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Transport and Utilization of D-Methionine and Other Methionine Sources in *Escherichia coli*

ROBERT J. KADNER

Department of Microbiology, The University of Virginia School of Medicine, Charlottesville, Virginia 22901

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The transport and utilization of D-methionine was investigated in several strains of *Escherichia coli* K-12. Wild-type cells exhibit a single transport system with a K_m of 1.16 μM. This activity exhibits a specificity similar to that of the uptake of L-methionine. The activity toward the D-isomer and the high-affinity uptake of L-methionine are lost in strains mutant in *metD*, along with the ability to utilize D-methionine as methionine source. Both activities respond identically to gene dosage of *metD* and are both restored in revertants or transductants. However, although L-methionine is a potent inhibitor of D-methionine uptake, D-methionine has little or no effect on the uptake of the L-isomer. No mutants altered in the uptake of only one of the two isomers were found in a screening. Regulation of both activities was similar in their response to the internal methionine pool, and some evidence was suggestive of partial repressive control of these activities. The evidence is most consistent with the role of the *metD* product as a common step for two methionine-specific uptake systems, but other gene products may represent the initial substrate binding sites. This system also appears to be involved in the uptake of *N*-acetyl methionine and methionine sulfoxide and methionine sulfoximine. The uptake of the keto analogue of methionine, α-keto-γ-methiol butyrate, appears to be mediated by a separate system specific for α-keto straight-chain acids 5- to 6-carbon units in length.

The enteric bacteria frequently possess multiple transport systems for most of the common amino acids (10, 22). The genetic and kinetic characterization of the uptake and utilization of L-methionine by *Escherichia coli* has revealed the operation of two separate transport systems (13, 15). The high-affinity system, with a K_m of 0.13 μM, is missing in strains mutant in *metD*; a low-affinity system with a K_m near 20 μM is altered in strains mutant at an as yet unlocalized site distinct from *metD*. The high-affinity system, and probably the low-affinity one, depends on phosphorylated compounds, presumably adenosine 5'-triphosphate, as the energy source for active transport (16). Both systems also are regulated, in part, by the level of the internal methionine pool by a phenomenon termed transinhibition, analogous to feedback inhibition (14).

The growth properties of the parental and mutant strains were correlated with the substrate specificities of these uptake systems. Auxotrophic requirements for methionine can be satisfied by a variety of compounds, including methioninyl peptides, *N*-acetyl methionine, and the α-keto analogue α-keto-γ-methiol butyrate. In addition, D-methionine can be utilized (6, 18). This is in contrast to the situation with most amino acids for which the D-isomer will not satisfy the auxotrophic requirement. Mutants that do respond to specific D-amino acids have been isolated; these are altered to exhibit either increased uptake of the D-isomer or increased activity of the enzyme activity responsible for the conversion of the D- to the L-isomer (17, 18, 19). The transport systems for the D-isomers of histidine (1) or leucine (23) are one of the normal systems for the entry of the L-isomer.

Mutants altered at a single site and lacking the high-affinity L-methionine uptake system were unable to utilize *N*-acetyl methionine or D-methionine (15). Only the high-affinity component of L-methionine uptake was competitively inhibited by *N*-acetyl methionine or methioninyl peptides. As originally shown by Piperno and Oxender (22), L-methionine uptake is only poorly inhibited by D-methionine, if at all. Since these *metD* mutants are unable to utilize D-methionine, it was of importance to characterize D-methionine transport and to determine the biochemical lesion in these mutants. This paper will demonstrate a close, but not exact, relationship between the uptake sys-

tems for the two isomers. To attempt to define the identity of these transport activities, the biochemical criteria were supplemented with a number of genetic tests, including the construction of strains diploid for *metD* and the isolation of a number of methionine analogue-resistant mutants, many of which were altered in transport.

Finally, Cooper (6) showed that cells could utilize D-methionine if they had been grown in the presence of D-methionine, but not if grown with the L-isomer. The role of transport processes in this regulation was investigated.

MATERIALS AND METHODS

Bacterial strains. Most of the studies described here used the derivatives of the *E. coli* K-12 strain KBT001, which are described in Table 1. Phenotypic characterization of *metD* mutants is based on their inability to utilize D-methionine and the absence of the high-affinity L-methionine uptake system. Genetic characterization is based on the inheritance of the ability to utilize D-methionine among 85 to 95% of the recombinants receiving *proC*$^+$ from HfrH. This linkage clearly distinguishes *metD* from *dadA*, which also results in the inability to utilize D-methionine (25).

The F′ used for dominance studies was KLF4, which extends from the origin of HfrH to *proA* and carries *metD*; it was obtained from B. Low. Strains TJC22 (*metD*$^+$) and TJC13 (*metD*) were obtained from W. Epstein and were used for comparison with the putative *metD* strains described here. There is no significant difference between the properties of TJC13 and those of the mutants isolated here.

TABLE 1. *List of bacterial strains*

Strain	Genotype	Source
KBT001	*thi leuB proC purE trp lysA metE ara xyl lacZ strA tonA*	
RK4205	As KBT001, but *metE*$^+$ by transduction	
RK4211	As RK4205, but *metD*	
RK4214	As KBT001, but *lys*$^+$ *recA*	
RK4215	As KBT001, but *metD lys*$^+$ *recA*	
RK4227	KLF4/RK4214; F′ *leu*$^+$ *metD*$^+$/*leu metD*$^+$ *recA*	
RK4228	KLF4/RK4215; F′ *leu*$^+$ *metD*$^+$/*leu metD recA*	
RK4101	*thi leuB proC lysA metE argH lac strA tonA*	P. Bassford
RK4226	As RK4101, but *metJ* (?)	
RK4229	As RK4226, but *metE*$^+$ by conjugation	
RK4230	As RK4226, but *metE*$^+$ *metJ*$^+$ by conjugation	
TJC22	*proA metB argI his str mal*	W. Epstein
TJC13	*proA metB metD argI his str mal xyl lac*	W. Epstein
E15	*thi phoA* Hfr	E. Lin
KL209	*thi* Hfr	B. Low
H	*thi* Hfr Hayes	B. Low

Cells were cultivated in minimal medium A of Davis and Mingioli (7), supplemented with glucose (0.5%), thiamine (1 μg/ml), adenine (40 μg/ml), and required amino acids (100 μg/ml). When specified, vitamin B_{12} was added to a final concentration of 5×10^{-8} M; cells were grown in B_{12} long enough to exhaust methionine pools and to ensure that they were dependent on B_{12} for growth. Cells were grown into the mid-logarithmic phase at 37°C in a gyratory incubator.

Isolation of analogue-resistant mutants. Methionine analogue-resistant mutants were selected in the *metE* strains KBT001 and RK4101 with the use of B_{12} as methionine source. Of the analogues tested at concentrations of less than 500 μg/ml, only methionine sulfoximine and α-methyl methionine were efficient growth inhibitors and were routinely used at concentrations of 50 and 500 μg/ml, respectively. The parental strains were inoculated into replicate tubes of minimal medium with 5×10^{-8} M B_{12} as methionine source and incubated overnight. A portion from each tube was plated onto minimal agar plates containing 5×10^{-8} M B_{12} and the appropriate analogue. Resistant colonies appearing were purified by single-colony isolation and then tested for their response to both analogues and to D-methionine. Only one mutant of each type from each selective plate was retained.

Assays. The transport assay procedure has been described (13) and uses cells washed twice and resuspended to a density of about 0.85 mg of protein per ml of medium A containing glucose (0.5%) and, unless otherwise specified, chloramphenicol (100 μg/ml). All transport assays were at 23°C, and initial rates were measured by filtration and washing of cells after their exposure for 0.20 min to labeled substrate. Specificity of transport was measured in like fashion by the addition of cells to the labeled substrate and the unlabeled inhibitor at the specified concentration.

The intracellular distribution of labeled metabolites of methionine was determined by filtration of cells exposed to the appropriate labeled substrate for various time periods ranging from 0.3 to 5 min. Washed cells on the filter were extracted either with boiling water, as previously described (13), or with ice-cold 70% ethanol, according to Montie and Montie (20). The extract was dried by lyophilization. Portions were applied to Whatman 3MM paper and resolved by descending chromatography with butanol-acetic acid-water (2:1:1). The distribution of radioactivity was measured in a Packard radiochromatogram scanner, and the migration of standards was detected with ninhydrin.

Cystathionase assays were carried out with toluene-treated cells, as described by Holloway et al. (11).

Chemicals. L-[U-^{14}C]methionine was obtained from New England Nuclear Corp. D-[1-^{14}C]methionine was synthesized by Calbiochem. Most of the other chemicals were from Sigma. The contents of

preparations of D- and L-methionine were analyzed on a Beckman amino acid analyzer.

RESULTS

Transport of D-methionine. Experiments to examine the ability of D-methionine to serve as methionine source revealed that methionine auxotrophs achieved the same growth yield (130 μg [dry weight]/μg of methionine) with D-methionine as with the L-isomer or the α-keto analogue. In addition, for these three sources, the growth rate was constant and independent of the concentration of supplement. As already mentioned, strains mutant in *metD* are unable to respond to D-methionine, but their growth responses to L-methionine and α-keto-γ-methiol butyrate were the same as the wild type. These results indicated that normal strains possess an efficient transport system for D-methionine.

The transport of D-methionine was determined by the uptake of carboxyl-labeled D-methionine into cells in the presence of glucose and chloramphenicol. This isomer was accumulated into cells against a concentration gradient. Almost all of the intracellular label extracted with boiling water had the chromatographic mobility of methionine. No radioactivity was expected to be associated with the polyamines since the labeled carboxyl group is lost in their formation. No significant amounts of label were found with the chromatographic mobility of α-keto-γ-methiol butyrate ($R_f = 0.9$).

The initial rate of uptake was a hyperbolic function of the external concentration in the range 0.20 to 20 μM, indicating the presence of but a single system (Fig. 1). The apparent K_m for uptake was 1.16 μM in strains KBT001 (*metE*), RK4205 (*metE*$^+$), and E15 (*metE*$^+$). The V_{max} in strains KBT001 and RK4205 was 330 pmol/μl of cell water × min, and 575 pmol/μl × min in strain E15. The *metD metE*$^+$ strain RK4211 exhibited very low rates of D-methionine uptake. Identical results were obtained with a *metD* strain, TJC13, obtained from W. Epstein, as well as with other *metD* strains isolated here. It was previously shown that these strains also lack the high-affinity L-methionine uptake systems (13) but retain normal levels of the low-affinity uptake system.

The substrate specificity of the D-methionine transport system was determined through the effect of unlabeled analogues on the uptake of the labeled methionine isomers (Table 2). There was no effect on the uptake of either isomer by any of the other amino acids found in proteins. Seleno-methionine was as effective an inhibitor as methionine. A number of methionine-containing dipeptides, amides, and esters

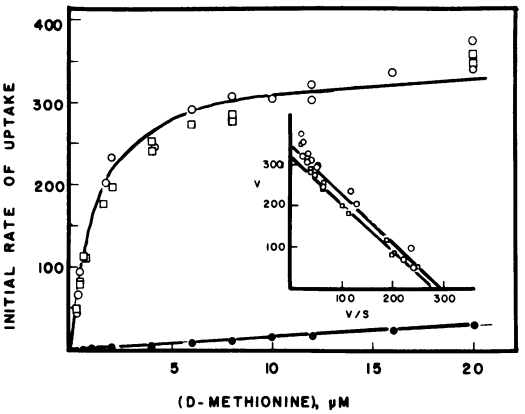

FIG. 1. *Initial rate of D-methionine uptake as a function of external concentration. The initial rate of D-[^{14}C]methionine uptake, as determined at 0.20 min, was measured at increasing substrate concentrations for washed cells of strains KBT001 (□), RK4205 (○), and RK4211 (metD) (●). Each point represents the average of duplicate determinations in picomoles accumulated per microliter of cell water × min. The line drawn is the theoretical curve for a system with $K_m = 1.16$ μM and $V_{max} = 330$.*

were competitive inhibitors of the uptake of both isomers. Whereas the uptake of D-methionine could be almost completely abolished by these compounds, the uptake of L-methionine could only be inhibited 70 to 80% by them, owing to the presence of the low-affinity L-methionine uptake system. This system is insensitive to these compounds, as shown by their lack of inhibition of L-methionine uptake in *metD* mutants. Carboxyl-substituted methionine competed with either isomer on all uptake systems. The inhibition constants for the methionyl dipeptides, amides, and esters were in the range of 2 to 8 μM and were similar with either isomer as substrate. Major differences were noted with methionine sulfoximine and methionine sulfoxide. These were very poor inhibitors of L-methionine uptake but were considerably more inhibitory against D-methionine. These results suggested that the binding sites for the two isomers might not be identical.

The effect of the presence of one methionine isomer on the uptake of the other yielded similar results (Fig. 2). The uptake of D-methionine was competitively inhibited by L-methionine with a K_i of 0.13 μM, which is also the K_m for the uptake of the L-isomer through the high-affinity system. In contrast, and in agreement with previous reports (22), D-methionine was a very poor inhibitor of L-methionine uptake. The competitive inhibition observed had a K_i in the range of 150 to 200 μM and could result from a minor (less than 1%) contamination of D-methi-

TABLE 2. *Effect of methionine analogues on the uptake of* L- *and* D-*methionine*[a]

Addition	Concn (μM)	Rate of uptake (% of control) of:	
		L-Methionine	D-Methionine
N-acetyl-L-methionine	10	59	24
	80	27	13
	200		6
N-acetyl-D-methionine	80	81	40
	200		8
Glycyl-L-methionine	10	39	47
	80	33	18
L-Methionyl glycine	20	14	17
	80	4	9
L-Methionine ethyl ester	10	45	17
	80	16	10
	200		3.6
L-Methionine sulfoxide	40	100	49
	200	71	19
L-Methionine sulfoximine	80	98	64
	200		40
DL-Norleucine	80	100	65
Methioninol	80	88	89
DL-Ethionine	40	97	76
Seleno-L-methionine	20	8	18
S-methyl cysteine	20	89	90
α-Methyl methionine	60	89	94
α-Keto-γ-methiol butyrate	80	86	78

[a] The initial rate of uptake by washed cells of strain RK4205 was measured in duplicate after 0.20-min exposure to the labeled methionine isomer and the addition at the indicated concentration. The final concentration of labeled L-methionine (100 Ci/mol) was 0.78 μM; that of D-methionine was 4 μM. All values are the average of at least four determinations, each in duplicate.

onine by the L-isomer. This lack of inhibition was not explicable by the assumption that one L-methionine uptake system was sensitive to the D-isomer while the other system was not, since little inhibition was observed no matter what proportion of substrate was transported by the high-affinity system.

Related to this was the demonstration that intracellular label from D-methionine was rapidly chased from cells upon addition of D-methionine (3 mM), L-methionine (2 μM), or the α-keto analogue (200 μM) to uptake medium containing 3.3 μM labeled D-methionine. The half-time for the exponential loss of label under these conditions was 1.3 to 1.8 min. In contrast, the intracellular label derived from L-methionine was chased by the addition of excess L-methionine or α-keto-γ-methiol butyrate, but not by the addition of D-methionine.

Energy poisons had essentially identical effects on the uptake of the two isomers. Treatment with sodium fluoride (100 mM), sodium azide (10 mM), or dinitrophenol (1 mM) in the presence of glucose resulted in only partial decreases in the rate of uptake and the steady-state level. Accumulation was abolished by exposure to fluoride plus azide, fluoride plus dinitrophenol, or *N*-ethylmaleimide (1 mM).

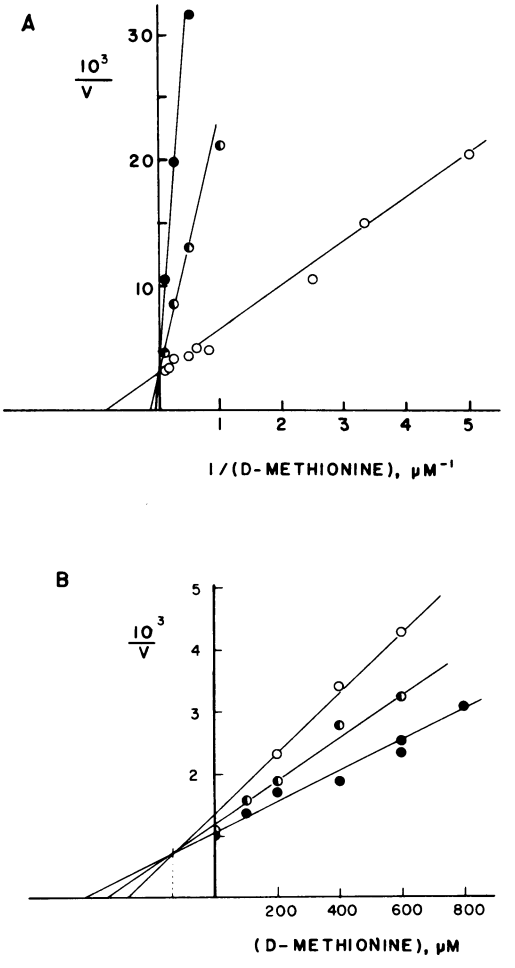

FIG. 2. *Inhibition of methionine uptake. (A) presents a double reciprocal plot of the effect of* L-*methionine on the uptake of* D-*[*^{14}*C]methionine. The concentration of* D-*methionine was varied at unlabled* L-*methionine concentrations of 0* (○), *0.5* (◐), *or 2.5* (●) *μM. Each point is the average of duplicate 0.2-min uptake values. Strain RK4205, grown in minimal medium in the absence of methionine, was used. (B) shows the effect of* D-*methionine on* L-*[*^{14}*C]methionine uptake. The effects of the increasing inhibitor concentrations shown along the abscissa on the initial rate of uptake of* L-*methionine at 0.78* (○), *2* (◐), *or 4* (●) *μM were determined in duplicate. The point of intersection of the experimental lines projects on the ordinate to a value almost equal to* V_{max}, *and the projection on the abscissa is equal to* $-K_i$.

Genetic control of D-methionine uptake. These results indicated that the uptake systems for the two methionine isomers were related but not identical. Genetic evidence already presented showed that mutants altered only in *metD* lost both the high-affinity L-methionine uptake system and the ability to utilize D-methionine for growth. These strains were also defective in uptake of D-methionine. All of 17 revertants of the *metD* strain RK4209, which were selected for regaining the ability to grow on D-methionine, had regained wild-type levels of transport of both methionine isomers.

A second indication that *metD* codes for a step common to the transport of both isomers came from the transport activities of strains diploid for the *metD*-containing region of the chromosome (Table 3). The diploid strain RK4227 (*metD*$^+$/*metD*$^+$) had 2.8- and 3.5-fold higher initial rates of L- and D-methionine uptake, respectively. The increase in L-methionine uptake activity affected only the high-affinity component, and the uptake of D-methionine in this strain was characterized by a higher V_{max}, but the same K_m, relative to the haploid strain. The diploid strain RK4228, with only one functional *metD*$^+$ allele, exhibited essentially the same rate of uptake as the haploid strains. Thus, the *metD*$^+$ allele is dominant and the *metD* locus is clearly involved in a step of the uptake of both isomers.

Mutants altered in methionine transport were obtained by penicillin selection in the presence of D-methionine or by selection for resistance to the methionine analogues, α-methyl methionine or methionine sulfoximine. Seven independent mutants specifically unable to grow with D-methionine as methionine source were obtained after nitrosoguanidine mutagenesis and penicillin selection. These mutants utilized L-methionine and its α-keto analogue, but not D-methionine or *N*-acetyl methionine. All lacked D-methionine and high-affinity L-methionine uptake. Conjugal crosses between three of these strains and HfrH revealed that the locus conferring the ability to utilize D-methionine in these strains was located between *leu* and *proC* on the chromosome map. These mutants were identical in all respects to the *metD* strains already described.

TABLE 3. *Effect of metD gene dosage on methionine uptake*

Strain[a]	Relevant genotype	Relative initial rate of uptake[b] of:	
		L-Methionine	D-Methionine
RK4214	*metD*$^+$	1.00	1.00
RK4215	*metD*	0.11	0.04
RK4227	*metD*$^+$/*metD*$^+$	2.79	3.52
RK4228	*metD*$^+$/*metD*	1.11	1.10

[a] Cells were grown in minimal growth medium with L-methionine (100 μg/ml). The two diploid strains were grown without leucine to maintain selection for the presence of the episome.

[b] Washed cells were exposed to labeled L-methionine (2 μM, 100 Ci/mol) or D-methionine (4 μM, 100 Ci/mol) for 0.20 min before filtration and washing. The initial rates of uptake, in terms of picomoles per microliter of cell water × minute, for strain RK4214 were 540 for L-methionine and 370 for D-methionine.

The transport properties of 62 methionine analogue-resistant mutants (half selected on one analogue, half on the other) were determined. Of 31 α-methyl methionine-resistant isolates, 25 were also resistant to methionine sulfoximine; 8 of the 32 methionine sulfoximine-resistant mutants were also resistant to α-methyl methionine. All of the mutants resistant to both analogues were unable to utilize D-methionine. Each of these mutants had depressed initial rates of uptake of both methionine isomers, down to 5 to 15% of the parental rate for D-methionine and 41 to 49% for L-methionine. The genetic lesion in five representative mutants of this class was located between *leu* and *proC* in the region expected for *metD*, as judged by the genetic criteria mentioned above. None of the mutants resistant to either methionine analogue alone had depressed uptake of either isomer (at least no lower than 80% of the wild-type rate). Three mutants resistant only to methionine sulfoximine had markedly increased levels of uptake of both isomers. The characterization of these strains is described below. No mutants were specifically altered in the uptake of either isomer alone.

Regulation of D-methionine uptake. In accord with the findings of Cooper (6), the rate of uptake of D-methionine was elevated in cells grown in the presence of D-methionine over that in cells grown in L-methionine (data not shown). However, the uptake of L-methionine responded similarly to the growth conditions. Uptake of either isomer into cells grown without methionine supplementation was also higher than that in cells grown with L-methionine, suggestive of a repression process. On the other hand, the rate of incorporation of either isomer into trichloroacetic acid-insoluble material was very similar regardless of the methionine supplementation (data not shown). This was interpreted as indicating that the primary level of control by the growth conditions on the utilization of methionine was at the transport process. We have previously shown that the uptake of L-methionine is regulated by the in-

ternal methionine pool by a form of feedback inhibition (14). This same regulation also appeared to apply to the uptake of D-methionine (14). A pertinent example of this type of regulation of methionine uptake was provided by those mutants resistant to methionine sulfoximine that had elevated levels of uptake of both methionine isomers. A representative member of this group, RK4226, was studied further. The rates of uptake of proline, arginine, and histidine in this mutant were close to those in the parental strain, RK4101 (0.71, 0.77, and 0.68, respectively), and glutamine uptake was 1.8 times higher in the mutant. Relative to the parental strain, the uptake of L-methionine was elevated three- to fivefold, and that of D-methionine was elevated 8- to 12-fold. Considerable variation in the uptake in this mutant was noted, in contrast to the reproducibility usually observed with other strains.

This elevated uptake was not due to decreased efflux activity or the inability of the system to be regulated by feedback inhibition. The level of cystathionase, a methionine biosynthetic enzyme, was more than 10-fold higher in extracts of the mutant than in extracts from the parental strain. This behavior was reminiscent of that in strains altered in *metJ*, the regulatory gene for the methionine biosynthetic enzymes, both with respect to the elevated expression of the genes of the biosynthetic pathway (9, 24) and in the elevated levels of the transport seen in *metJ* mutants that are also methionine auxotrophs (14).

The genetic location of the alteration in this strain was estimated by conjugal crosses with the Hfr strain, KL209, with selection for arg^+ or $metE^+$ recombinants. Of 200 arg^+ recombinants, 32% were $metE^+$ and 100% were sensitive to methionine sulfoximine. Of 200 $metE^+$ recombinants, 79% were arg^+ and 77% were sensitive to methionine sulfoximine. These results, and the distribution of recombinant classes, were most consistent with the location of the mutation conferring resistance between the markers *argH* and *metE*, and closer to *argH*. This is the published location of *metJ* (24), but the unambiguous assignment of this mutation to *metJ* is unwarranted. Evidence that resistance to methionine sulfoximine was associated with the altered regulation of the methionine biosynthetic pathway was provided by the levels of cystathionase in selected $metE^+$ recombinants. All of four analogue-sensitive recombinants (of which two were arg^+) had cystathionase levels typical of wild-type strains grown in minimal medium. This level of activity was repressed 5- to 7-fold by growth in the presence of methionine. In contrast, all of three analogue-resistant recombinants had elevated cystathionase levels (averaging 7.5-fold higher than that in the sensitive strains), and this level was independent of methionine supplementation. These results might indicate that the expression of the transport system is regulated by the same system controlling the biosynthetic enzymes.

An alternative proposal (14) states that, since *metJ* product also controls the *S*-adenosyl methionine (SAM) synthetase (11), which represents the major route of cellular utilization of methionine, this elevated transport activity results from the relief of feedback inhibition in this methionine auxotroph, which has elevated rates of methionine utilization. To test this proposal, the *metE metJ* double mutant (strain RK4226) was made proficient for methionine biosynthesis either by supplementation with B_{12} or by conjugal transfer of the $metE^+$. The response of the levels of cystathionase or methionine uptake activity to the growth conditions was similar in both series (Table 4). As mentioned previously, the level of uptake in strain RK4226 is variable from day to day, presumably reflecting the changing level of the methionine pool as a function of the time the cells had been depleted for methionine. Allowing the endogenous synthesis of methionine in *metJ* strains did reduce the uptake activity, but never down to the level of $metJ^+$ strain. As in the other experiments, the level of D-methionine uptake paralleled that of L-methionine. These results do verify that a major portion of the regulation of the transport activity is at the level of the transport system's activity rather than on its production.

Utilization of α-keto-γ-methiol butyrate. These results indicated a clear, although not exact, relationship between the uptake of the two methionine isomers, and that the *metD* product is involved in both uptake processes. It was then of interest to investigate the route of entry of the α-keto analogue of methionine. Based on the effective utilization of this compound for the satisfaction of auxotrophic requirements of methionine transport mutants and on its lack of competition for methionine uptake (at least initially), it was unlikely that this compound entered via a methionine transport system (14). Evidence concerning its transport was sought from growth experiments, based on the assumption that compounds sharing its transport system would specifically inhibit the growth of cells on limiting amounts of the keto analogue.

The growth of the methionine auxotroph KBT001 was measured with 66.7 μM L-methionine, D-methionine, or α-keto-γ-methiol butyr-

ate, either by themselves or in the presence of 1 mM concentrations of α-ketobutyrate, α-ketovalerate, α-ketocaproate, or α-ketoisocaproate. Two of these α-keto acids, α-ketocaproate and α-ketovalerate, extended the doubling time with α-keto-γ-methiol butyrate but had little effect on growth with L- or D-methionine (Table 5). This inhibition was competitive. Only a small portion of this inhibition could be due to competition for the intracellular transaminase, as shown by the fact that the inhibitory acids only slightly decreased the growth rate on D-methionine, whose conversion to L-methionine must utilize the same transaminase.

None of the other α-keto acids tested caused this selective inhibition of growth on α-keto-γ-methiol butyrate. The presence of α-ketobutyrate produced a lag before the initiation of growth at the normal rate with all three methionine sources. This lag was overcome by the addition of isoleucine plus valine, but not by isoleucine alone. The complete inhibition of growth by α-ketoisovalerate was fully reversed by isoleucine and is a reflection of the sensitivity of *E. coli* K-12 strains to valine. The selective inhibition by α-ketocaproate or α-ketovalerate was not reversed by the addition of isoleucine plus valine. No inhibition was observed with α-ketoisocaproate (which could serve as leucine source), pyruvate, α-ketoglutarate, or valerate; no β-keto acids were tested.

These results indicate that *E. coli* possesses a transport system for α-keto normal acids with 5- and 6-carbon equivalents. It was apparently not affected by a C_5 normal acid, a C_4 α-keto acid, a C_5 α-keto dicarboxylic acid, or branched-chain C_5 or C_6 α-keto acids, which appear to utilize a separate transport system. Another point of evidence for these separate transport systems came from the finding that the growth inhibition by α-ketoisovalerate (100 μM) was reversed by the addition of α-ketoisocaproate or α-ketocaproate, but the utilization of α-keto-γ-methiol butyrate was inhibited only by α-ketocaproate.

DISCUSSION

Experiments described in this paper were concerned with the multiplicity and specificity of transport systems for methionine and its analogues. There are two major uptake systems for L-methionine operative at concentrations below 50 μM. Based on competition experiments in the wild-type and mutant strains, the high-affinity system appears to have the broadest substrate specificity. It is, however, difficult to reconcile the genetic and kinetic results in terms of a single substrate binding site. It is clear that there is a single locus, *metD*, that is involved in the function of the high-affinity L-methionine uptake system and is also necessary for the utilization of D-methionine, methionine sulfoxide, and *N*-acetyl methionine. The ability to utilize D-methionine depends on its transport, which is the function lost in *metD* mutants. It is not possible to identify the *metD* product as the initial substrate binding site, although its level appears to be rate limiting, as shown by the increased uptake in strains diploid for *metD*. However, the competition between L- and D-methionine for transport suggests separate binding sites. The D-methionine site appears also to be involved in the binding of the sulfoxide and sulfoximine of methionine.

It was not possible to obtain mutants altered in the uptake of either isomer alone. As origi-

TABLE 4. *Methionine uptake in metJ strains*

Strain	Relevant genotype	Growth supplement[a]	Initial rate of uptake[b] of:		Relative cystathionase level[c]
			L-Methionine	D-Methionine	
RK4101	*metE*	Met	45	42.3	1.0
		B_{12}	63	60.4	2.8
		Met + B_{12}	38.9	16.5	1.3
RK4226	*metE metJ*	Met	205.6	542	21.5
		B_{12}	98.8	80.1	38.5
		Met + B_{12}	158.7	305	38.8
RK4229	*metE*$^+$ *metJ*		69.7		31.2
		Met	92.9		29.9
RK4230	*metE*$^+$ *metJ*$^+$		98.8		10.0
		Met	46.3		1.0

[a] Cells were grown in minimal growth medium with methionine (100 μg/ml) or B_{12} (5×10^{-8} M). Exponentially growing cells were washed twice in medium A containing glucose and chloramphenicol.

[b] L-Methionine was present in the assay at 2 μM; D-methionine was present at 4 μM. Uptake rate is expressed as picomoles of substrate per microliter of cell water in 0.20 min.

[c] Cystathionase levels are based on units of enzyme activity per milligram of protein, relative to the level in wild-type cells grown in the presence of methionine.

TABLE 5. *Effect of α-keto acids on utilization of methionine sources*

Inhibitor	Concn (mM)	Doubling time (min)[a]		
		L-Methionine	D-Methionine	α-Keto-γ-methiol butyrate
None		55	58	64
α-Ketovalerate[b]	0.33	57L[c]	61L	86L
	1.0	67L	74L	140L
α-Ketocaproate[b]	0.5			98
	1.0	59	65	155
α-Ketobutyrate	1.0	62L	70L	71L
+ isoleucine	0.67	57L		
+ valine + isoleucine	0.67	57		
α-Ketoisovalerate	1.0	>300	>300	>300
+ isoleucine	0.67	55		
α-Ketoisocaproate	1.0	58	61	76
Pyruvate	1.0	56	52	59
α-Ketoglutarate	1.0	50	51	60
Valerate	1.0	56	58	64

[a] Washed cells of strain KBT001 were inoculated into side-arm flasks containing minimal growth medium with the indicated methionine sources (0.67 mM) and the putative inhibitors. The doubling times were measured from the linear portion of semilogarithmic plots of the optical density of the culture measured at 420 nm. The doubling time in the absence of methionine source was greater than 400 min. There was no effect on the utilization of any of the three methionine sources from the presence of arginine, isoleucine plus valine, and/or the aromatic amino acids.

[b] Growth rates were determined with α-ketocaproate or α-ketovalerate at 0, 0.5, and 1.0 mM in the presence of α-keto-γ-methiol butyrate as methionine source at concentrations from 0.2 to 2.0 mM. Both analogues were competitive inhibitors of growth with respect to the latter compound. The K_i values for these analogues were in the range of 50 to 100 μM.

[c] The symbol L indicates that the onset of growth in these cultures was delayed by at least 60 min relative to the control culture.

nally shown by Ayling and Bridgeland (2) in *Salmonella typhimurium*, those analogue-resistant mutants resistant to both α-methyl methionine and methionine sulfoximine are defective in methionine uptake. The uptake of both isomers is defective, although the low-affinity L-methionine uptake system was still functional. The uptake of glutamine was essentially normal in these strains. Those mutants resistant only to one of the analogues had normal transport of methionine and were probably altered in intracellular aspects of methionine metabolism. Several mutants resistant to methionine sulfoximine had elevated levels of methionine uptake and will be discussed later.

With respect to the mechanism of methionine uptake, Montie and Montie (20) have recently shown that in *Yersinia pestis* and *E. coli* the methionine transported into the cell accumulated as SAM rather than as free methionine. This led to their proposal that methionine uptake is a group translocation process dependent on the function of a membrane-bound SAM synthetase. This mechanism would fit nicely with many of our results concerning the energy source for methionine accumulation, the regulation by feedback inhibition, and the decreased rates of methionine uptake in *metK* mutants deficient in SAM synthetase. They correctly pointed out that our earlier experiments on the contents of the intracellular pool after transport were in error owing to the fact that the extraction with boiling water would lead to decomposition of SAM.

There are, however, certain objections to the operation of a group translocation mechanism. We attempted to extract the intracellular pool after methionine uptake by methods as close to those described by Montie and Montie as possible. The radiochromatograms clearly revealed the formation of SAM from labeled methionine. However, in our hands, from the earliest time of collection, free methionine still represented at least 40% of the total intracellular soluble label. The material migrating with $R_f = 0.07$ to 0.13, presumably SAM, never amounted to more than 15% of the total label. In several experiments, we found that the distribution of radioactivity on chromatograms differed in the two controls used. In the first case, when labeled methionine was added to cold 70% ethanol and carried through all the operations as the experimental samples, a characteristic pattern was obtained in which 75% of the label migrated as methionine and 20% as oxidized forms or homocysteine (R_f of 0.34 to 0.40). When the label was added to a tube containing cold 70% ethanol and a filter containing the same number of cells as used in the experimental conditions, a pattern more similar to that seen in the experimental situation was obtained. Ethanol extraction also resulted in the recovery of labeled material migrating more rapidly than methionine. We were thus unable to confirm the initial appearance of label from methionine as SAM. It was possible that the low-affinity system represented this group translocation process. However, the distribution of labeled metabolites from methionine was similar in the *metD* strain tested, although the total level of uptake was much lower.

Several other findings were not consistent with this hypothesis either. The efficient uptake of D-methionine would appear to conflict with its inability to serve as a substrate for SAM synthetase (11). Ayling and Bridgeland (2) showed that although *S. typhimurium metK* mutants had somewhat lower rates of methionine uptake than the parental strain, these SAM synthetase-deficient strains exhibited elevated levels of uptake if also blocked in methionine biosynthesis. For these reasons, we continue to hold that methionine is transported intact and unaltered into the cell.

This paper provided further information on the regulation of methionine uptake activity. No essential differences were noted between the uptake of L- and D-methionine in terms of the response of the uptake system to the internal pool or growth conditions. It is clear that there is a feedback inhibitory control operating independently of cell growth. Another example was seen of a marked elevation of transport activity under conditions of pool depletion. There was evidence from the study of the methionine sulfoximine-resistant *metJ* mutant of a partial repression-derepression control of the level of the uptake system. Considerable variation in the methionine pool is expected in *metJ* mutants owing to the relationship between the elevated levels of biosynthesis and metabolic conversion. Pool sizes in *metJ* strains are variable (5). The operation of a repression process is also consistent with the results of Ayling and Bridgeland (2), showing elevated transport in a *metA metK* double mutant. This would be consistent with our results, which indicate that the transport system is subject to partial repression by the *metJ* product, the same controlling element as for the biosynthetic genes.

The basis for the methionine sulfoximine resistance of *metJ* mutants is not clear. This analogue probably serves in the cell as a glutamine analogue. In *S. typhimurium*, complete resistance to this analogue requires loss of both methionine and glutamine uptake (3). Glutamate synthetase and glutamine synthetase appear to be the primary sites of growth inhibition by this analogue (4). It is probable that the overproduction of methionine in the *metJ* strain confers resistance to the sulfoximine by interference with its uptake. It should be noted that different classes of mutants are obtained upon selection for analogue resistance depending on whether the strain is prototrophic or conditionally auxotrophic for methionine.

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