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Molybdenum Cofactor Requirement for Biotin Sulfoxide Reduction in *Escherichia coli*

ALICE DEL CAMPILLO-CAMPBELL AND ALLAN CAMPBELL*

Department of Biological Sciences, Stanford University, Stanford, California 94305-2493

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The *bisC* gene of *Escherichia coli* is tentatively identified as the structural gene for biotin sulfoxide reductase by the isolation of *bisC*(Ts) mutants that make thermolabile enzyme. The products of four other *E. coli* genes (*chlA*, *chlB*, *chlE*, and *chlG*) are also needed for enzymatic activity. Mutations previously assigned to the *bisA*, *bisB*, and *bisD* genes belong to genes *chlA*, *chlE*, and *chlG*, respectively. The biotin sulfoxide reductase deficiency of a *chlG* mutant is partially reversed by the addition of 10 mM molybdate to the growth medium. Mutational inactivation of the *chlD* gene reduces the specific activity of biotin sulfoxide reductase about twofold. This effect is reversed by the addition of 1 mM molybdate to the growth medium. The specific activity of biotin sulfoxide reductase is decreased about 30-fold by the presence of tungstate in the growth medium, an effect that has been observed previously with nitrate reductase and other molybdoenzymes. The specific activity of biotin sulfoxide reductase is not elevated in a lysate prepared by derepressing a λ *c*I857 *chlG* prophage. Whereas biotin sulfoxide reductase prepared by sonic extraction of growing cells is almost completely dependent on the presence of a small heat-stable protein resembling thioredoxin, much of the enzyme obtained from lysates of thermoinduced λ *c*I857 lysogens does not require this factor.

d-Biotin-*d*-sulfoxide is a spontaneous oxidation product of the vitamin *d*-biotin. Like many other organisms, *Escherichia coli* can reduce biotin sulfoxide to biotin. Thus, biotin auxotrophs can utilize biotin sulfoxide in place of biotin. Analysis of mutants unable to grow on biotin sulfoxide revealed the existence of four genes (*bisA-bisD*) that are necessary for biotin sulfoxide reduction (11). Operationally, each of these genes is defined by a cluster of closely linked mutations conferring similar phenotypes. We do not know whether all of the mutations assigned to one such gene belong to a single complementation group.

Extracts from wild-type cells can reduce biotin sulfoxide to biotin in vitro, whereas extracts of *bis* mutants lack this activity. The enzyme has been purified 50-fold. Its activity in vitro requires a heat-stable thioredoxin-like protein that is present in both wild-type and mutant extracts (9). Its subunit structure is unknown. Until the present work, no information was available as to which, if any, of the *bis* genes are structural genes for the enzyme.

The original isolation of *bis* mutants employed heavy mutagenesis with subsequent screening. Direct selection for *bis* mutants, allowing easy recovery of spontaneous mutations, was accomplished by the use of strains in which the *lacZ* gene is fused to the biotin operon and hence repressible by biotin (3). In a wild-type culture, repression can be achieved by the addition of biotin sulfoxide instead of biotin, because biotin sulfoxide is reduced intracellularly to biotin, which represses. Selection for derepressed derivatives (Lac^+ on biotin sulfoxide plates) yields *bis* mutants.

The study of such mutants reported in this paper indicates that at least one of these genes (*bisC*) is a structural gene for the enzyme. It has also provided strong evidence that the *bisA* and *bisB* genes are in fact identical to the *chlA* and *chlE* genes, whose function in nitrate reduction may be to form a molybdenum-containing cofactor. Additional data on the effect of tungstate on biotin sulfoxide activity and of mutations in other genes are presented here, all consistent with the idea that biotin sulfoxide reductase and nitrate reductase (EC 1.7.99.4) require a common, molybdenum-containing cofactor.

MATERIALS AND METHODS

Chemicals and media. Tryptone medium, eosin methylene blue indicator medium with galactose or xylose as fermentable substrate, and basic synthetic medium, with 0.4% glucose or lactose as carbon source, have been described previously (3). L medium for preparation of lysates and transduction by phage

P1 was as described previously (22). Tetracycline and mitomycin C were purchased from Sigma Chemical Co., St. Louis, Mo.

Bacterial strains and genetic terminology. Bacterial strains are listed in Table 1. The experiments reported in this paper indicate that the *bisA* gene is identical to *chlA*, *bisB* is identical to *chlE*, and *bisD* is identical to *chlG*. We have deliberately refrained from anticipating this conclusion in our nomenclature and have retained instead mutant designations based on the manner of isolation. In future work, these *bis* mutations may be renamed as alleles of the appropriate *chl* gene.

TABLE 1. Bacterial strains

Strain	Genotype[a]	Origin or reference
BM1161	F⁻ *araD129 ΔlacU89 thi rpsL Φ(bioA-lacZ)301*	3
DD38	F⁺ *bioA24 bisD21*	11
DD66	F⁺ *bioA24 bisB28*	11
DD120	F⁻ *bioa24 thr xyl mtl leu his pro arg thi lac ara rpoL tsx* Mt^{rb}	11
DD130	F⁺ *bioA24 bisA29*	11
DD132	F⁺ *bioA24 bisA31*	11
DD136	F⁺ *bioA24 bisA35*	11
RK7-36	Hfr KL96 *gal chlB36*	V. Stewart[c]
RK4940	F⁻ *araD139 ΔlacU169 non rpsL gyrA thi zif-200*::Tn*10*	V. Stewart[c]
RK5200	F⁻ *araD139 ΔlacU169 non rpsl gyrA thi chlA200*::Mu cts	V. Stewart[c]
RK5201	F⁻ *araD139 ΔlacU169 non rpsL gyrA thi chlE201*::Mu cts	V. Stewart[c]
RK5203	F⁻ *araD139 ΔlacU169 non rpsL gyrA thi chlA203*::Mu cts	V. Stewart[c]
RK5218	F⁻ *araD139 ΔlacU169 non rpsL gyrA thi chlE215*::Mu cts	V. Stewart[c]
RK5221	F⁻ *araD139 ΔlacU169 non rpsL gyrA thi chlA218*::Mu cts	V. Stewart[c]
RK5255	F⁻ *araD139 ΔlacU169 non rpsL gyrA thi chlE237*::Mu cts	V. Stewart[c]
RK5257	As RK4940, *chlB207*::Mu cts	V. Stewart[c]
R823	F⁺ *gal*⁺ *bio*⁺ *galK2 galT1 bio*$^{+d}$	5
S1134	As BM1161, *bisB41*	3
S1138	As BM1161, *bisB42*	3
S1177	F⁺ *bioA24 bisB21* (λ *bisD*⁺ *cI857*)	Lysogen of DD38
S1178	As S1177	Lysogen of DD38
S1186	F⁺ *bioA24 bisB28* (λ *imm434*)	Lysogen of DD66
S1187	F⁺ *bioA24 bisC37* (λ *imm434*)	Lysogen of DD133 of reference 11
S1198	F⁻ *galK2 galT1 rpsL* (λ *dg imm434*) (λ::*coli* cI857)	Strain W3350 (3) lysogenized by λ*dg imm434* and a random phage from λ *Eco*RI pool
S1228	F⁻ *trp lac thr metF ilv lys his xyl rpsL tsx zhj428*::Tn*10*	Tc^r transductant of χ1097 of R. Curtiss[e]
S1231	F⁺ *bioA24 bisB28 galK1 galT2 zbh-428*::Tn*10* (λ *imm434*)	Tc^r transductant of S1186[e]
S1234	F⁺ *bioA24 zbh-428*::Tn*10* (λ *imm434*)	Tc^r transductant of S1186[e]
S1235	As BM1161, *zbh-428*::Tn*10*	Tc^r transductant of S1134[e]
S1236	As BM1161, *bisB41 zbh-428*::Tn*10*	Tc^r transductant of S1134[e]
S1294	As BM1161, *bisC113*(Ts)	*bis*(Ts) mutant of BM1161
S1295	As BM1161, *bisC114*(Ts)	*bis*(Ts) mutant of BM1161
S1296	As BM1161, *bisC117*(Ts)	*bis*(Ts) mutant of BM1161
S1297	As BM1161, *bisC105*(Ts)	*bis*(Ts) mutant of BM1161
S1298	As BM1161, *bisC120*(Ts)	*bis*(Ts) mutant of BM1161
S1299	As BM1161, *bisC115*(Ts)	*bis*(Ts) mutant of BM1161
S1300	As BM1161, *bisC118*(Ts)	*bis*(Ts) mutant of BM1161
S1301	As BM1161, *bisC119a*(Ts)	*bis*(Ts) mutant of BM1161
S1302	As BM1161, *bisB121*(Ts)	*bis*(Ts) mutant of BM1161
SA291	F⁻ Δ(*gal-chlA*) *his rpsL*	3
SA302	F⁻ *his* (λ cI857)	S. Adhya
SA322	F⁻ *his* Δ(*chlD-pgl*) (λ cI857)	S. Adhya (derivative of SA302)

[a] Tn*10* insertions into a region, but not into a known gene, are named according to the system of Kleckner et al. (16). Thus, insertions *zbh-428*, *zhj-42*, and *zif-200* are located at 17, 79, and 85 min, respectively, on the genetic map of *E. coli*.

[b] Mt^r, Mitomycin resistant.

[c] Strains from collection of R. D. Kadner, University of Virginia.

[d] R823 has a tandem chromosomal duplication of *gal-bio*.

[e] Tc^r, Tetracycline resistant because Tn*10* is present.

Phage λ *Eco*RI libraries. The λ781 *bisD* isolates used in this work came from a pool of λ phages containing cloned *Eco*RI fragments of *E. coli* DNA. This library (derived from phage λ781 by E. Selker and R. Gunsalus, laboratory of C. Yanofsky, this Department) was the same one described previously (2). The *chlB* phages came from an *Eco*RI pool of *Salmonella typhimurium* DNA cloned into λgt *c*I857 (26), from the laboratory of R. W. Davis, Department of Biochemistry, Stanford University.

Mutagenesis. Mutagenesis with Mu *cts* and Tn*10* were as described previously (3, 22). For localized mutagenesis of the *bisA-bisB* region by ethyl methane sulfonate, a drop of the mutagen was placed on a lawn of the Tn*10*-carrying strain S1235 on medium selective for *bis* mutants (synthetic lactose medium with a repressing concentration, 390 nM, of biotin sulfoxide). Mutant colonies appeared around the drop at a frequency roughly 10-fold higher than the spontaneous rate seen on a nonmutagenized plate. Colonies from such plates were pooled, grown in L broth, and infected with phage P1. The resulting lysate was then used to transduce Tn*10* into BM1161, selecting on agar containing 15 μg of tetracycline per ml. Transductants were scored for Bis phenotype by replica plating onto synthetic dextrose medium with 16 nM biotin sulfoxide as the biotin source.

Transduction by phage P1. The procedure for transduction was as described previously (22). For transduction of Tn*10*, cells were held in liquid an additional 60 min after the addition of sodium citrate to allow full gene expression before plating on tetracycline agar.

Preparation of extracts. Sonic extracts were prepared as described previously (9). Where enzyme was assayed in phage lysates, lysis was induced by one of two methods, depending on the prophage genotype. Lysogens of λ *c*1857 were heated to 43°C for 30 min and then shaken at 37°C until lysis was complete. Lysogens of λ cI^+ were induced with mitomycin C (8).

Biotin sulfoxide reductase. Biotin sulfoxide reductase was assayed as described previously (9), except that 8 μM flavin adenine dinucleotide was added to the incubation mixture and that various assay temperatures were used, as indicated in the tables.

Nitrate reductase. Strains were scored as Nit^+ or Nit^- by stab assay (11). Quantitative assays of enzyme extracted from S1302 and its parent BM1161 were performed on the cell envelope fraction, isolated, and assayed as described previously (20). In these experiments, the various fractions were assayed for biotin sulfoxide reductase as well as nitrate reductase, confirming that biotin sulfoxide reductase remains in the soluble fraction under the same conditions that nitrate reductase is found in cell envelopes.

RESULTS

Isolation and mapping of *bis* mutations. Various types of *bis* mutations were selected in different experiments: (i) spontaneous temperature-sensitive mutations (Bis^+ at 30°C, Bis^- at 43°C); (ii) bacteriophage Mu *cts* insertions; (iii) mutations induced by ethyl methane sulfonate; and (iv) spontaneous *bis* mutations.

To facilitate genetic mapping, specialized transducing phages for the various *bis* genes were sought, starting from a pool of *E. coli* *Eco*RI fragments cloned into λ. This lysate was used to transduce various *bis* mutants to Bis^+ (selecting for growth on minimal glucose agar supplemented with 16 nM biotin sulfoxide) and also screened directly for Bis^+ feeding plaques on lawns of Bio^- Bis^- strains on minimal glucose medium supplemented with 780 nM biotin sulfoxide. Both methods yielded several isolates carrying one of the *bis* genes (*bisD*). We have not yet succeeded in isolating λ phages that carry *bisA*, *bisB*, or *bisC*. A defective λ *bisA* phage was isolated by Dykhuizen (11), but has since been lost. We also isolated (from an *Eco*RI pool of *Salmonella typhimurium* DNA cloned into λ) a λ *chlB* phage. Below, we show that, although *chlB* mutants have not been found in selections for *bis* mutants, they have a Bis^- phenotype.

Rapid mapping of *bisA*, *bisB*, and *bisC* mutations was facilitated by the use of strains in which the tetracycline resistance transposon Tn*10* had inserted near these loci. To prepare such strains, we first infected the Bis^+ strain R823 with phage NK55 by the method of Kleckner et al. (15) for the isolation of Tn*10* insertions. Many tetracycline-resistant survivors were pooled. We then grew phage P1 on this pool and selected for cotransduction of Tn*10* and bis^+ into a recipient strain carrying a *bis* mutation. By this method, we isolated two Tn*10* insertions linked to *bisC* and two others linked to *bisA* and *bisB*. Table 2 shows the results of P1 transduction using one insertion of each type.

Dykhuizen (11) divided those *bis* mutations that are linked to *gal* into two groups, *bisA* and *bisB*. The *bisA* mutations showed about 24% cotransduction with *gal* and lay under the *gal-bio* deletion of strain SA291, whereas *bisB* mutations showed about 3% cotransduction with *gal* and 15% cotransduction with the SA291 deletion. As seen in Table 2, the cotransduction frequency with *zbh-428*::Tn*10* is 80 to 100% for *bisA* and 10 to 20% for *bisB*. As the numbers in Table 2 are ratios of colony counts on different media, a cotransduction frequency greater than 80% is indistinguishable from complete linkage. Therefore, in some experiments individual tetracycline-resistant transductants were picked and scored for Bis phenotype. With a *zbh-428*::Tn*10* donor and a *bisA29* recipient, 113 of 120 (or 94%) of the tetracycline-resistant transductants were Bis^+. In the reverse cross (*bisA29 zbh-428*::Tn*10* donor and bis^+ recipient), 48 of 78 (or 62%) of the tetracycline-resistant transductants were Bis^-.

Classification of new mutations as *bisB* rather than as *bisA* is illustrated by the *bisB41* mutation of strain S1134. The cotransduction frequency of 11% is in the range typical of *bisB* mutations.

TABLE 2. Cotransduction of *bis* mutations with Tn*10*

Donor	Relevant genotype	Recipient	Relevant genotype	Cotransduction frequency[a] of Tn*10-bis*
S1234	*bioA24 bis*$^+$ *zbh-428*::Tn*10*	DD130	*bioA24 bisA29*	0.89
		DD132	*bioA24 bisA31*	0.80
		DD136	*bioA24 bisA35*	0.83
		S1186	*bioA24 bisB28*	0.22
		S1134	Φ (*bioA-lacZ*)*301 bisB41*	0.11
		S1187	*bioA24 bisC37*	0
S1231	*bioA24 bisB28 zbh-428*::Tn*10*	DD130	*bioA24 bisB29*	0.89
		S1186	*bioA24 bisB28*	0
		S1134	Φ (*bioA-lacZ*)*301 bisB41*	0
S1235	Φ (*bioA-lacZ*)301 *bis*$^+$ *zbh-428*::Tn*10*	DD132	*bioA24 bisA31*	1.2
		S1134	Φ (*bioA-lacZ*)*301 bisB41*	0.11
		S1138	Φ (*bioA-lacZ*)*301 bisB42*	0.16
S1236	Φ (*bioA-lacZ*)301 *bisB41- zbh-428*::Tn*10*	DD132	*bio24 bisA31*	0.88
		S1134	Φ (*bioA-lacZ*)*301 bisB41*	0
		S1138	Φ (*bioA-lacZ*)*301 bisB42*	0.003
S1228	*bis*$^+$ *zhj-428*::Tn*10*	DD130	*bioA24 bisA29*	0
		S1187	*bioA24 bisC37*	0.31

[a] Computed as the ratio of colonies on tetracycline-biotin sulfoxide agar to colonies on tetracycline-biotin agar. Tetracycline-biotin sulfoxide agar is minimal glucose agar plus tetracycline (15 μg/ml) and 16 nM biotin sulfoxide. Tetracycline-biotin agar contains 16 nM biotin instead of biotin sulfoxide. Both media contained thiamine hydrochloride (1 μg/ml). Whereas the actual cotransduction frequency of course cannot exceed unity, the measured value occasionally does (as in the transduction of DD132 by S1235) because of statistical fluctuations in colony counts.

Moreover, no Bis$^+$ tetracycline-resistant transductants were observed when the donor carried the *bisB28* mutation. Likewise, a *bisB41 zbh-428*::Tn*10* donor gives no Bis$^+$ tetracycline-resistant transductants with a *bisB28* recipient. The *bisB41 zbh-428*::Tn*10* donor S1236 has been used routinely to classify new mutations (such as *bisB42*), because the donor contains the same *bio-lac* fusion as the new mutants that we have isolated.

Dykhuizen (11) established the gene order *gal-attλ-bio-uvrB-bisA-bisB*. P1 transductions into recipients marked at *gal*, *attλ*, *bio*, and *bis* place *zbh-428*::Tn*10* to the right of *bio* and probably to the right of *bisA* (data not shown). The *zbh-428*::Tn*10* insertion confers no obvious phenotype other than tetracycline resistance. Specifically, it does not make the cell sensitive to UV light or affect its ability to reduce biotin sulfoxide or nitrate.

The *zhj-428*::Tn*10* insertion is linked to *bisC* (Table 2). Dykhuizen (11) established the gene order *mtl-xyl-glyS-bisC*. Use of a *xyl-zhj-428*::Tn*10* donor established the order (*zhj-428*::Tn*10*)*-xyl-bisC*, with 50% cotransduction of *xyl* with *zhj-428*::Tn*10* (data not shown). We routinely use this *xyl* donor in scoring new mutations, so that the order as well as the cotransduction frequency is verified for each *bisC* mutation.

Biotin sulfoxide reductase from temperature-sensitive mutants. We have mapped nine spontaneous *bis*(Ts) mutations, of which eight lie in *bisC* and one lies in *bisB*. No *bisA*(Ts) or *bisD*(Ts) mutations have yet been found.

With all *bis*(Ts) mutations tested, extracts from cells grown at 30°C were active, whereas extracts from cells grown at 43°C showed reduced activity. With some *bisC*(Ts) mutants (*bisC113*, *bisC114*, *bisC117*, Table 3), the enzyme produced at 30°C was more thermolabile than the wild-type enzyme. The enzyme from other mutants (*bisC105*, *bisC115*, *bisC119*, *bisC120*, *bisC121*) was at least as stable as the wild-type enzyme. We infer that mutations in the first group directly alter the structure of the enzyme, although the technical possibility that they affect some other component of the extract has not been rigorously excluded. Hence, *bisC* appears to be the structural gene for the enzyme or a subunit thereof. The enzyme from the one temperature-sensitive (*bisB*) mutant (*bisB121*) does not differ significantly from wild type enzyme in its thermostability.

Identity of some *bis* genes with previously characterized *chl* loci. Dykhuizen (11) noted that three of the four *bis* loci were closely linked to genes for nitrate reductase (called *chl* genes because most mutants deficient in nitrate reductase are chlorate resistant). One mutation (*bisD21*) appeared to cause both a Bis$^-$ and a Chl^r (Nit$^-$) phenotype. The two properties disappeared together on reverse mutation, and both were partially suppressed when transferred into

TABLE 3. Biotin sulfoxide reductase from *bis*(Ts) mutants[a]

Strain	Relevant genotype[b]	Growth temp (°C)	Biotin sulfoxide reductase assayed at 30°C[c]	% Activity (assayed at 40°C/ assayed at 30°C)[d]
BM1161	*bis*$^+$	30	44	42
		43	47	
S1294	*bisC113*(Ts)	30	22	16
		43	0.47	
S1295	*bisC114*(Ts)	30	23	12
		43	0.27	
S1296	*bisC117*(Ts)	30	22	14
		43	0.25	
S1297[e]	*bisC105*(Ts)	30	44	68
		43	31	
S1298[e]	*bisC120*(Ts)	30	26	49
		43	18	
		30	83	
S1299	*bisC115*(Ts)	43	0.83	50
S1300	*bisC118*(Ts)	30	18	37
		43	0.32	
S1301	*bisC119*(Ts)	30	32	50
		43	0.56	
S1302	*bisB121*(Ts)	30	17	50
		43	0.38	

[a] In these experiments, no flavin adenine dinucleotide was added to the assay tubes.
[b] All strains are derivatives of BM1161, which is Bio$^-$ due to the Φ (*lacZ-bioA*)*301* deletion.
[c] Specific activity in milliunits per milligram of protein, where units are defined as micrograms of biotin formed per 15 min (9).
[d] In a separate series of experiments, the same enzyme preparations from 30°C growth that are shown here were assayed in parallel at 30°C and at 40°C.
[e] S1297 and S1298 grow slowly on biotin sulfoxide at 43°C. They are Lac$^+$ on Lac agar with 390 nM biotin sulfoxide at 43°C. Genetic mapping of the temperature-sensitive mutations to the *bisC* gene was based on scoring their Lac phenotype.

a different genetic background. In this paper (see below), we present additional verification that *bisD21* is in fact a single mutation.

Many of Dykhuizen's *bisA* and *bisB* mutants were Nit$^-$ as well. A possible explanation was that these mutants were really double mutants, bearing mutations in a *bis* gene and in a nearby *chl* gene. The mutagen used, nitrosoguanidine, is known to cause closely linked multiple mutations (4). Genetic mapping placed the *bisA* mutations close to the *chlA* gene and *bisB* close to *chlE*. This explanation seemed supported by the isolation of some *bisA* and *bisB* mutations with no obvious effect on nitrate reductase, of a deletion strain that scored in complementation studies as *bisA*$^+$ but *chlA*$^-$ and of a specialized transducing phage that scored as *chlE*$^+$ but *bisB*$^-$.

The development of methods for selective isolation of *bis* mutants (3) allowed us to recover spontaneous mutants, most of which should differ from the Bis$^+$ parent by a single mutation. Some of these turned out to affect both Bis and Nit phenotypes. For example, we reported earlier (3) that strain S1134 was Nit$^-$ as well as Bis$^-$. Another isolate, S1138, although not completely Nit$^-$, produced noticeably less nitrite than did the wild type, as judged by the intensity of color in our standard stab test. Both S1134 and S1138 carry *bisB* mutations (Table 2). One of our temperature sensitive mutations, *bisB121*, determined a complex phenotype. Whereas strains carrying the mutation were Bis$^+$ at 30°C and Bis$^-$ at 43°C, the effect of temperature on nitrate reduction was the reverse. They scored as fully Nit$^+$ at 43°C and only weakly Nit$^+$ at 30°C. Nitrate reduction by the *bisB121* strain S1302 depended strongly on the composition of the growth medium (data not shown). The biotin sulfoxide reductase isolated from S1302 did not differ from the wild-type enzyme in its thermosensitivity (Table 3). The nitrate reductase extracted from S1302 was also indistinguishable from wild-type nitrate reductase in its relative activity at different temperatures (data not shown). The effect of this mutation therefore appears to be regulatory.

The occurrence of spontaneous mutants simultaneously deficient in both Bis and Nit led us to reexamine the possibility that single mutations, rather than coincident double mutations, were inactivating both functions. Several findings support this interpretation.

(i) Those *bisB* mutants originally scored by

Dykhuizen as Nit$^+$ all appeared, on reexamination, to be partially deficient in nitrate reduction, as judged by the intensity of color generated in the standard stab assay. Furthermore, some Nit$^-$ *bisB* mutants, such as *bisB28*, were found to revert to Bis$^+$ and Nit$^+$ in a single step (data not shown).

(ii) Eight Bis$^-$ mutants recovered after infection of BM1161 by bacteriophage Mu *c*ts have been analyzed genetically. Five of these mutations have been mapped at *bisA*, and three have been mapped at *bisC*. All five *bisA* mutants were Nit$^-$ as well as Bis$^-$, and the two traits were inseparable in P1 transduction. In all eight mutants, the Bis$^-$ phenotype and the thermosensitivity imparted by the Mu *c*ts prophage were completely linked, as expected for a Mu insertion within the *bis* gene. Assay of biotin sulfoxide reductase from the three *bisC*::Mu strains showed a specific activity that was low (about 4% of the wild-type control) but not zero.

(iii) A collection of Mu insertions in various chlorate genes was kindly provided to us by Valley Stewart of the University of Virginia (Table 1). As these strains were Bio$^+$, we could not test their Bis phenotype by growth on biotin sulfoxide. However, enzyme assays of three *chlA*::Mu strains (RK5200, RK5203, RK5221), three *chlE*::Mu strains (RK5201, RK5218, RK5255), one *chlB*::Mu strain (RK5237) and one strain (RK7-36) with a *chlB* mutation, *chl-36*, showed that these strains made no biotin sulfoxide reductase (less than 1% of wild-type activity). As *bis* mutations located near *chlB* had not appeared in our previous screening, we wanted to verify that the Bis$^-$ phenotype of RK7-36 was caused by the *chl-36* mutation. For this purpose, we transduced Tn*10* into RK7-36, using as donor strain RK4940, which carries a Tn*10* insertion linked to *chlB*. Of the tetracycline-resistant transductants, 13 of 18 were Nit$^+$. One of these transductants was assayed for biotin sulfoxide reductase and found to have full wild-type activity. Furthermore, a Bio$^-$ derivative of RK7-36 was prepared by transduction of Tn*10* into RK7-36, using as donor strain S1234, which carries a Tn*10* insertion linked to the *bioA24* mutation. This transductant had a Bis$^-$ phenotype and reverted in one step to Bis$^+$ Nit$^+$. This *bioA24 chlB* transductant was used for the isolation of the λ *chlB* phage mentioned earlier.

(iv) To obtain more *bisA* and *bisB* mutations, our usual selection for *bis* mutations was applied to the BM1161 derivative S1235, which carries a Tn*10* insertion close to *bisA*. Mutant colonies that were Lac$^+$ on plates containing 390 nM biotin sulfoxide were pooled, and phage P1 grown on the mixed culture was used to transduce Tn*10* into BM1161. Both unmutagenized and ethyl methane sulfonate-mutagenized cultures were used. This procedure yielded nine mutants that were analyzed by transduction of Tn*10* from the mutants into BM1161. Of these, three carried spontaneous *bisA* mutations, five carried ethyl methane sulfonate-induced *bisA* mutations, and one carried an ethyl methane sulfonate-induced *bisB* mutation. All nine were Nit$^-$ as well as Bis$^-$, and the Nit and Bis traits were inseparable by transduction. Bis$^+$ revertants selected from the eight *bisA* mutants as well as from one of Dykhuizen's *bisA* mutants, DD132, were all found to be Nit$^+$ as well and Bis$^+$.

Effects of molybdate and tungstate. Some (perhaps all) of the genes *chlA*, *chlB*, *chlE*, and *chlG* function in the biosynthesis of a molybdenum-containing cofactor which is a component of nitrate reductase. It is characteristic of molybdoenzymes that growth in the presence of the molybdate antagonist tungstate sharply reduces their specific activity in extracts (17, 23). As shown in Table 4, the specific activity of biotin sulfoxide reductase was decreased about 30-fold by growth in 10 mM tungstate.

Inactive nitrate reductase in tungstate-grown cells can be reactivated in vivo by the addition of molybdate to a culture in which new enzyme synthesis is inhibited by chloramphenicol (23). We observed no reactivation of biotin sulfoxide reductase under these conditions, nor have we achieved a significant degree of reconstitution of activity from extracts of tungstate-grown cells by any in vitro treatment (data not shown).

Another gene that functions in molybdenum utilization for nitrate reduction is *chlD*. Mutational inactivation of *chlD* causes partial resistance to chlorate and lowers nitrate reductase activity during growth on ordinary media. Supplementation of the medium with 1 mM molybdate relieves the defect (25). Bio$^-$ strains deleted for the *chlD* gene (such as SA322, Table 4) can grow with biotin sulfoxide as biotin source, but form less biotin sulfoxide reductase than the *chlD*$^+$ control. Supplementation of the medium with 1 mM molybdate completely reversed the defect (Table 4).

Effect of *bisD* dosage on enzyme activity. One reason for wanting λ *bis* phages, besides their use in genetic mapping, is the possibility of increasing the dosage of a particular *bis* gene by phage replication. Accordingly, we assayed biotin sulfoxide reductase both in λ *bisD* cI857 lysogens and in lysates made from thermoinduction of such strains. The specific activity of extracts made by induction was about 50 mU/mg of protein compared with a value of 30 mU/mg for the uninduced control or 50 mU/mg for a nonlysogenic *bis*$^+$ strain such as BM1161 (Table 4). We thus observe no significant increase in biotin sulfoxide reductase after induction, indi-

TABLE 4. Effect on biotin sulfoxide reductase of tungstate or molybdate in growth medium

Strain	Relevant genotype	Growth conditions[a]	Biotin sulfoxide reductase[b]
BM1161	*bis*$^+$	37°C	54
		10 mM Na tungstate, 37°C	1.8
SA302	*bis*$^+$ *chlD*$^+$	30°C	56
		1 mM Na molybdate, 30°C	51
SA322	*bis*$^+$ Δ(*chlD*)	30°C	31
		1 mM Na molybdate, 30°C	94
DD38	*bisD21*	37°C	0.11
		1 mM Na molybdate, 37°C	0.32
		10 mM Na molybdate, 37°C	4.1
		100 mM Na molybdate, 37°C	2.3

[a] Cultures were grown in tryptone broth supplemented with tungstate or molybdate as indicated.
[b] Specific activity in milliunits per milligram of protein, as in Table 3.

cating either that the *bisD* product is not made efficiently under these conditions, or that it is not rate limiting for enzyme synthesis or activity.

The validity of this conclusion rests on the assumption that the λ *bisD* phage really carries the *bisD* gene, rather than suppressing the effect of the *bisD* mutation by some other means. To test this assumption, we mapped the determinant carried by the *bisD* phage at the *bisD* locus (between *thr* and *leu*) as follows. The *bisD21* strain DD38 was lysogenized by λ *bisD*$^+$ *c*I857 to yield a strain (S1177) of presumed genotype *bioA24 bisD21* (λ *bisD*$^+$ *c*I857). To select for loss of the prophage, strain S1177 was streaked on complete medium (eosin methylene blue medium with galactose) at 43°C, and 20 independent thermoresistant survivors were picked and purified by restreaking. Of these, 8 of 20 were Bis$^+$ and 12 of 20 were Bis$^-$. This is the expected result if the λ *bisD*$^+$ phage lysogenized by crossing over with its chromosomal homolog (the principal route of lysogenization by a phage in which the *int* gene and *att* site are substituted by bacterial DNA as expected for the λ781 cloning vector) and is lost by crossing over within the same homologous region. To obtain direct evidence that the homology is at the *bisD* locus, we wanted to know whether the *bisD* mutation had in fact been replaced by its wild type homolog in the Bis$^+$ segregants rather than being suppressed by some event occurring elsewhere in the genome.

This goal was accomplished as follows. Phage P1 was grown on two of the thermoresistant segregants, one of which was Bis$^+$ and the other of which was Bis$^-$. The lysates thus prepared were used for transduction into strain DD120, which is *bio*$^-$ *thr*$^-$ *leu*$^-$, to see whether the *bisD21* mutation had been replaced in the Bis$^+$ segregant. All of the Thr$^+$ Leu$^+$ transductants from the Bis$^+$ donor were Bis$^+$, whereas most of those from the Bis$^-$ donor were Bis$^-$ (Table 5). We conclude that the *bisD21* allele had been replaced in the Bis$^+$ segregant. Therefore, the *bisD21*$^+$ allele must have been present in the λ *bisD*$^+$ phage. Because it forms "feeding plaques," we assume that the λ *bis*$^+$ phage contains the entire *bisD* gene.

All the transductants from Table 5 and all the thermoresistant segregants from S1177 were tested for Nit as well as Bis. The two traits coincided in every case, verifying that a single mutation generates both phenotypes.

As a final check, we isolated a λ *bisD21* phage as a non-feeding plaque in a lysate of λ *bisD*$^+$ grown on a *bisD21* host. To show that this phage carried a mutant *bisD* gene rather than no *bisD*

TABLE 5. Transduction from Bis$^+$ and Bis$^-$ derivatives of *bisD21* (λ *bisD*$^+$ *c*I857)[a]

Donor	Selection	Transductants per phage	Fraction of transductants that were Bis$^+$
Bis$^+$	*thr*$^+$ *leu*$^+$	5×10^{-8}	20/20
	thr$^+$	8×10^{-6}	20/20
Bis$^-$	*thr*$^+$ *leu*$^+$	1×10^{-7}	4/20
	thr$^+$	2×10^{-5}	10/20

[a] Recipient is DD120 (Table 1), whose relevant genotype is *bioA24 thr*$^-$ *leu*$^-$. Transductions were performed on synthetic medium supplemented with the other amino acids required by DD120.

TABLE 6. Factor-independent activity of lysates prepared by phage induction

Strain	Relevant genotype	Type of extract[a]	Protein (mg)[b]	Sp act[c]	
				No added factor	Added factor[d]
S1178	*bisD21* (λ *bisD21* cI857)	Sonic	0.11	5	ND
			0.82	33	ND
			1.92	39	ND
S1178	*bisD21* (λ *bisD21* cI857)	Phage induction	0.02	55	ND
			0.05	46	ND
			0.10	56	ND
			0.26	57	ND
			0.77	62	ND
S1178	*bisD21* (λ *bisD21* cI857)	Sonic	0.82	33	84
S1178	*bisD21* (λ *bisD21* cI857)	Phage induction	0.1	40	40
			0.062	37	24
S1198	(λ *imm434*) (λ cI875)	Sonic	0.1	9	54
S1198	(λ *imm434*) (λ cI875)	Phage induction	0.062	50	24

[a] Sonic extracts were prepared from cells grown at 30°C. Extracts prepared by phage induction were induced by heating to 43°C for 15 min and then shaken at 37°C until lysis was complete.

[b] Milligrams of protein of extract in the 0.25-ml assay tube.

[c] Milliunits per milligram of protein, as in Table 3. ND, Not determined.

[d] The added factor was a sonic extract of the *bisD21* mutant DD38 which had been heated for 1 min at 92°C and had a specific activity (when assayed alone) of about 0.1. The amount of protein added in the factor was 1.7 mg (first three entries) or 0.9 mg (last two entries).

gene at all, we tested its ability to rescue the *bisD21*$^+$ allele from a *bisD*$^+$ host. The phage was spotted on a lawn of the Bis$^+$ segregant from S1177 (Table 5). Among progeny phage obtained by thermoinduction of cells taken from the center of the spot and grown in broth, 0.30% (17 of 5,704) were feeder (Bis$^+$) plaques, whereas the control lysate (λ *bisD21* on the Bis$^-$ segregant) contained 0 of 2,536 Bis$^+$ plaques. When a random nonfeeding plaque from the λ *Eco*RI *E. coli* pool was cycled in the same manner, 0 of 12,151 Bis$^+$ plaques were found.

We conclude that the λ *bisD*$^+$ phage does in fact carry the *bisD* gene, although amplification of the gene through induction of the lysogen does not lead to an increase in biotin sulfoxide reductase.

Effect of λ induction on biotin sulfoxide reductase. In the course of the λ *bisD* studies, an interesting effect of λ induction on the properties of the enzyme came to light. In some extracts of *bis*$^+$ strains, biotin sulfoxide reductase activity depends on the presence of a heat-stable thioredoxin-like protein (9). This dependence is manifested by a decrease in specific activity on dilution of the extract. The activity of a diluted extract can be restored by the addition of an undiluted heated extract obtained from either a wild-type or a Bis$^-$ strain.

As shown in Table 6, extracts prepared by induction of λ lysogens were not dependent on the heat-stable factor. Sonic extracts of uninduced cultures of the same strains show the previously observed dependence on added factor. Further experiments (data not shown) have demonstrated that (i) the same factor independence is observed when a *bis*$^+$ lysogen of λ *c*$^+$ is induced with mitomycin rather than heating; (ii) no activity is observed when λ lysogens of strains carrying *bisA*, *bisB*, *bisC*, or *bisD* mutations are induced; (iii) the enzyme formed by phage induction is completely stable to heating 1.5 min at 60°C, a treatment that reduces the activity of a sonic extract to 14% of its original value; (iv) enzyme prepared by phage induction of the *bisC114*(Ts) lysogen S1295 is also stable to heating 1.5 min at 60°C; (v) when heated for 1.5 min at 70°C, wild-type enzyme prepared by phage induction retains 66% activity, whereas enzyme from S1295 retains only 30% activity; (vi) a sonic extract is not stabilized against heating by mixing with an inactive lysate prepared by phage induction of a *bisC* lysogen; and (vii) when mixtures of wild-type and *bisC114* enzymes prepared by phage induction were heated, the resulting activity was that expected if the two were inactivated independently.

These results suggest that biotin sulfoxide reductase can exist in at least two molecular forms, and that the enzyme obtained by inducing a λ lysogen is largely in a factor-independent form that is more thermostable than the factor-dependent form. The factor-independent form might either utilize an alternative reaction pathway not requiring factor or contain factor tightly bound to the enzyme. Studies of dilution curves of various extracts suggest that both forms exist in growing cells, but that the factor-independent

form is preferentially produced or preferentially extracted when enzyme is prepared by λ induction (data not shown).

DISCUSSION

Enzymes from diverse biological sources contain molybdenum in the form of a dissociable molybdenum-containing factor whose chemical structure (where known) is a pterin-molybdenum complex (6, 14). Molybdenum-containing factors from various organisms are functionally interchangeable and may be physically identical, although the factor from certain bacterial nitrogenases is distinct and contains iron as well as molybdenum (24). It is characteristic of molybdoenzymes that the amount of active enzyme is drastically reduced in cells grown in high concentrations of tungstate or vanadate (17, 23). The apparent mechanism is competition between tungstate and molybdate for some site on the factor or the enzyme. Inactive enzyme from tungstate-grown cells contains tungsten in place of molybdenum in the case of some enzymes, but contains neither metal in others.

The best-characterized molybdoenzyme of *E. coli* is nitrate reductase, an integral membrane protein with several components including the reductase itself (coded by the *chlC* gene), cytochrome b_1, and the molybdenum-containing factor (19). The *chlD* product is implicated in the initial stages of molybdenum assimilation; the *chlA* gene product appears to function in the biosynthesis of the molybdenum-containing factor into the enzyme (18, 25). Little is known about the functions of *chlE* and *chlG*. It was suggested at one time, on very indirect evidence, that the *chlE* gene product was cytochrome b_1, but this idea has apparently been withdrawn by its author (18, 19). Deletions or Mu insertions have been isolated in most of the *chl* genes. Bacteria bearing such alterations are viable. Therefore, none of these genes performs an essential cellular function.

Our finding that the specific activity of biotin sulfoxide reductase is depressed in tungstate-grown cells suggests that it is a molybdoenzyme. This interpretation is supported by the genetic results. The most economical hypothesis is that nitrate reductase and biotin sulfoxide reductase require a common molybdenum-containing cofactor, and that the *chlA*, *chlB*, *chlE*, and *chlG* genes contribute to both enzymes solely by making products that function in the synthesis or attachment of the cofactor. In media unsupplemented with excess molybdate, the *chlD* product is also needed for full activity of both enzymes. The effect of *chlD* deficiency on nitrate reductase activity is much more drastic than that on biotin sulfoxide reductase. We have also seen that the biotin sulfoxide reductase activity of a *chlG* mutant (DD38) is partially reversed by growth in 10 mM molybdate. As this is the only *chlG* strain examined, it would be premature to interpret this result with respect to *chlG* function.

Strictly speaking, our data do not prove that these *chl* genes are identical to the corresponding *bis* genes, only that single mutations of known nature can inactivate both functions simultaneously. Polarity or protein-protein interactions could in principle generate the same result. However, the original evidence that the genes are different was not compelling. The argument depended mainly on the phenotypes of deletion mutants or of cells carrying specialized transducing phages (11). As the Bis and Nit functions are scored under different growth conditions, an adjacent deletion might well eliminate one phenotype and not the other by placing the gene under control of a promoter different from the normal one.

The relationship between nitrate reductase and other cellular functions affected by *chlA*, *chlB*, *chlD*, *chlE*, and *chlG* functions is incompletely understood. The structural genes for nitrate reductase (*chlC*) and formate dehydrogenase (*chlF*) are closely linked, but distinct (10). Many observations indicate that these two proteins interact with each other in vivo. Formate dehydrogenase is found in two distinct molecular forms, both of which contain selenium (7). One is membrane bound and inducible by nitrate; the other is soluble and repressed by nitrate. Most mutations in *chlC* and *chlF* do not render the cell chlorate resistant, suggesting that a separate molybdoenzyme may be able to reduce chlorate to chlorite (13). The case of biotin sulfoxide reductase may be of interest because there is no indication that it is associated in any way with nitrate reductase. Biotin sulfoxide reductase is soluble and constitutive, whereas nitrate reductase is membrane bound and inducible. This emphasizes the possibility that, regardless of what interactions may take place among individual enzymes, the only element common to all the activities affected by the *chlA*, *chlB*, *chlD*, *chlE*, and *chlG* genes may be the molybdenum cofactor. If the only role of these five genes is in molybdenum cofactor synthesis, then the cofactor must be unique (not duplicated by the products of any other *E. coli* genes) and not required for any indispensable *E. coli* enzymes.

Our failure to reconstitute active biotin sulfoxide reductase by adding molybdenum or cofactor to inactive preparations, either in vitro or in vivo (in the presence of chloramphenicol), has precluded a more direct demonstration that molybdenum is required for activity. However, the results do not cast doubt on this conclusion

either, because similar difficulties have been encountered in other systems. For example, no in vivo reconstitution of *E. coli* cofactor able to complement *Neurospora crassa* nitrate reductase in vitro was observed in the presence of chloramphenicol, although active cofactor could be regenerated by incubation of the extract mixture with 20 mM Na_2Mo0_4 (1).

Biotin sulfoxide reductase is one of a number of enzymes (generally not molybdoenzymes) that require a small heat-stable protein factor for maximal activity in vitro. It is not known how many species of such thioredoxin-like molecules are present in *E. coli*. Mutational evidence shows that thioredoxin is required in vivo for replication of bacteriophage T7 and that another protein, thioredoxin reductase, which regenerates thioredoxin by reaction with NADPH, is needed for methionine sulfoxide reduction (12, 21). Mutants lacking either thioredoxin or thioredoxin reductase can reduce biotin sulfoxide in vivo and yield extracts containing heat-stable factor (9). Hence biotin sulfoxide reduction does not depend on the unique thioredoxin species functional in T7 replication. There is no genetic evidence that the thioredoxin-like factor plays any part in biotin sulfoxide reduction in vivo. Experiments reported here indicate that dependence on added factor is not absolute, and that enzyme obtained from λ lysates is largely in a factor-independent form. This factor independent activity is unlikely to represent a totally different enzyme from biotin sulfoxide reductase because of the common genetic requirements for the two activities.

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