINDING, INDUCER RESPONSE, OLIGOMERIZATION, AND POSITIVE CONTROL*

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CysB is a tetrameric LysR-type transcriptional regulator that acts as an activator of cys regulon genes and as an autorepressor. Positive control of cys genes requires the presence of the inducer N-acetylserine. Following random and site-directed mutagenesis of the cysB gene, 20 CysB variants were isolated. Six single amino acid substitutions within the N terminus of CysB abolished the DNA-binding ability of the protein. Seven mutations in the central region of CysB affected its response to the inducer. Four of these CysB mutants retained repressing activity, but lost their activating function in vivo. Their DNA binding characteristics were consistent with an inability to respond to acetylserine by a qualitative change in the DNA-protein interaction. Three of the single residue substitutions resulted in constitutive activity of CysB. The electrophoretic mobility of the complex formed by one of the CysB* variants with the cysP promoter suggested a dimeric state of this protein. Characteristics of six truncated CysB variants lacking 5–30 C-terminal residues indicated the involvement of the C terminus in the DNA binding, oligomerization, and stability of CysB. The single substitution Y27G resulted in the CysB** variant, able to bind DNA and to respond to the inducer by a qualitative change in the DNA-protein complex, but defective in the positive control of the cysP promoter.

LysR-type transcriptional regulators (LTTRs), initially reported by Henikoff et al. (1), compose probably the largest family of prokaryotic regulatory proteins. Since the last systematic review (2), in which some 50 LTTRs were mentioned, the family has expanded to over 100 members identified in diverse bacterial genera. In Escherichia coli K12, the overall repertoire of DNA-binding transcriptional regulators, estimated as 314 proteins (3), includes 45 LTTRs (18 known and 27 predicted). All LTTRs investigated so far are DNA-binding proteins that positively regulate transcription of target genes, and most of them also repress their own expression. The common family features are the similar size of the molecule (between 300 and 350 amino acids), the formation of either homodimers or homotetramers, the presence of the helix-turn-helix (HTH) motif in the N-terminal region, and the requirement for a small molecule that acts as coinducer (2). The homology between LTTRs is generally high, suggesting their evolution from a common distant ancestor and the possibility of their similar tertiary structure. The highest sequence similarity exists within the 66 N-terminal amino acids, containing a HTH motif and proposed to function as a DNA-binding domain. The central region of the LysR-type proteins shows the lowest sequence homology between family members. Mutational studies performed on some proteins suggest that two subdomains of this region (amino acids 95–173 and 196–206) can be engaged in coinducer recognition/response (2, 4–6). The regions involved in multimerization of LTTRs are not well defined. Some experimental results obtained for NahR (7), AmpR (8), and OxyR (9) indicate involvement of the C-terminal domain in oligomer formation. Several LysR-type proteins, including CysB, have been suggested to interact with the C-terminal domain of the RNA polymerase α-subunit (10). However, to date, no study has clarified the details of the putative contact(s) of any LTTR with the RNA polymerase. Structural characterization of LysR-type proteins has been delayed by the fact that many of the family members are insoluble when overexpressed and difficult to obtain in highly purified form.

CysB, a LysR family member, is an essential positive factor for activity of most cys genes engaged in the assimilatory sulfate pathway via cysteine biosynthesis (see Ref. 11 for a review). High-level expression of these genes, composing the cysteine regulon, requires, in addition to CysB, the presence of an inducer (N-acetyl-L-serine) and sulfur limitation. CysB acts also as a repressor of its own gene (12, 13). The interactions of CysB with responsive promoter regions are unusually complex (11, 14). DNA regions identified as “CysB-binding sites” show poor sequence homology. In general, they share a configuration of imperfect dyad symmetry between 19-bp half-sites, but the number, spacing, and arrangement of half-sites vary in particular promoters. Wild-type CysB is a tetramer of identical 36-kDa subunits in solution (15), and it also binds to DNA as a tetramer (16). In positively regulated promoters (i.e. cysK and cysF), CysB binds in the absence of inducer and occupies a large DNA region including three half-sites (16–18). This mode of DNA-protein interaction results in DNA bending by ~100° and is unfavorable for transcriptional activation of the promoter. N-Acetylserine is thought to induce a conformational change in CysB; this change allows the protein to interact preferentially with “activatory sites,” composed of two half-sites spaced by 1–2 bp and localized just upstream of the ~35 regions of the
Construction of the ΔcysB Mutant—Deletion within the cysB gene was created by excision of the 821-bp {\textsc{NruI}}/{\textsc{HpaI}} fragment encompassing the promoter region and 216 codons of the cysB open reading frame (see Fig. 1) from plasmid pMH148, containing the 2.8-kilobase pair EcoRI/EcoRV fragment of pJOH2 cloned in pUC19 EcoRI/HinCl. The fragment was tagged by insertion of a Kan r cassette excised from pUC4KIXX as a 1.35-kilobase Smal fragment. The resulting plasmid, pMH161, was linearized and transformed into \textit{E. coli} JC7623. Kan r transformants were selected, and correct replacement of the cysB gene in the genomic DNA was confirmed by PCR analysis. The ΔcysB:kan mutation was transferred to other \textit{E. coli} strains (see Table \textit{I}) by P1-mediated transduction.

Oligonucleotides and Plasmid Constructions—The \textit{3}'-coordinates indicated for all \textit{cysB}-specific oligonucleotides used in this study are consistent with those of the published \textit{cysB} sequence (27), where \textit{+1} indicates \textit{A} in the ATG start codon. Nucleotides that had been changed to create restriction sites, stop codons, or specific mutations are shown in boldface. Primers ECCC8 (\textit{GGAATTC}TCAATGCGATGTTAAACA-G, \textit{+1} and 295) and ECCC9 (AAACTCAGCGCGCTTAATAGACAGC-3, \textit{+116}, complementary strand) served to amplify the promoterless wild-type \textit{E. coli} \textit{cysB} gene from the \textit{pJOH1} plasmid. The fragment obtained was cleaved with \textit{HindIII}/Spfl and inserted into pBR322 \textit{HindIII}/Spfl to give plasmid pMH147. Plasmids pMH189, pMH191, pMH193, pMH195, pMH222, pMH223, pMH224, pMH225, pMH226, pMH228, and pMH229 are derivatives of pMH146, containing random mutations and contain point mutations within \textit{cysB} (see Table \textit{II}). Plasmids pMH190, pMH192, pMH194, pMH196, and pMH197 were obtained from pMH189, pMH191, pMH193, pMH195, and pMH147, respectively, by excision of the \textit{HindIII}/Spfl fragments (containing the \textit{cysB} region) and their insertion into \textit{HindIII}/Spfl-cleaved pACYC184. Primers ECCC8 (\textit{GGAATTC}TCAATGCGATGTTAAACA-G, \textit{+1} and 295) and ECCC9 (AAACTCAGCGCGCTTAATAGACAGC-3, \textit{+116}, complementary strand) served to amplify the promoterless \textit{cysB} gene on the \textit{pJOH1} template, and the PCR product was cleaved with EcoRV/PstI and placed under the pua promoter in pTre99A cleaved with EcoRV/PstI to give plasmid pMH199. Plasmid pMH199 served for overproduction of WT \textit{cysB}. To overproduce \textit{cysB} mutant proteins, plasmids pMH200, pMH202, and pMH205 were obtained by place-}

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**—The \textit{E. coli} strains and basic plasmids used in this study are listed in Table \textit{I}. All strains were grown at 37 \textdegree\text{C} in either LB medium or sulfate-free minimal medium (24) supplemented with glucose (0.2%), tryptophan (4 \textmu g/ml), and a sulfur source. Sulfate (0.5 mM) was used as a sulfur source in solid media, and were used according to the manufacturers’ recommendations. The dideoxy chain termination method (26) was used to sequence the \textit{cysB} region from plasmid templates. Most sequences were obtained using an ABI PRISM 377 DNA sequencer, and some plasmids were sequenced manually using a sequencing kit and \textit{α}-\textsuperscript{32}P-dATP (both from Amersham Pharmacia Biotech).
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inserted into pNM482 cleaved with BamHI/PstI to give pMH302. The cysB::lacZ region was excised from pMH302 as a 5.5-kilobase EcoRI/StuI fragment and cloned in the low-copy vector pHSG576 cleaved with EcoRI/StuI to give pMH303. Primers ECCR3 and ECCR11 (GGTCTGTCGCCTCTTCGGA-3′, +148, complementary strand) served to amplify the cysB promoter fragment from the pMH1122 template.

Primers ECCP1 (ATTGATGGCGGCAGCACACT) and ECCP2 (GACCGCCGAGTGACTCTTCT, complementary strand) were used to amplify the cysB promoter region (from positions +321 to +72 relative to the transcription start site) from the pMH1122 template.

RESULTS

Isolation and Initial Characterization of Random cysB Mutants—Assuming that the lack of positive control function of CysB can result from mutations impairing DNA-binding ability, inducer response, or RNA polymerase contacts, we used the cysPT::lacZ cysB strain (EC2266) (Table I) for selection of cysB mutants contained on plasmids. Since the expression of β-galactosidase in EC2266 is under the control of the CysB-dependent cysB promoter, the strain yielded white colonies on LB agar plates containing X-gal and dark-blue colonies in the presence of plasmid pMH147 carrying WT cysB. After transformation of EC2266 with several samples of mutagenized pMH147, 32 white or pale-blue transformant colonies were selected. To determine the location and nature of the mutations, the cysB genes contained on plasmids were sequenced entirely. This allowed us to exclude mutants resulting from generation of an early termination codon within the cysB open reading frame (with the exception of the Q5Ter mutant, which served as a control in some experiments) and several mutants resulting from identical nucleotide change. Ten plasmids containing cysB with mutations that caused single residue substitutions within the CysB protein (Table II) were analyzed further. None of these plasmids complemented the Cys− phenotype of two cysB strains tested (Table II). To distinguish between repressing and non-repressing cysB mutants, the corresponding plasmids were screened for their ability to affect the expression of the translational cysB::lacZ fusion, encoded by the low-copy number plasmid pMH303 in the ΔcysB background (EC2549 strain). As shown in Table II, the presence of WT cysB reduced the expression of β-galactosidase in this strain to ~4%. Decreased levels of cysB::lacZ expression (2.6–5.6%) were also observed with four plasmids encoding CysB variants M160I, T196I, A244V, and A247E. The same mutants cloned in pA-CYC184 reduced to ~5% the expression of the chromosomal fusion cysB::Mad1Ap,lacZ in strain EC1171 (data not shown). We concluded that repressing CysB proteins M160I, T196I, A244V, and A247E (positions of mutations shown in Fig. 1) expressed from these plasmids retained a DNA-binding ability. The remaining six CysB variants with single residue substitutions in the N-terminal portion of the protein (region 11–48 containing the HTH motif and its close vicinity; see Fig. 1) were defective to various extents in the repression of the cysB::lacZ fusion (Table II). These variants were therefore assumed to be impaired in DNA binding. The repression activities of plasmids carrying cysB mutants were also measured in strain EC1250 containing pMH303. The β-galactosidase activity in this strain represents partially repressed level of cysB::lacZ expression resulting from the activity of chromosomally encoded WT CysB; this value has been estimated as ~2.5 times lower than that in the EC2549 strain containing pMH303. The presence of plasmids encoding some of the non-repressing CysB variants (E11K, S20I, and T22I) in the pMH303-containing EC1250 strain resulted in elevation of the cysB::lacZ expression to 230–270% of the level characteristic for the strain (Table II). The effect observed could be consistent with the assumption of negative dominance of CysB proteins.
expressed from plasmids over chromosomally encoded WT CysB. In contrast, a plasmid expressing no CysB (Q5Ter mutant) and plasmids encoding CysB variants I48T, L44T, and I48T, E41K showed no such dominant-negative effect, as they did not express from plasmids over chromosomally encoded WT CysB monomers and formation of inactive mixed hetero-

plasmid Relevant genotype and characteristics Ref. or source

strain

E. coli strains

DH5α supE44 ΔlacZΔM15 lacY1ΔΔ (6005lacZΔM15) hisD17 recA1 endA1 gyrA96 thi-1 relA1 Laboratory collection

NK1 trpE5 leu-6 thi hsdR mB cysB N. M. Kredich 31

JC7623 F′ thr-1 ara-14 leu-6 proA2 lacY1 sbcC201 tss-33 gaK1K2 sbcB15 his4 recC22 recB21 rpsL31 xyl-5 mtl-1 argE3 thi-1

EC1250 MC1400 trp-3 12

EC2275 EC1250 cysB Trp+ P1(NK1) × EC1250

EC2249 EC1250 cysB Trp+ This study

EC2256 EC1250 cyaPT329-lacZ imm14 32

EC2266 EC2256 cysB Trp+ P1(NK1) × EC2256

EC2259 EC2256 cysB Trp+ P1(EC2549) × EC2256

EC1171 EC1250 cysB::Mud1(Ap, lacZ) Laboratory collection

NR4548 mutD1 zaf-13: Tn10 33

Plasmids

pBR322 Cloning vector, Ap’ Tet’ Amersham Pharmacia Biotech

pACYC184 Cloning vector, Cm’ Tet’ New England Biolabs, Inc.

pNM482 Vector for construction of lacZ fusions, Ap’ lacZY’ 34

pHS676 Cloning vector (low-copy number), Cm’ 35

pUC6K1XX ‘Ran’ B’le’ cartridge Amersham Pharmacia Biotech

pTcr99A Expression vector, Ap’, trc promoter, pBR322 origin Amersham Pharmacia Biotech

pJH1 Ap’ cysB (E. coli) cloned in pBR322 on EcoRV/HpaI fragment 27

pMH147 Ap’ cysB (E. coli) cloned in pBR322 on HindIII/Spal fragment This study

pMH199 Ap’, promoterless cysB (E. coli) cloned in pTcr99 under prv This study

pMAH91 Ap’, cysB 301 cloned in pBR322 32

pMAH2 Ap’, cysB 302 cloned in pBR322 32

pMAH3 Ap’, cysB 303 cloned in pBR322 32

pMH303 Cm’, cysB::lacZ cloned in pHS676 This study

pMH1822 Ap’, E. coli cysPT’ region cloned on 2.2-kb EcoRV fragment in pUC18 HincII Laboratory collection
single substitution I33N (37). It seems that some arrangement of subunits between CysB I33N and C-terminally truncated CysB variants allows formation of a protein able to positively control the cysP promoter. Our attempts to overproduce CysB mutants lacking >16 C-terminal residues for in vitro experiments failed, which may suggest an instability of these CysB variants. It is therefore possible that the C terminus contributes to the overall stability of the protein, and the instability of the C-terminally truncated CysB variants might explain the observed abortive complementation of the CysB I33N mutation.

DNA Binding Characteristics of Repressing and Constitutive CysB Variants—E. coli WT CysB and those CysB mutants that displayed repressing activity in vivo were cloned in the pTrc99A vector and overexpressed, and obtained extracts were fractionated as described under “Materials and Methods.” The extent of CysB overproduction in particular preparations was estimated by SDS-polyacrylamide gel electrophoresis (Fig. 3).

The DNA binding characteristics of the E. coli WT CysB preparation used in this work (referred to as E. coli WT CysB) were tested by EMSAs with a 393-bp DNA fragment containing the E. coli cysP promoter region (pCysB). The complexes formed by E. coli WT CysB with pCysP, shown in Fig. 4A, could be identified by strict correspondence to those formed by highly purified S. typhimurium CysB, analyzed in detail previously (16, 18). When a single CysB tetramer binds to pCysP, it induces DNA bending of $-100^\circ$ in the absence of acetylserine and of $-50^\circ$ in its presence, giving rise to “slow” (C1s) and “fast” (C1f) primary complexes, respectively. The C1f (fast) primary complex represents a preferential interaction of the CysB tetramer with the
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single activatory site located just upstream of the −35 region of the promoter. The inducer responsiveness of CysB can therefore be monitored by EMSA as a qualitative (mobility) and quantitative (amount) alteration of the primary complex (Fig. 4A, compare lanes 2 and 4 and lanes 5 and 6). We used EMSA to test three non-repressing CysB variants (A244V, A247E, and T196I) for DNA binding characteristics with \( p_{\text{cysP}} \). The results of this analysis showed that all three proteins formed complexes with \( p_{\text{cysP}} \) (Fig. 4, B and C), but the mobilities of C1 complexes were unaffected by acetylserine. For CysB variants A244V and T196I, these mobilities were exactly the same as that of WT CysB in the absence of inducer (Fig. 4, B, compare lanes 3 and 5–7; and C, compare lanes 3 and 8–10). In the case of CysB A247E, the C1 complex showed an intermediate mobility between slow and fast, irrespective of the presence of acetylserine (Fig. 4C, lanes 5–7), although the amount of complex was higher with the inducer. It should be stressed that none of the tested mutants, T196I, A244V, and A247E, was able to respond to the inducer by a qualitative change in the DNA-protein complex (reflected by complex mobility), which is critical for transcriptional activation of the promoter by WT CysB. Defects in inducer responsiveness observed in DNA binding studies correlated with the inability of corresponding CysB mutants to activate expression of the cysP promoter (as the cysPT::lacZ fusion) in vivo. Earlier studies on the autoregulatory function of S. typhimurium CysB (19) revealed that acetylserine reduces the affinity of the protein for a particular “repressory” binding site in the cysB promoter region. We have shown by EMSA (Fig. 5) that interaction of E. coli WT CysB with \( p_{\text{cysP}} \) displayed the same characteristics, i.e. there was less complex formed by a given amount of CysB in the presence of acetylserine than in its absence. In contrast, CysB mutant derivatives T196I, A244V, and A247E formed similar amounts of complex with the cysP promoter fragment, irrespective of the presence of inducer. This result further confirmed the involvement of CysB residues 196, 244, and 247 in response to the inducer and the non-inducible character of the T196I, A244V, and A247E variants.

We also analyzed three cysB mutants obtained previously in our laboratory and characterized as causing constitutive expression of sulfite reductase and O-acetylserine sulfhydrolase in E. coli (32). Sequencing of these mutants (cysB201, cysB302, and cysB303) identified the single amino acid substitutions A227D, Y164N, and Y197S, respectively, in encoded proteins (Fig. 1 and Table II). Plasmids carrying cysB mutants displayed wild-type repressing and activating functions in vivo in tests summarized in Table II, but they conferred constitutive (not inhibited by cysteine) expression of the cysPT::lacZ fusion in strain EC2266 (data not shown). Two of the CysB variants, Y164N and A227D, were overproduced, and they were characterized in EMSA with the cysP promoter fragment. The CysB Y164N protein formed a fast primary complex (C1f) with \( p_{\text{cysP}} \) in the absence of acetylserine, and the mobility of this complex corresponded exactly to that of the complex formed by WT CysB in the presence of inducer (Fig. 4D, compare lanes 4 and 5). It therefore seems that CysB Y164N was able to adapt the conformation suitable to interact with an activatory site in \( p_{\text{cysP}} \) without the aid of the inducer. However, an influence of acetylserine on CysB Y164N could be noticed as a quantitative effect on C1 complex formation and a further subtle increase in complex mobility (Fig. 4D, compare lanes 5 and 6). This result suggests that CysB Y164N, albeit active in vivo without the inducer, can still respond to it. It is possible that acetylserine induces some conformational change in CysB Y164N allowing formation of a complex in which DNA is bent less than in the fast complex formed by WT CysB. The complex formed by the other CysB variant, A227D, showed an unexpectedly higher electrophoretic mobility than complexes formed by WT CysB and CysB Y164N (Fig. 4D, lanes 7 and 8), and acetylserine affected neither the mobility of this “super fast” complex (C1f) nor its amount. A secondary complex formed by CysB A227D was detected (Fig. 4D, lane 8; and E, lane 5), whose mobility was comparable to that of WT CysB without inducer. The secondary complex (C2) formed by WT CysB is shown in Fig. 4D (lane 3), and a similar C2 complex formed by the CysB T196I variant is shown in Fig. 4C (lane 9). As established by previous stoichiometry studies (16), the C2 complex of WT CysB results from binding of two CysB tetramers in two separate binding sites within \( p_{\text{cysP}} \). Considering the differences in mobility between complexes formed by the A227D variant and those of WT CysB, it is tempting to conclude that the A227D protein might bind DNA as a dimer, forming a super fast primary complex.

Among the C-terminally truncated CysB variants, CysB K320Ter was not tested further, as its phenotypic characteristics were similar to those of WT CysB. Of further truncated CysB mutants, only one, CysB N309Ter, could be overproduced to an amount sufficient for DNA binding experiments. The corresponding extract enriched with CysB N309Ter was tested in EMSA using the cysP promoter. As shown in Fig. 4E (lanes 7–9), this protein was able to bind \( p_{\text{cysP}} \), forming a complex of much higher mobility than that of WT CysB. Interestingly, the mobility of this super fast complex (C1f) was comparable to that of the complex formed by CysB A227D. The plasmid encoding CysB N309Ter (and other plasmids encoding trun-
cated CysB variants) displayed no dominant-negative effect on the Cys phenotype of WT strain EC1250 (Table II) and did not reduce the β-galactosidase level in the EC2256 strain (data not shown). The lack of a dominant-negative effect on WT CysB in vivo can be most easily explained by an oligomerization defect in CysB N309Ter and the other C-terminally truncated variants. Consequently, we can hypothesize that CysB N309Ter bound DNA as dimers because complexes formed by these proteins with the cysP promoter displayed a similar mobility (see also "Discussion"). However, in contrast to CysBc A227D, CysB N309Ter could not perform all functions of WT CysB in vivo, as it was unable to complement the cysteine requirement of the ΔcysB mutant strain.

Construction and Characteristics of the Putative CysBpc Mutant—One of the objectives of this study was to identify single amino acid substitution(s) in CysB that result in the loss of transcriptional activation of the cys promoter, but not loss of both DNA binding and inducer response. By these criteria, none of the cysB mutants described above could be regarded as defective specifically in "positive control" function. In one of the attempts to isolate a CysBpc mutant (where pc is positive control), we took advantage of the recent study on LysR-type GcvA protein (36) suggesting that single residue substitutions F31L and F31A in the recognition helix of the HTH motif are responsible specifically for the positive control function of GcvA. Since Phe31 of GcvA corresponds to Tyr27 of CysB in alignment of the putative HTH regions of these proteins, we introduced the mutation changing Tyr27 of CysB to Gly by site-directed mutagenesis. Consequently, we can hypothesize that CysB N309Ter and CysBc A227D bound DNA as dimers because complexes formed by these proteins with the cysP promoter displayed a similar mobility (see also "Discussion"). However, in contrast to CysBc A227D, CysB N309Ter could not perform all functions of WT CysB in vivo, as it was unable to complement the cysteine requirement of the ΔcysB mutant strain.

**FIG. 4.** Binding of CysB protein variants to the cysP promoter region as measured by EMSA. The 5' -labeled 393-bp DNA fragment extending from positions −321 to +72 relative to the transcription start site of cysP was incubated with the indicated amounts of particular CysB preparations (CysB-enriched protein extracts shown in Fig. 3) and 10 mM O-acetyl-L-serine (OAS) where indicated. Use of O-acetyl-L-serine instead of N-acetyl-L-serine is explained under "Materials and Methods." Mixtures were run on 5% polyacrylamide gels, and the radiolabeled bands were visualized by autoradiography. A, DNA binding characteristics of wild-type E. coli CysB (wtE.c) and purified S. typhimurium CysB (wtS.t); B–E, DNA binding of E. coli WT CysB and CysB variants with the indicated mutations. Primary complexes (C1s (slow), C1f (fast), and C1f* (super fast)) and secondary complexes (C2) are described under "Results." No shifted band was detected with a control extract obtained from cells containing vector pTrc99A (data not shown).

**FIG. 5.** Binding of CysB protein variants to the cysB promoter region in EMSA. The 5'-labeled 308-bp DNA fragment containing the E. coli cysB promoter region (from positions −143 to +165 relative to the translation start codon) was used as a probe. Experimental conditions are identical to those described in the legend to Fig. 4. OAS, O-acetyl-L-serine.
tagesis (see “Oligonucleotides and Plasmid Construction”). The plasmid expressing CysB Y27G showed the wild-type level of repression of the cysP promoter region as measured by EMSA. Conditions of the experiment are similar to those described in the legend to Fig. 4. Transcription runoff assays were performed with the cysP promoter (fragment 321 to +72), the indicated amounts (μg/ml) of CysB proteins (wild-type purified S. typhimurium CysB (wt S.t.) and extracts enriched with WT E. coli CysB (wt E.c.) or CysB Y27G), and 10 μM O-acetyl-l-serine (OAS) or 1 μM N-acetyl-l-serine (NAS) where indicated. Transcripts were sized according to the sequencing reaction performed with the pMH1822 template and the 5-32P-labeled primer ECCP2 (lanes A, C, G, and T).

**DISCUSSION**

In this study, we analyzed the properties of 20 newly isolated CysB mutants to define roles of particular protein regions (see Fig. 1 for proposed functional map of CysB).

**Regions Important for DNA Binding**—In addition to the previously identified mutations S34R (14) and I33N (37), six new mutations were obtained that are clustered in region 11–48. All of these mutations impaired the ability of CysB to negatively autoregulate and also to activate the cysP promoter. Therefore, it seems clear that the predicted helix-turn-helix motif (residues 18–38) of CysB (13) and its close vicinity are crucial for the DNA-binding function. Most of the N-terminal residues identified in CysB as important for DNA binding are highly conserved among LTTRs. It was proposed (9) that highly conserved amino acids contact conserved base pairs of a generic TNR2 motif present in the binding sites for most LTTRs (38). Some CysB residues (i.e. Glu11 and Thr22) are poorly conserved in other LysR family members; these residues may therefore confer the specificity of DNA-CysB interactions. We found that a C-terminal region of CysB may be also critical for the DNA-binding function, as removal of 19–30 C-terminal residues
resulted in loss of repressing ability of the protein \textit{in vivo}. The possible involvement of the C-terminal region in DNA binding places CysB in one subclass of LTRs along with AmpR (8), NahR (7), and OxyR (9), in contrast to other subclass of LTRs, \textit{i.e.} MetR (39) and Nac (40), which tolerate substantial C-terminal truncations. The role of the C-terminal end of CysB in DNA binding may be indirect, as truncations of 19 and more residues apparently affect the stability of the protein and possibly its oligomerization state (see below).

**Regions Important for Oligomerization**—Four mutations affecting the response of CysB to acetylserine fit in regions 95–173 and 196–206, respectively (Fig. 1), suggested to be responsible for the inducer response in several LTRs (2). Three remaining mutations were localized closer to the C-terminal part of the protein. The importance of this additional region (residues 227–255) for inducer recognition/response is also illustrated by mutations described more recently for OxyR (constitutive variants A233VT and G253K) (4) and NahR (variant R248C) (5). The amino acid residues whose substitution results in either non-inducible or constitutively active CysB variants are presented in the model of \textit{E. coli} CysB (Fig. 7). As can be seen, all these residues (perhaps with the exception of Met\textsuperscript{160}) are localized in the neighborhood of the cavity, which is believed to be an acetylserine-binding pocket (20).

We performed molecular modeling to compare the structure of the WT CysB dimer with that of either non-inducible or constitutive CysB variants. However, the observed differences were not of essential meaning, so one cannot expect fundamental structural changes in the protein caused by the particular mutations studied in this work.

**Regions Important for Inducer Recognition/Response**—It was shown previously that substitutions T149M/P and W166R result in constitutive or partially constitutive activity of CysB, respectively (41). We have identified seven additional residues of CysB (Fig. 1) that may contribute to inducer binding and response. Non-inducible CysB variants were unable to respond to the inducer, acetylserine, by the conformational change that allows the transition of the slow DNA-protein complex to the fast complex, of which only the latter represents the structure required for transcriptional activation of the \textit{cysP} promoter (16, 18). The interaction of these CysB variants with the \textit{cysB} promoter region was also unaffected by acetylserine, in contrast to wild-type CysB, whose binding to \textit{p\textsubscript{cysP}} was inhibited by the inducer. One of the constitutive CysB variants examined in this study (CysB\textsuperscript{Y164N}) formed the fast complex with the \textit{cysP} promoter region independently of the presence of inducer. The characteristics of this mutant correlated well with those described for CysB\textsuperscript{T149M} (41). More interestingly, the other CysB\textsuperscript{Y} variant, A227D, formed a super fast complex with the \textit{cysP} promoter, suggesting a different oligomerization state of this mutant protein compared with that of WT CysB. Although attempting to evaluate the nature of the super fast complex is speculative without stoichiometry data, two arguments allow us to favor the possibility that the protein binds DNA as a dimer. First, a similar super fast complex was also observed with the CysB N309Ter variant, whose other characteristics suggested an oligomerization defect. Second, another LysR family protein, OxyR, may serve as a precedent, as its wild-type form is tetrameric, but the constitutive A233V variant binds DNA as a dimer (9). The fact that CysB\textsuperscript{Y} A227D was fully functional \textit{in vivo} also suggests that the tetrameric structure of CysB may be nonessential for the activatory function of the protein.

Four mutations affecting the response of CysB to acetylserine fit in regions 95–173 and 196–206, respectively (Fig. 1), suggested to be responsible for the inducer response in several LTRs (2). Three remaining mutations were localized closer to the C-terminal part of the protein. The importance of this additional region (residues 227–255) for inducer recognition/response is also illustrated by mutations described more recently for OxyR (constitutive variants A233VT and G253K) (4) and NahR (variant R248C) (5). The amino acid residues whose substitution results in either non-inducible or constitutively active CysB variants are presented in the model of \textit{E. coli} CysB (Fig. 7). As can be seen, all these residues (perhaps with the exception of Met\textsuperscript{160}) are localized in the neighborhood of the cavity, which is believed to be an acetylserine-binding pocket (20).

We performed molecular modeling to compare the structure of the WT CysB dimer with that of either non-inducible or constitutive CysB variants. However, the observed differences were not of essential meaning, so one cannot expect fundamental structural changes in the protein caused by the particular mutations studied in this work.

**Regions Important for Oligomerization**—The standard test for negative \textit{trans}-dominance (42) is frequently used to identify residues involved in oligomerization of multimeric transcriptional regulators. Examples of LysR family members, NahR (7), OxyR (9), and GcvA (36), also suggest the correlation between the oligomerization defect and the inability of the mutant protein to exert a dominant-negative effect (also called a “poisoning effect”) on the activity of the wild-type counterpart produced by the cell. For CysB, the negative dominance was clearly seen with some non-repressing (non-binding) variants (with mutations in region 11–22) as well as with non-inducible variants (Fig. 1). In contrast, three non-repressing mutants (substitutions in region 41–48) and all of the C-terminally truncated CysB variants did not display a dominant-negative effect. One truncated CysB variant (CysB N309Ter) bound to the \textit{cysP} promoter, and the mobility of the resulting primary complex was consistent with a dimeric rather than a tetrameric form of the protein. The most likely interpretation of these findings indicates the involvement of the 16 C-terminal residues in oligomerization of CysB (as was also suggested for OxyR and NahR), but also the importance of a region encompassing Leu\textsuperscript{41}–Ile\textsuperscript{48}. It is tempting to speculate that region 41–48, composing part of the DNA-binding domain, is important for appropriate contacts between two CysB monomers whose HTH motifs interact directly with dyadic activatory binding sites. An alternative explanation for the lack of a dominant-negative effect of the E41K, L44L, and I48T variants...
assumes that the mutant proteins possess the ability to oligomerize, but that mixed CysB oligomers are still functional (i.e., the wild-type monomers are dominant to the mutant monomers with respect to DNA binding). Another suggestion emerging from our study is that interactions between subunits within the CysB tetramer may be affected by an interaction with the inducer. Such a possibility is inferred by the characteristics of the CysB\textsuperscript{A227D} variant, most probably dimeric as discussed earlier. A model proposed for WT CysB (37) assumed that C-terminal portions of the four subunits maintain the tetramer in an inactive state and that acetylserine disrupts these contacts to release the protein surface for interaction with the RNA polymerase. The characteristics of the CysB\textsuperscript{A227D} variant would be consistent with this model if one assumes that the A227D mutation disrupts C-terminal contacts between subunits and that the resulting protein is active without an inducer. It has to be pointed out that if both CysB\textsuperscript{A227D} and CysB N309Ter bind DNA as dimers, the arrangement of subunits in these dimers may not be equivalent. The electrophoteric mobilities of the primary complexes formed by these proteins with the cysP promoter were similar but not identical, and the N309Ter variant (in contrast to CysB\textsuperscript{A227D}) apparently was not active with all CysB-dependent cys gene promoters in vivo, as it was unable to complement the ΔcysB mutant.

Perhaps the characteristics of the above CysB variants closely resemble those of dimeric OxyR mutants, for which the possibility of dimerization via either the “dimerization domain” or the “tetramerization domain” was suggested (9).

**Region Involved in the Positive Control Function**—The single amino acid substitution Y27G in CysB (Fig. 1) resulted in a phenotype expected for a CysB\textsuperscript{Y27G} mutant: the Y27G protein was able to bind DNA and to respond to the inducer by a qualitative change in the DNA-protein complex, but it was still defective in functional regulators belonging to other families, i.e. Acl (43) and Fis (44) proteins.

CysB protein is likely to contact the C-terminal domain of the RNA polymerase α-subunit, as the rpmA341 mutation, changing K271E in the α-peptide, specifically affects CysB-dependent expression of the cysPTWA operon (45). It is possible that recruitement of RNA polymerase by CysB involves a direct protein-protein contact between the C-terminal domain of the RNA polymerase α-subunit and the putative activating region of CysB, in which Thr\textsuperscript{27} seems to be essential.

In conclusion, the results of this study highlight the regions of CysB that are critical for DNA binding, inducer response, oligomerization, and positive control and demonstrate that these functions can be genetically separated. Further work will continue to focus on the purification and detailed biochemical characterization of selected CysB variants for better recognition of CysB subunit interactions. Isolation of additional mutants for further characterization of the CysB activating region is also in progress.
Functional Dissection of the LysR-type CysB Transcriptional Regulator: REGIONS IMPORTANT FOR DNA BINDING, INDUCER RESPONSE, OLIGOMERIZATION, AND POSITIVE CONTROL

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