JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 276–279 Vol. 179, No. 1
0021-9193/97/$04.00+0

Binding of L-Branched-Chain Amino Acids Causes a Conformational Change in BkdR

KUNPULI T. MADHUSUDHAN,[1] NING HUANG,[1] EMORY H. BRASWELL,[2] AND JOHN R. SOKATCH[1]*

Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190,[1] and Molecular and Cell Biology Department and National Analytical Ultracentrifugation Facility, Biotechnology Center, University of Connecticut, Storrs, Connecticut 06269-3149[2]

Received 29 April 1996/Accepted 12 October 1996

BkdR is the positive transcriptional activator of the inducible *bkd* operon of *Pseudomonas putida*. Evidence is accumulating that L-branched-chain amino acids are the inducers of the operon, and the data obtained in this study show that they induce a conformational change in BkdR. Addition of L-branched-chain amino acids increased the susceptibility of BkdR to trypsin with the cleavage between Arg-51 and Gln-52 on the C-terminal side of the DNA-binding domain. L-Valine also caused an increased fluorescence emission intensity and produced significant changes in the circular dichroism spectrum of BkdR. Analytical ultracentrifugation confirmed earlier data obtained from gel filtration that BkdR was a tetramer with a Stokes radius of 32 ± 3 Å and an axial ratio of 2:1.

The branched-chain keto acid dehydrogenase multienzyme complex is the second enzyme in the catabolic pathway of branched-chain amino acids. Branched-chain keto acid dehydrogenases from *Pseudomonas putida* (27), *Pseudomonas aeruginosa* (20), *Bacillus subtilis* (15), bovine kidney (22), and rabbit liver (21) are composed of three components but four polypeptides. The *bkd* operon (3–5) of *P. putida* encodes the four polypeptides of the complex.

Regulation of expression of catabolic pathways in pseudomonads is not well understood, except that cyclic AMP is not involved (23, 26). Recently, a novel protein, CRC, which plays a role in carbon catabolite repression has been isolated from *P. aeruginosa* (16). Expression of branched-chain keto acid dehydrogenase of *P. putida* is induced in media containing D- or L-branched-chain amino or keto acids (19). The expression of branched-chain keto acid dehydrogenase is repressed by ammonium ion, glucose, and succinate (29), so expression of this operon is under complex regulation.

BkdR is the positive regulator of the *bkd* operon of *P. putida* and belongs to the Lrp family of proteins (18). Lrp, the leucine-responsive protein, is a global transcriptional regulator of *Escherichia coli* and is a dimer with about 3,000 copies per cell (6). In contrast, there are about 25 to 40 copies of BkdR per cell in *P. putida*, and the molecular weight of BkdR estimated by gel filtration indicated that BkdR is a tetramer (17). BkdR binds specifically to about 100 to 110 bp of DNA in the *bkdR-bkdA1* intergenic region with four potential binding sites in this region (17) based on similarity to Lrp-binding consensus sequences (10, 25, 30). Twelve monomers or three tetramers of BkdR bind per mol of substrate DNA (11), which explains why such a large segment of DNA is protected from DNase I by BkdR. L-Branched-chain amino acids change the DNase I protection pattern by enhancing the appearance of hypersensitive sites in the protected region (17).

Assuming that branched-chain amino or keto acids are the inducers of the *bkd* operon, one mechanism by which an inducer could affect transcription would be through a conformational change of BkdR. The present paper describes the effects of L-branched-chain amino acids on the secondary structure of BkdR. Additional data are provided about the tetrameric structure of BkdR and the Stokes radius of the tetramer.

L-Branched-chain amino acids enhance the proteolytic digestion of BkdR. Limited proteolysis of BkdR by trypsin was markedly enhanced in the presence of 50 mM L-valine, producing 14- and 6-kDa peptides (Fig. 1), suggesting that a conformational change had been induced. However, substrate DNA (17) had no effect on proteolysis. In separate experiments (results not shown), 50 mM L-leucine and L-isoleucine also enhanced the sensitivity of BkdR to digestion by trypsin, but α-ketoisovalerate, L-glutamate, and L-alanine did not.

To identify the cleavage site, about 100 μg of BkdR was digested by 0.3 μg of trypsin in a total volume of 180 μl with the same buffer and conditions described in the previous paragraph except that the buffer included 50 mM L-valine. At the end of 90 min, the reaction was stopped by adding an equal volume of 2× loading buffer. Peptides were separated electrophoretically by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 10 to 20% gradient gels and transferred to a Trans-Blot membrane (13). The band corresponding to the 14-kDa peptide was excised, and the N-terminal amino acid sequence was determined by microsequencing at the University of Oklahoma Health Sciences Center Molecular Biology Resource Facility. The sequence of the first 10 amino acids was Gln Gln Val Thr Leu Leu Ser Pro Glu Ala. Therefore, the cleavage site was between Arg-51 and Gln-52 on the C-terminal side of the putative helix-turn-helix of BkdR (17) yielding a DNA-binding domain and a C-terminal domain.

It was possible to quantitate the effect of L-valine by measuring the amount of radioactivity in the digestion products resulting from the digestion of [^{35}S]BkdR prepared as described elsewhere (11). Preliminary experiments demonstrated that radioactivity determined by the phosphorimager was directly proportional to the amount of BkdR. The radioactivity in the low-molecular-weight bands was determined, and the data were plotted as S/V versus S, where S is the concentration of L-valine (in this case from 1 to 20 mM) and V is the radioactivity in the digestion products (Fig. 2). In this plot, the negative intercept of the x axis is equal to $-K_m$. The average of three separate experiments produced a K_m of 2.5 mM. This is

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190. Phone: (405) 271-2227, ext. 244. Fax: (405) 271-3092. E-mail: john-sokatch@uokhsc.edu.

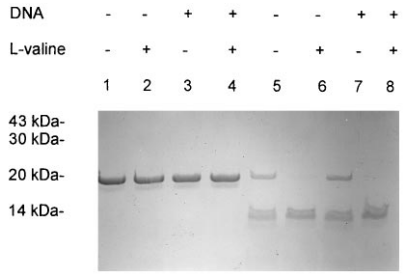

FIG. 1. Effect of L-valine on tryptic digestion of BkdR. All reaction mixtures contained 18 μg of BkdR (≈1 pmol) in final volume of 65 μl. Other components were added as follows: L-valine, 32.5 μmol (50 mM); substrate DNA, 800 μg (≈3.6 pmol); trypsin, 0.1 μg. Substrate DNA was a 354-bp PCR fragment extending from nucleotide 1286 to 1639 which includes the *bkdR-bkdA1* intergenic region. Nucleotide numbering is the same as that in GenBank sequence M57613. Samples were taken from tubes 1 to 4 before addition of trypsin and from tubes 5 to 8 45 min after the addition of trypsin. The digestion conditions are taken from those described in reference 9. The molecular mass markers are shown at the left.

the concentration of L-valine which resulted in a half-maximal rate of proteolysis of BkdR by trypsin resulting from the change in conformation of BkdR.

Fluorescence emission spectrum of BkdR plus L-valine. Changes in the fluorescence emission spectrum of BkdR in the presence of L-branched-chain amino acids confirmed that a conformational change had occurred in BkdR. Excitation at 278 nm resulted in an emission maximum at 305 nm (Fig. 3). BkdR lacks tryptophan but contains 2.5 mol% of tyrosine (18), which accounts for the emission maximum at 305 nm (7). Addition of 50 mM L-valine caused a 16% increase in intrinsic fluorescence emission with no change in the emission maximum nor in the shape of the spectrum. Addition of D-valine did not affect the UV spectrum. The change in fluorescence emission is relatively small because of the absence of tryptophan in BkdR.

CD spectroscopy. Addition of L-branched-chain amino acids resulted in a large change in the circular dichroism (CD) spectrum (Fig. 4). The CD spectrum in the absence of L-valine was characterized by a single large negative peak at 283 nm with shoulders at 275 and 293 nm (Fig. 4). Addition of 50 mM

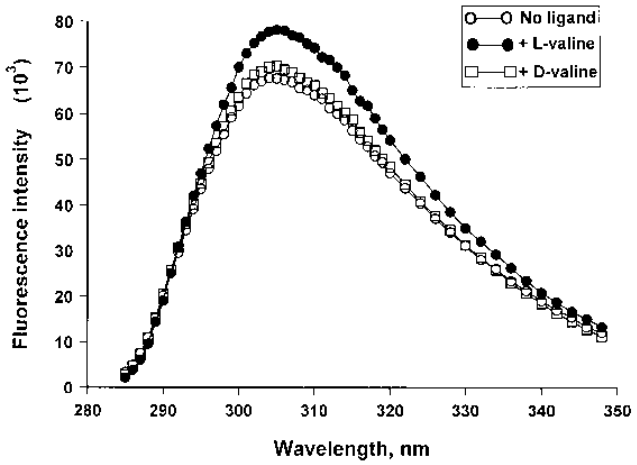

FIG. 3. Fluorescence emission spectra of BkdR. The concentration of BkdR was 0.3 mg ml^{-1} in 50 mM potassium phosphate (pH 7.5)–200 mM NaCl–10% glycerol. Spectra were recorded in an SLM-8000 spectrofluorometer (SLM-Aminco Instruments Inc., Urbana, Ill.) with excitation at 278 nm. Ligand concentration, 50 mM.

L-valine resulted in a reduction of $[\theta]_{mrw}$ by almost half with peaks at 275, 283, and 293 nm and a shoulder at 268 nm. Addition of D-valine to BkdR gave a CD spectrum identical to the spectrum with no ligand. The peak at 283 nm and the shoulders at 275 and 290 nm are likely due to conformational changes in the region of tyrosine residues. There was no change in the far CD spectrum from 200 to 250 nm caused by the addition of L-branched-chain amino acids.

The secondary structure of BkdR deduced from the CD spectra by Prosec consists of 11% α-helix and 48% β-turns, with the rest being aperiodic structure with no β-sheets. Assuming that about 20 amino acids compose the helix-turn-helix (2), this would constitute about 12% of the total protein. The figure of 11% α-helix obtained from analysis of the CD spectra suggests that the only helical structure in BkdR is the DNA-binding domain. Lrp, a homologous protein, has been divided into DNA-binding, activation, and ligand-binding domains by

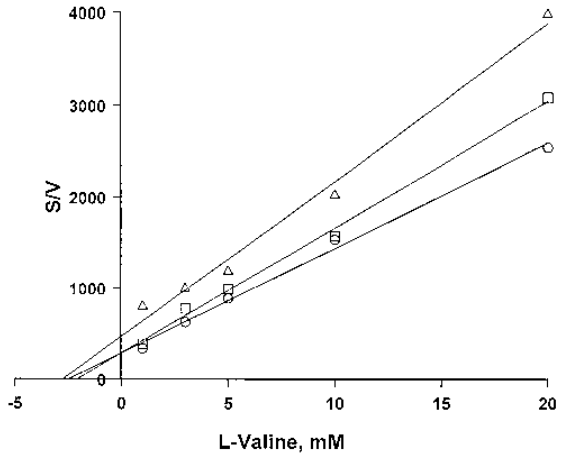

FIG. 2. Plot of *S*/*V* versus concentration of L-valine. *S* is the concentration of L-valine, and *V* is the amount of radioactivity in the 14- and 6-kDa digestion products detected by phosphorimaging. △, ○, and □ represent the results of three separate experiments.

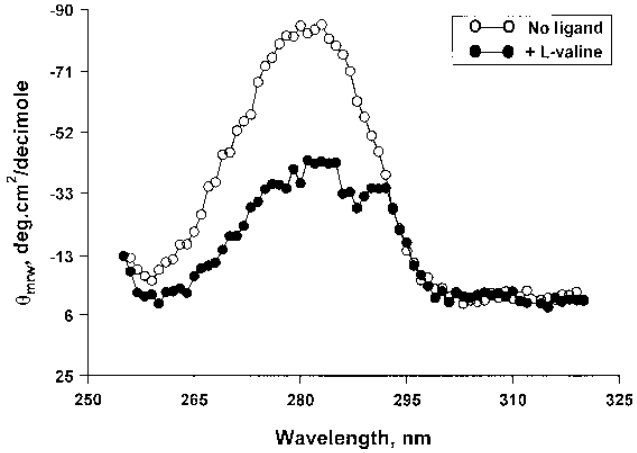

FIG. 4. Effect of L-valine (50 mM) on the CD spectrum of BkdR. The concentration of BkdR was 2.14 mg ml^{-1} dissolved in 50 mM sodium phosphate buffer, pH 7.5, containing 200 mM NaCl and 18% glycerol. The spectra were read at 27°C in a model 62DS AVIV CD spectrophotometer. The mean residue weight for BkdR was 113.976.

cataloging the effects of mutations on the action of Lrp (24), and this will probably be true also of BkdR.

Analytical ultracentrifugation of BkdR. The molecular weight of BkdR obtained by gel filtration indicated that BkdR was a tetramer (17), which was somewhat surprising since Lrp is a dimer (6). In order to confirm this finding, the molecular weight of BkdR was determined by sedimentation equilibrium. Portions of a solution containing 1 mg of BkdR ml^{-1} were dialyzed against 10 μM Tris-HCl (pH 8.0)–1 μM EDTA–200 mM NaCl–10% glycerol (nondenaturing buffer) and the same buffer containing 0.1% SDS with 0.1% mercaptoethanol (denaturing buffer). The final concentrations of BkdR in the nondenaturing buffer were 0.05, 0.1, and 0.2 mg ml^{-1}, and the final concentrations of BkdR in the denaturing buffer were 0.2 and 0.4 mg ml^{-1}. Sedimentation equilibrium experiments were performed (1) at 20°C at 16 and 24 krpm for the BkdR solutions in nondenaturing buffer and 26 krpm for those solutions in the denaturing buffer by using a Beckman model E analytical ultracentrifuge. The data were fitted globally by NONLIN (12) and analyzed with various assumed models of molecular association. NONLIN yields the model of association, the association constants, and the molecular weight of the smallest associating molecule. The value of $\bar{\nu}$, which is necessary for the calculation of molecular mass, was 0.745 ml g^{-1} and was calculated from the amino acid content (8).

The association model which best fits the sedimentation equilibrium data is one in which BkdR is mainly a tetramer in solution which has a tendency to further dimerize to an octamer. In the absence of SDS, the smallest associating molecule had a molecular mass of 84 ± 2 kDa. This is higher than that expected for the tetramer calculated from the amino acid composition (73.4 kDa). In order to obtain the theoretical molecular mass from the sedimentation equilibrium data, $\bar{\nu}$ would have to be about 0.71 ml/g. Since the theoretical value of $\bar{\nu}$ applies only to aqueous solutions, it is possible that 10% glycerol, which is used to keep BkdR in solution (17), causes a decrease in its actual value.

Sedimentation equilibrium data obtained from BkdR dissolved in buffer containing SDS and β-mercaptoethanol were best fitted by a weak monomer-dimer equilibrium in which the smallest associating molecule had a molecular mass of 21.4 ± 0.4 or 18.4 ± 0.4 kDa with a $\bar{\nu}$ of 0.71 ml/g. However, the ratio of the molecular masses obtained in the absence of SDS and mercaptoethanol to that in the presence of SDS and mercaptoethanol is about 4 (3.93 ± 0.17) no matter which value of $\bar{\nu}$ is used. Therefore, under these conditions, the tetramer is dissociated to a monomer which has a small tendency to dimerize.

Sedimentation velocity experiments were run on the protein solutions (0.4 mg/ml) which had been dialyzed against buffer and buffer containing 0.1% SDS. These experiments were performed in a Beckman XLA analytical ultracentrifuge at 60 krpm at 20°C. The concentration gradient was monitored by radial absorption measurements at 280 nm, and the data were analyzed by the "g^* (s)" method (28).

Velocity sedimentation of BkdR in buffer in the absence and presence of SDS produced s values of 5.44 ± 0.4 and 1.55 ± 0.11, respectively. Assuming that these values (obtained at a protein concentration of 0.4 mg/ml) are close to the zero concentration values, one can take them to be approximately the $s_{20,w}$ values of the tetramer and monomer, respectively. Because the dissociation of the tetramer is not complete in SDS alone, the latter value is only approximate. Assuming that the molecule can be represented as a hydrated prolate ellipsoid with the usual hydration factor of 1.33 g per g of protein, the two values produce axial ratios of 2:1 ± 1 and 8:1 ± 1 for the tetramer and monomer, respectively. The computed Stokes radius for the tetramer is 32 Å and for the monomer is 27.5 Å. However, the axial ratio and Stokes radius of the monomer were obtained in buffer with SDS, which denatures protein, so these numbers cannot be compared with those for the tetramer.

Role of BkdR in transcription of the *bkd* operon. The *bkd* operon is induced by growth on DL-branched-chain amino and branched-chain keto acids (19). This means that at least one class of these compounds is the inducer and very likely binds to BkdR effecting a change which allows transcription to occur. Evidence is accumulating that the inducers are the L-branched-chain amino acids. In addition to the data presented here, L-branched-chain amino acids enhance the appearance of hypersensitive sites in the DNase I protection pattern, while D-branched-chain amino acids and branched-chain keto acids have no effect (17). This report shows that one of the changes brought about by L-branched-chain amino acids is a conformational change in BkdR. The prediction can be made that L-branched-chain amino acids should be required for transcription of the *bkd* operon, and these experiments are under way.

The fact that the concentration of L-branched-chain amino acids required to effect a change in the conformation of BkdR is in the millimolar range (Fig. 2) suggests that the *bkd* operon will be induced at the same concentration. This fits with the role of branched-chain keto acid dehydrogenase in the cell. Because the *bkd* operon is not induced until the intracellular concentration of branched-chain amino acids approaches the millimolar range, there are no gene products to interfere with the biosynthesis of leucine via α-ketoisovalerate.

Although Lrp and BkdR are homologous proteins, Lrp is a dimer and BkdR is a tetramer. Lrp activates transcription of the *bkd* operon in a *bkdR*-null mutant (18); therefore, it was important to firmly establish that BkdR is a tetramer. Mobility through gel filtration columns is affected by the shape of the molecule as well as the molecular weight. However, analytical ultracentrifugation does not suffer from this shortcoming. The results of the analytical ultracentrifugation studies reported here demonstrate clearly that BkdR is a tetramer with some tendency to form an octamer. We have also found that chromatography of BkdR in 5 mM L-valine did not affect the mobility of BkdR in filtration through Sephadex G-150. Therefore, valine does not affect the association properties of BkdR in a major way. In addition, BkdR recovered from the gel filtration column was active in the gel shift assay, so the tetramer is active in binding DNA. A single BkdR-DNA complex was obtained under a number of conditions with a stoichiometry of 12 monomers or 3 tetramers per mol of substrate DNA (11). Therefore, all the evidence indicates that it is the BkdR tetramer which binds DNA. It is still possible that the tetramer could dissociate to dimers after binding substrate DNA as does the Lac repressor (14), but this remains to be determined.

ACKNOWLEDGMENTS

This research was supported by Public Health Service Research Grant DK 21737 to J.R.S. and NSF grant BIR 931373 to E.H.B.

We also thank Richard Taylor for generous assistance with the use of the AVIV spectrophotometer.

REFERENCES

1. **Advant, S. J., E. H. Braswell, C. V. Kumar, and D. S. Kalonia.** 1995. The effect of pH and temperature on the self-association of recombinant human interleukin-2 as studied by equilibrium sedimentation. Pharm. Res. **12:**637–641.
2. **Brennan, R. G., and B. W. Mathews.** 1989. The helix-turn-helix DNA binding motif. J. Biol. Chem. **264:**1903–1906.
3. **Burns, G., T. Brown, K. Hatter, J. M. Idriss, and J. R. Sokatch.** 1988. Similarity of the E1 subunits of branched-chain-oxoacid dehydrogenase from

Pseudomonas putida to the corresponding subunits of mammalian branched-chain-oxoacid and pyruvate dehydrogenases. Eur. J. Biochem. **176:**311–317.
4. **Burns, G., T. Brown, K. Hatter, and J. R. Sokatch.** 1988. Comparison of the amino acid sequences of the transacylase components of branched chain oxoacid dehydrogenase of *Pseudomonas putida*, and the pyruvate and 2-oxoglutarate dehydrogenases of *Escherichia coli*. Eur. J. Biochem. **176:**165–169.
5. **Burns, G., T. Brown, K. Hatter, and J. R. Sokatch.** 1989. Sequence analysis of the *lpdV* gene for lipoamide dehydrogenase of branched chain oxoacid dehydrogenase of *Pseudomonas putida*. Eur. J. Biochem. **179:**61–69.
6. **Calvo, J. M., and R. G. Mathews.** 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. Microbiol. Rev. **58:**466–490.
7. **Chen, R. F., H. Edelhoch, and R. F. Steiner.** 1969. Fluorescence of proteins, p. 171–244. *In* S. J. Leach (ed.), Physical principles and techniques of protein chemistry, part A. Academic Press, New York, N.Y.
8. **Cohn, E. J., and J. T. Edsall.** 1943. Density and apparent specific volume of proteins, p. 370–381. *In* E. J. Cohn and J. T. Edsall (ed.), Proteins, amino acids and peptides as ions and dipolar ions. Rheinhold Publishing Corporation, New York, N.Y.
9. **Cui, J., and R. L. Somerville.** 1993. The TyrR protein of *Escherichia coli*, analysis by limited proteolysis of domain structure and ligand-mediated conformational changes. J. Biol. Chem. **268:**5040–5047.
10. **Cui, Y., Q. Wang, G. D. Stormo, and J. M. Calvo.** 1995. A consensus sequence for binding of Lrp to DNA. J. Bacteriol. **177:**4872–4880.
11. **Huang, N., K. T. Madhusudhan, and J. R. Sokatch.** 1996. Stoichiometry of BkdR to substrate DNA in *Pseudomonas putida*. Biochem. Biophys. Res. Commun. **223:**315–319.
12. **Johnson, M. L., J. J. Correia, D. A. Yphantis, and H. R. Halvorson.** 1981. Analysis of data from analytical ultracentrifuge by nonlinear least-squares techniques. Biophys. J. **36:**575–588.
13. **LeGendre, N., M. Mansfield, A. Weiss, and P. Matsudaira.** 1993. Purification of proteins and peptides by SDS-PAGE, p. 71–101. *In* P. Matsudaira (ed.), A practical guide to protein and peptide purification for microsequencing, 2nd ed. Academic Press, San Diego, Calif.
14. **Lewis, M., G. Chang, N. C. Horton, M. A. Kercher, H. C. Pace, M. A. Schmuacher, R. G. Brennan, and P. Lu.** 1996. Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. Science **271:** 1247–1254.
15. **Lowe, P. N., J. A. Hodgson, and R. N. Perham.** 1983. Dual role of a single multienzyme complex in the oxidative decarboxylation of pyruvate and branched chain 2-oxoacids in *Bacillus subtilis*. Biochem. J. **215:**133–140.
16. **MacGregor, C. H., J. A. Wolff, S. K. Arora, P. B. Hylemon, and P. V. Phibbs, Jr.** 1992. Catabolite repression control in *Pseudomonas aeruginosa*, p. 198–206. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas* molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
17. **Madhusudhan, K. T., N. Huang, and J. R. Sokatch.** 1995. Characterization of BkdR-DNA binding in the expression of the *bkd* operon of *Pseudomonas putida*. J. Bacteriol. **177:**636–641.
18. **Madhusudhan, K. T., D. Lorenz, and J. R. Sokatch.** 1993. The *bkdR* gene of *Pseudomonas putida* is required for expression of the *bkd* operon and encodes a protein related to Lrp of *Escherichia coli*. J. Bacteriol. **175:**3934–3940.
19. **Marshall, V. P., and J. R. Sokatch.** 1972. Regulation of valine catabolism in *Pseudomonas putida*. J. Bacteriol. **110:**1073–1081.
20. **McCully, V., G. Burns, and J. R. Sokatch.** 1986. Resolution of branched-chain oxo acid dehydrogenase complex of *Pseudomonas aeruginosa*. Biochem. J. **233:**737–742.
21. **Paxton, R., and R. A. Harris.** 1982. Isolation of rabbit liver branched chain α-ketoacid dehydrogenase and regulation by phosphorylation. J. Biol. Chem. **257:**14433–14439.
22. **Pettit, F. H., S. J. Yeaman, and L. J. Reed.** 1978. Purification and characterization of branched-chain α-ketoacid dehydrogenase complex of bovine kidney. Proc. Natl. Acad. Sci. USA **75:**4881–4885.
23. **Phillips, A. T., and L. M. Mulfinger.** 1981. Cyclic adenosine 3′,5′-monophosphate levels in *Pseudomonas putida* and *Pseudomonas aeruginosa* during induction and carbon catabolite repression of histidase synthesis. J. Bacteriol. **145:**1286–1292.
24. **Platko, J. V., and J. M. Calvo.** 1993. Mutations affecting the ability of *Escherichia coli* Lrp to bind to DNA, activate transcription, or respond to leucine. J. Bacteriol. **175:**1110–1117.
25. **Rex, J. H., B. D. Aronson, and R. L. Somerville.** 1991. The *tdh* and *serA* operons of *Escherichia coli*: mutational analysis of the regulatory elements of leucine-responsive genes. J. Bacteriol. **173:**5944–5953.
26. **Siegel, L. S., P. B. Hylemon, and P. V. Phibbs, Jr.** 1977. Cyclic adenosine 3′,5′-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3′,5′-monophosphate phosphodiesterase in *Pseudomonas* and *Bacteroides*. J. Bacteriol. **129:**87–96.
27. **Sokatch, J. R., V. McCully, and C. M. Roberts.** 1981. Purification of a branched-chain keto acid dehydrogenase from *Pseudomonas putida*. J. Bacteriol. **148:**647–652.
28. **Stafford, W. F.** 1992. Boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile. Anal. Biochem. **203:**295–301.
29. **Sykes, P. J., G. Burns, J. Menard, K. Hatter, and J. R. Sokatch.** 1987. Molecular cloning of genes encoding branched-chain keto acid dehydrogenase of *Pseudomonas putida*. J. Bacteriol. **169:**1619–1625.
30. **Wang, Q., and J. M. Calvo.** 1993. Lrp, a global regulatory protein of *Escherichia coli*, binds co-operatively to multiple sites and activates transcription of *ilvIH*. J. Mol. Biol. **229:**306–318.