A Genetical Study of the Feedback-sensitive Enzyme of Methionine Synthesis in *Salmonella typhimurium*

By K. F. CHATER*

Genetics Department, The University, Birmingham 15

AND R. J. ROWBURY

Department of Botany and Microbiology, University College London, London W.C.1

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SUMMARY

The homoserine-*O*-transsuccinylase activity of three kinds of methionine-excreting mutants of *Salmonella typhimurium* was examined. In a *metI* mutant the enzyme was resistant to inhibition by methionine or its analogue α -methylmethionine. Feedback inhibition in a *metJ* and a *metK* strain was normal. *metI* was dominant to *metI*⁺ only when coupled in the *cis* position with the wild-type allele of the closely-linked *metA* (homoserine-*O*-transsuccinylase) gene, and a deletion analysis of nine *metI* mutations showed that they were all located within the *metA* gene. Thus both the regulatory and catalytic sites of homoserine-*O*-transsuccinylase are specified by a single polypeptide species. An estimate was made of the length of the *metA* gene, based on recombination data.

INTRODUCTION

The first step specific to methionine biosynthesis in *Salmonella typhimurium* and *Escherichia coli* is the *O*-succinylation of homoserine (Rowbury, 1964; Rowbury & Woods, 1964). Mutations in *S. typhimurium* resulting in the loss of homoserine-*O*-transsuccinylase activity are located in the *metA* gene, which is cotransduced with a second methionine structural gene (*metH*) (Childs & Smith, 1969) and with genes controlling the biosynthesis of purines (*purD, H*) and thiamine (*thi*) (Sanderson, 1967). Sensitive regulation of methionine synthesis is achieved by the feedback inhibition of homoserine-*O*-transsuccinylase activity by methionine and its important derivative *S*-adenosylmethionine, either singly or (more effectively) in combination (Rowbury, 1964; Lee, Ravel & Shive, 1966). False feedback inhibition by the methionine analogue α -methylmethionine has been demonstrated, and since this substance is unable to replace methionine in protein synthesis, its addition to growing cultures rapidly inhibits further cell division (Schlesinger, 1967; Rowbury, 1968; Smith, 1968).

Mutants of *Salmonella typhimurium* that were resistant to α -methylmethionine and overproduced methionine were described by Lawrence, Smith & Rowbury (1968). Genetical analysis of these mutants revealed two groups, one of which was defective in *metK*, a locus situated far from any of the known methionine genes in the linkage map, the other group being designated *metI*. *metI* mutations were all more than 95% cotransduced with *metA*, and it was considered probable that they possessed a homoserine-*O*-transsuccinylase that was not subject to inhibition by methionine or its

* Present address: John Innes Institute, Colney Lane, Norwich, NOR 70F.

analogue. Preliminary deletion mapping experiments led Lawrence *et al.* (1968) to speculate that *metA* and *metI* might be separate cistrons, *metA* specifying a catalytically active subunit of the enzyme and *metI* a regulatory subunit. This idea was reinforced by the data of Ayling & Chater (1968), who found that three independent pairs of *metA* and *I* mutations had the same orientation relative to outside markers.

The present account includes biochemical evidence that *metI* mutations do indeed give rise to feedback inhibition resistance, and genetical evidence, from complementation and deletion mapping experiments, that they occur *within* the *metA* cistron. A preliminary account of some of the genetical experiments has appeared elsewhere (Chater, 1970*b*).

METHODS

Media. The media and most of the supplements used are given in an accompanying paper (Chater, 1970*a*). Additional supplements to minimal media were adenine HCl and L-tryptophan (20 µg./ml.). Abbreviations used are: NA, nutrient agar; NB, nutrient broth; MA, minimal agar; MM, liquid minimal medium.

Organisms. The nomenclature is that of Sanderson (1967). *Escherichia coli* 1553/KLF10 was provided by K. Brooks Low, and is described in an accompanying paper (Chater, 1970*a*). All other strains were derived from *Salmonella typhimurium* strain LT2. Those previously described were: *metA*15, *A*54, *A*229 and *A*309, *metA*22tryB2, and *metA*43purE11 (Smith & Childs, 1966); *argF*111, *argF*111*metI*708, *metA*746*metI*706, *metB*23*metI*708 and *metB*23*metI*744 (Ayling & Chater, 1968); *metI*701, *I*702, *I*703, *I*704, *I*705, *I*706, *I*708, and *I*712 (Lawrence *et al.* 1968); *argF*111*rec-301*pyr (Chater, 1970*a*), and *metB*23*metK*747 (Chater, 1969). Mutant *metI*749 was provided by P. D. Ayling, and strain *metA*7 was obtained by transduction of the double mutant *metA*7*cysA*21 (Smith & Childs, 1966) to cysteine independence. The maintenance of stock cultures, and the propagation, assay and maintenance of transducing phage P22, were as described by Smith (1961). Cultures were grown at 37° unless otherwise stated. Growth experiments in MM were essentially as described by Lawrence *et al.* (1968).

Assay of homoserine-O-transsuccinylase activity. The incubation mixture and assay system for estimating the [¹⁴C]homoserine-dependent accumulation of [¹⁴C]-O-succinylhomoserine by a cell suspension are described in the legend to Table 1.

Episome transfer and the detection of haploid segregants. The methods used are described in an accompanying paper (Chater, 1970*a*).

Transduction. Transduction and recombinant analysis were as described by Ayling & Chater (1968), except in the deletion analysis of *metI* mutants, when the normal method would have involved the use of too much expensive α-methylmethionine. Instead, use was made of the methionine excretion of *metI* mutants, by analogy with a similar study of histidine feedback inhibition-resistant mutants of *Salmonella typhimurium* (Sheppard, 1964). Eighteen h. NB cultures of recipients were harvested by centrifugation and resuspended in culture volumes of T2 buffer (adsorption medium: Hershey & Chase, 1952), and donor phage added at a multiplicity of infection of 5. The transduction mixture was incubated at 37° for 5 min. before the addition of up to 1 ml. per tube to tubes containing 10 ml. of molten minimal soft agar maintained at 45°. The contents of each tube were then poured into a Petri dish containing a thin solidified layer (10 ml.) of MA. Plates were incubated at 37° for 48 h., and at room temperature (20 to 25°) for a further 24 h. Upon examination with a binocular micro-

scope at low magnification it was possible to distinguish methionine-excreting from non-excreting colonies: excretion resulted in growth of the background of methionine-requiring recipient cells, visible as a fuzzy edge to the excreting colony, while the boundaries of non-excreting colonies were smooth and clearly defined. Up to 300 colonies per plate could be scored. The reliability of the technique was established in reconstruction experiments with artificial mixtures of *metI* and *I*⁺ strains in the presence of a large excess of a *metA* deletion mutant. In addition, as further confirmation of the scoring in all experiments, all apparently non-excreting colonies were restreaked on MA, and their resistance to α -methylmethionine tested by replica plating. To

Table 1. *The effect of methionine and α -methylmethionine on the synthesis of O-succinylhomoserine by strains metB23 and metB23metI708*

Organisms (0.25 mg. dry wt./ml.) were incubated at 37° in a mixture consisting of glucose minimal medium (0.75 ml.), [¹²C]homoserine (800 nmoles), and [¹⁴C]homoserine (0.2 μ Ci), with additions as stated in the table, and water to 1.5 ml. After 15 min., tubes were heated in a boiling water-bath, and cell debris was removed by centrifugation. [¹⁴C]-O-Succinylhomoserine in the supernatant fluids was estimated as follows: 0.5 ml. aliquots were treated with 0.1 ml. M-KOH, and heated on a water-bath at 100° for 1 min. to convert O-succinylhomoserine to N-succinylhomoserine. Duplicate aliquots were left untreated. Treated and untreated samples were added to 5 cm. columns of Dowex-50 resin (H⁺ form), and eluted with water. N-Succinylhomoserine passes through the column, while unreacted [¹⁴C]homoserine is retained. 3.5 ml. of each eluate was collected, of which 1 ml. was added to 15 ml. of scintillation fluid (0.5 % butyl PBD in toluene-triton X 100; 3:2, v/v) and counted for 5 min. in a Packard Tri-Carb Liquid Scintillation Spectrometer 3320. [¹⁴C]-O-Succinylhomoserine was given by the difference between treated and untreated samples.

Additions	O-Succinylhomoserine formed (% of control)	
	<i>metB23</i>	<i>metB23metI708</i>
None (control)	100	100
L-Methionine		
0.02 mM	31	108
0.05 mM	8	86
0.25 mM	0	92
α -Methyl-DL-methionine		
0.001 mM	52	105
0.005 mM	5.5	101
0.02 mM	4	97
0.05 mM	3.5	90
0.25 mM	0	105
1.0 mM	0	103

confirm that any rare *metI*⁺ recombinants were not contaminants, any second auxotrophic marker present in the recipient was checked in the recombinants by replica plating. Where such markers were not available, the colonial morphology of the *metI* and *I*⁺ recombinants was examined, as this quality was sufficient to distinguish between the different recipients used, and was therefore unlikely to be shared with any chance contaminant.

RESULTS

Tests of the sensitivity of methionine analogue-resistant mutants to feedback inhibition by methionine. The assay of homoserine-O-transsuccinylase depending on the homoserine-dependent accumulation of [¹⁴C]-O-succinylhomoserine can be used only with

metB mutants, since the ester is further metabolized in other strains. The isolation of strains *metB23metI708*, *metB23metJ744* (Ayling & Chater, 1968) and *metB23metK747* (Chater, 1969) permitted the study of feedback inhibition in representatives of three classes of methionine analogue-resistant mutants. The effects of added methionine and α -methylmethionine on *O*-succinylhomoserine synthesis in the *metI* mutant and a *metI*⁺ strain are given in Table 1 and Fig. 1. External concentrations of methionine or α -methylmethionine giving effectively total inhibition of *O*-succinylhomoserine synthesis in *metI*⁺ cells had no effect at all on this activity in the *metI* mutant. Since *metB23metI708* cultures grew only in the presence of added methionine, one must assume that methionine could enter and be utilized by the cells, so that the only explanation for the observations in Table 1 and Fig. 1 is that the homoserine-*O*-transsuccinylase of *metB23metI708* was altered in such a way that it was no longer inhibited by methionine or α -methylmethionine.

Fig. 1. Time course for the synthesis of *O*-succinylhomoserine by strains *metB23* and *metB23metI708* in the presence and absence of methionine. Organisms (0.25 mg. dry wt./ml.) were incubated at 37° in the incubation mixture described in the legend to Table 1. At the stated intervals, samples were removed and assayed for [¹⁴C]-*O*-succinylhomoserine (see Table 1). ○, *metB23* in absence of methionine; ●, *metB23* in presence of 0.1 mM-methionine; △, *metB23metI708* in absence of methionine; ▲, *metB23metI708* in presence of 0.1 mM-methionine.

The rate of *O*-succinylhomoserine synthesis in the *metJ* and *metK* mutants was reduced in the presence of L-methionine (0.05 mM) to 7% and 5%, respectively, of that obtained in controls lacking methionine. Thus neither *metJ* nor *metK* mutations result in altered feedback sensitivity of homoserine-*O*-transsuccinylase.

Absence of complementation between metA and metI. Lawrence *et al.* (1968) suggested that *metA* and *I* might be separate genes. If they were, it should be possible to demonstrate complementation between them in a suitable strain. The genotype of the merodiploid required for such a test is *metA*⁺*I*⁻/*A*⁺*I*⁺. α -Methylmethionine-resistant growth of a strain of this genotype would indicate that the products of the *metA*⁺ and *I*⁻ alleles were complementing each other, i.e. *metA* and *I* were separate cistrons. The required merodiploid was obtained by transferring the episome KLF 10 (which carried the *metA* region of the *Escherichia coli* genome: Chater, 1970a), from

its *E. coli* 1553 host, via *Salmonella typhimurium* *argFIIIrec-301pyr*, into strain *metA746metI706*, with selection on MA for prototrophic heterogenotes. In addition, for control purposes, KLF 10 was transferred into strain *argFIII* to give the merodiploid *metA⁺I⁺/A⁺I⁺*, and into strain *argFIImetI708* to give the merodiploid *A⁺I⁻/A⁺I⁺*.

The growth of the heterogenotes and their haploid parents was followed in MM with and without the addition of α -methylmethionine (Fig. 2). As expected, the haploid *A⁺I⁺* strain (Fig. 2a) was sensitive to the analogue. The presence of KLF 10 in this strain (*A⁺I⁺/A⁺I⁺*; Fig. 2d) increased its resistance a little (probably due to a

Fig. 2. A test of complementation between *metA* and *metI*. The *Escherichia coli* episome KLF 10 (*argF⁺ metA⁺ metI⁺*) was transferred into various *Salmonella typhimurium* strains and the growth of the haploid parent strains and their heterogenote derivatives was followed, by extinction measurements, in minimal medium containing: Δ, no addition; ▲, DL- α -methylmethionine (1000 μ g./ml.); ○, L-methionine (20 μ g./ml.). The relevant genotypes (i.e. with respect to *metA* and *I*) are indicated on the graphs: where merodiploids were used, relevant episomal markers are given after a diagonal stroke.

metA gene dosage effect), though not to a level comparable with that of the *A⁺I⁻* strain (Fig. 2b), which, moreover, was unaffected by possession of the episome (*A⁺I⁻/A⁺I⁺*; Fig. 2e). Thus *metI* was dominant to *metI⁺* (as had been anticipated in the design of the complementation test). Figure 2f shows that the presence of the episome in the *A⁻I⁻* double mutant (giving the critical genotype *A⁻I⁻/A⁺I⁺*) permitted its growth in unsupplemented MM, while the haploid strain (Fig. 2c) gave only slow, leaky growth in MM unless methionine was added. However, the growth of the *A⁻I⁻/A⁺I⁺* strain was inhibited by α -methylmethionine to about the same extent as

was that of the A^+I^+/A^+I^+ strain (Fig. 2*d*). Thus the dominance of $metI^-$ to $metI^+$ suggested by Fig. 2*e* was apparently dependent upon its coupling in the *cis* position with $metA^+$. It was therefore concluded that the *metA* and *I* mutations used in the complementation test were probably located in the same cistron.

This conclusion is based on the assumption that the *Escherichia coli* and *Salmonella typhimurium* homoserine-*O*-transsuccinylases have not undergone enough changes during evolution from a presumed common ancestral form to prevent the efficient formation of mixed enzyme in hybrids. Two lines of evidence suggest that this assumption is justified. First, *E. coli*-*S. typhimurium* hybrid tryptophan biosynthetic enzyme complexes are easily obtained when extracts of the two organisms are mixed (Balbinder, 1964; Ito, 1969); and secondly, the expression of the histidine and methionine structural genes of *S. typhimurium* is efficiently controlled by regulatory elements derived from *E. coli* (Fink & Roth, 1968; Chater, 1970*a*). It seems that those sites of homologous enzymes from the two organisms that are concerned with quaternary interactions have undergone relatively little evolutionary divergence, so that the absence of complementation observed between *metA* and *I* is unlikely to be due to failure of the required quaternary interactions to take place.

Deletion mapping of metI mutations. The absence of complementation between *metA746* and *metI706* described above cast doubt on the suggestion of Lawrence *et al.* (1968) that *metA* and *I* were separate genes. To clarify the relationship between mutations causing *metA* and *metI* phenotypes, the locations of *metI* mutations in the *metA* deletion map were investigated. The original deletion map of Smith & Childs (1966) has been modified by the properties of a new deletion mutant, *metA596*, which gave prototrophic recombinants with *metA43* but not with *metA15* (D. A. Smith, personal communication). The resulting improvement in the map (Fig. 3) received support from the occurrence of prototrophic recombinants in the crosses *metA43* × *A54* or *A746*, and their absence in the crosses *metA15* × *A54* or *A746* (Chater, 1969). The criterion established by Smith & Childs (1966) for absence of recombination between two *metA* mutations was failure to produce prototrophic recombinants in three replicate reciprocal transduction crosses. Their crosses were performed by a spot technique in which crosses between non-allelic methionine mutants gave 40 to 100 recombinant colonies per spot. In order that the resolution of the mapping of *metI* mutations should be comparable with that obtained by Smith & Childs, at least 500 recombinant colonies were examined before absence of recombination between the donor and recipient mutant sites was concluded.

The results of crosses involving 9 *metI* mutants are presented in Table 2. Mutant *I702* gave no I^+ colonies among 506 recombinants obtained from a cross with *metA229*, a mutation located at one extremity of the deletion map (Smith & Childs, 1966). *A229* and *I702* were therefore placed in the same deletion group (x in Fig. 3). In only one case, in which *I705* was the donor, was an I^+ recombinant obtained in crosses with *metA7*, but so few prototrophic recombinants occurred with this recipient that no significance could be attached to the negative results, which are therefore not included in Table 2. The positive result with *I705* is, however, taken into account in the assignment of this mutation to deletion group x. The result for *I706* did not agree with that given by Lawrence (1967), who obtained one $metI^+$ colony among 90 recombinants from an *A43* × *I706* cross. No explanation can be offered for this disparity.

Fig. 3. A revised deletion map of the *metA* gene. The map is based on that of Smith & Childs (1966, and unpublished) and on data given in Results. Heavy unbroken lines represent regions deleted in *metA* deletion mutants (isolation numbers given at left end of each line). Heavy broken lines indicate regions whose existence is not certain. Deletion regions are not drawn to scale. Bold-face figures give the numbers of methionine auxotrophic (*Met*⁻) and feedback resistance (*Fbr*) mutations so far mapped in the various deletion regions (i to x). The total numbers mapped are also given (far right). The orientation of regions vi, vii and viii relative to the rest of the map has not been established.

Table 2. Deletion mapping of *metI* mutations

Phage P22 propagated on *metI* mutants was used to transduce *metA* deletion mutants to prototrophy. Recombinants were scored for inheritance of the *metI* phenotype (see Methods). In the table, the number of colonies with *metI*⁺ phenotype is followed in each case by the total number of colonies scored. Assignment to deletion groups refers to Fig. 3, and for *metI*702 and 705 utilizes additional information given in the Results. Parentheses indicate assignments based on negative results with fewer than 500 recombinant colonies.

Donor	No. of colonies with <i>metI</i> ⁺ phenotype among recombinants from cross with recipient					Deletion group
	<i>metA</i> 43	<i>metA</i> 15	<i>metA</i> 596	<i>metA</i> 22	<i>metA</i> 309	
<i>metI</i> 701	4/724	0/578	1/372	n.t.	n.t.	i or iii
<i>metI</i> 702	0/1162	1/103	n.t.	n.t.	n.t.	x
<i>metI</i> 703	13/1775	0/660	2/886	n.t.	n.t.	i or iii
<i>metI</i> 704	0/894	4/150	3/67	1/56	0/181	(viii)
<i>metI</i> 705	0/1934	0/287	3/217	14/82	11/577	x
<i>metI</i> 706	0/1830	4/439	1/144	9/45	23/648	iv, v, ix or x
<i>metI</i> 708	0/737	0/193	2/126	11/48	9/162	iv, v, ix or x
<i>metI</i> 712	0/660	0/236	2/243	10/69	26/579	iv, v, ix or x
<i>metI</i> 749	0/526	9/318	5/200	2/149	0/1065	viii

n.t. = Not tested.

DISCUSSION

The failure of methionine or α -methylmethionine to inhibit *O*-succinylhomoserine synthesis in a *metI* mutant (Table 1; Fig. 1) was attributable either to failure of these substances to penetrate the cells, or to an alteration of homoserine-*O*-transsuccinylase such that it was not sensitive to feedback inhibition. Two observations precluded the former interpretation. First, strain *metB23metI708* grew at the same rate as strain *metB23* on methionine at concentrations down to 0.02 mM (i.e. the lowest concentration used in these experiments: R. J. Rowbury, unpublished observations); and secondly, α -methylmethionine resistance was dominant to sensitivity in a *metI*⁻/*metI*⁺ heterogenote (Fig. 2e), which contrasts with the observation of Ames & Roth (1968) that histidine analogue resistance resulting from mutation of the histidine permease gene was recessive to sensitivity. Thus the *metI* gene product cannot be a component of the methionine transport system, so must be a component of homoserine-*O*-transsuccinylase. This conclusion has also recently been reached by D. A. Lawrence (personal communication), who has tested the feedback sensitivity of homoserine-*O*-transsuccinylase activity in cell-free extracts of four other *metI* mutants: in no case was the activity affected by methionine or α -methylmethionine.

Smith & Childs (1966) observed no abortive transduction, indicating no complementation, between strains bearing any of 37 *metA* point mutations, all of which were located within the *metA* deletion map; hence the map involves a single cistron only. As *metI* mutations were scattered within this map, and one of them failed to show complementation with a *metA* mutation, *metA* and *I* mutations must both result in alterations of the same polypeptide, which must specify both the substrate and the inhibitor binding sites of homoserine-*O*-transsuccinylase. Thus aspartate transcarbamylase in *Escherichia coli* remains the only case yet described in which the catalytic and regulatory sites of an enzyme are carried by distinct polypeptide species (Gerhart & Schachman, 1965). Deletion mapping of histidine feedback-resistant mutants of the *hisG* gene of *Salmonella typhimurium* (Sheppard, 1964) gave similar results to those described in the present work, though the clustering of most of the *hisG* feedback resistance mutations in a single region of the *hisG* deletion map is in contrast to the lack of gross clustering of methionine feedback resistance mutations in the *metA* map (Fig. 3). As *metI* mutations are now known to be situated within the *metA* gene, such mutations will in future publications be designated *metA* (e.g. *metI701* will become *metA701*). This accords with the convention suggested by Demerec, Adelberg, Clark & Hartman (1966).

Finally, by combining the results of the deletion mapping with those of three-point transduction crosses described by Ayling & Chater (1968) (which placed *metI706* between *metA746* and *metH*, and *A15* between *I749* and *thi*), the *metA* deletion map can be orientated unambiguously with respect to *metH* and *thi*. This orientation is indicated in Fig. 3. The estimated recombination frequency of 0.033 along the major part of the *metA* gene, i.e. that containing deletion regions iv to ix (Fig. 3), indicates that it occupies about 1/30th part of the average transduction fragment carrying it (assuming that recombination events occurred at random in the crosses analysed in Table 3).

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