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# Genetic Mapping with a Thiamine-requiring Auxotroph of *Escherichia coli* K-12 Defective in Thiamine Phosphate Pyrophosphorylase

TAKASHI KAWASAKI, TARO NAKATA, AND YOSHITSUGU NOSE
*Biochemical Institute, Kyoto Prefectural University of Medicine, Kyoto, Japan*

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The pathway of thiamine biosynthesis in *Escherichia coli* from its pyrimidine and thiazole moieties involves four enzymes (Y. Nose et al., J. Vitaminol. Kyoto **10**:105, 1964): hydroxymethylpyrimidine kinase (EC 2.7.1.49), phosphohydroxymethylpyrimidine kinase (EC 2.7.4.7), thiazole kinase (EC 2.7.1.50), and thiamine phosphate pyrophosphorylase (EC 2.5.1.3). A mutant of *E. coli* W auxotrophic for thiamine (70-23) was shown to be lacking hydroxymethylpyrimidine kinase (Y. Nose et al., J. Vitaminol. Kyoto **10**:105, 1964).

The genetic determinants specifying information for these four enzymes have not yet been mapped on the chromosome. The *thi* locus mapped on the chromosome map of *E. coli* (A. L. Taylor and M. S. Thoman, Genetics **50**:659, 1964) has been assumed to be a locus concerned with the synthesis of the thiazole moiety of thiamine. This is based on findings which show that two thiamineless mutants of *E. coli* K-12 (W4162 and AB1450, obtained from Y. Hirota of Osaka University), with or without other nutritional requirements, are auxotrophic for the thiazole moiety of thiamine as determined by requirement for growth (T. Kawasaki and Y. Nose, *unpublished data*). A similar finding was recently reported for *Salmonella typhimurium* (J. L. Parada and M. V. Ortega, J. Bacteriol. **94**:707, 1967).

As part of a study to locate the genetic determinants of the four enzymes in thiamine synthesis from its pyrimidine and thiazole moieties, mutants which require only thiamine, but not the pyrimidine or thiazole moiety, have been isolated from *E. coli* K-12 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (E. A. Adelberg, M. Mandel, and G. C. C. Chen, Biochem. Biophys. Res. Commun. **18**:788, 1965) and subsequent selection on minimal agar containing 0.01 μM thiamine. Among those mutants obtained, a mutant (KG167) and its Hfr strain (KG1673) were defective in thiamine phosphate pyrophosphorylase (Table 1). The activity of the other three enzymes was comparable with those in a mutant of *E. coli* W auxotrophic for the thiazole moiety (26-43), which was grown under the same condition of thiamine limitation as used for KG1673.

Mapping on the chromosome of *thi*, which contains the mutation affecting the enzyme described above, was carried out by crossing the Hfr KG1673 with appropriate $F^-$ strains according to procedures reported by M. Ishibashi, Y. Sugino, and Y. Hirota (J. Bacteriol. **87**:554, 1964). All strains used for mapping were derivatives of *E. coli* K-12; strains AB261, W4573, and AB1450 were kindly supplied by Y. Hirota. KG1673 was obtained from KG167 ($F^+$ *thi*$^-$ *lac*$^+$) by a three-step procedure. Strain KG1671 ($F^+$ *thi*$^-$ *lac*$^-$) was isolated from KG167 by nitrosoguanidine treatment, and then acridine orange treatment (Y. Hirota, Proc. Natl. Acad. Sci. U.S. **46**:57, 1960) was used to convert KG1671 to the corresponding $F^-$ strain (KG1672). A cross, KG1672 ($F^-$ *thi*$^-$ *met*$^+$ *lac*$^-$) × AB261 (Hfr *thi*$^+$ *met*$^-$ *lac*$^+$), and selection of recombinants on minimal eosine-methylene blue (EMB)-lactose-agar (J. Lederberg, Methods Med. Res. **3**:5, 1950) supplemented with 0.01 μM thiamine yielded KB1673, which transfers the chromosome in the same clockwise order (*pro ara xyl mal gal lac*) as the donor strain AB261.

In a preliminary experiment to locate the *thi* locus, W4573 ($F^-$ *ara*$^-$ *thi*$^+$ *xyl*$^-$ *mal*$^-$ *gal*$^-$ *lac*$^-$ *str-r*) was used as a partner in a cross with KG1673 (Hfr *ara*$^+$ *thi*$^-$ *xyl*$^+$ *mal*$^+$ *gal*$^+$ *lac*$^+$ *str-s*). Recombinants of *ara*$^+$ *str-r*, *xyl*$^+$ *str-r*, and *mal*$^+$ *str-r*) were selected on appropriate EMB-sugar-agar (J. Lederberg, Methods Med. Res. **3**:5, 1950) containing streptomycin (100 μg/ml) and were purified twice on the same agar medium. Overnight cultures of the recombinants in minimal medium (B. D. Davis and E. S. Mingioli, J. Bacteriol. **60**:17, 1950) supplemented with 0.01 μM thiamine were streaked on appropriate EMB-sugar-agar and also minimal agar with or without 0.01 μM thiamine. After overnight incubation at 37 C, unselected markers were scored. The *thi* locus was found to be linked 4.1, 22, and 3.4% to *ara*, *xyl*, and *mal* genes, respectively.

TABLE 1. *Deficiency of thiamine phosphate pyrophosphorylase activity in extracts of KG1673 (Hfr thi⁻)*[a]

| Strain | Hydroxymethylpyrimidine kinase | Phosphohydroxymethylpyrimidine kinase | Thiazole kinase | Thiamine phosphate pyrophosphorylase |
|---|---|---|---|---|
| *Escherichia coli* W, thiazoleless | 0.78 | 0.86 | 1.07 | 21.0 |
| KG1673 | 0.68[b] | 0.74[b] | 1.02[c] | 0 |

[a] The strains were grown in minimal medium in the presence of 0.01 μM thiamine. After harvesting and washing, the cells were disrupted by sonic oscillation and were centrifuged (15,000 × *g*, 15 min); the crude supernatant fluids were employed in the enzyme studies after dialysis for 8 hr against two changes of 200-fold volume of a medium containing 0.02 M tris(hydroxymethyl) aminomethane (Tris) buffer (*p*H 7.5) and 1 mM 2-mercaptoethanol. The enzyme assay methods employed were described previously (Y. Nose et al., J. Vitaminol. Kyoto **10**:105, 1964). The reaction mixtures used were as follows. I, hydroxymethylpyrimidine (HMP) kinase: 100 μM HMP, 10 μM thiazole phosphate; II, phospho-HMP kinase: 100 μM HMP phosphate, 10 μM thiazole phosphate; III, thiazole kinase: 100 μM thiazole, 10 μM HMP pyrophosphate; IV, thiamine phosphate pyrophosphorylase: 10 μM thiazole phosphate, 10 μM HMP pyrophosphate. In addition, each reaction mixture contained 50 mM Tris buffer (*p*H 7.5), 20 mM ATP (except for assay of enzyme IV), 20 mM $MgCl_2$, and the extracts (5 mg of protein for assay of enzymes I, II, and III, and 1 mg for enzyme IV) in a final volume of 5 ml. After incubation at 37 C (for 60 min in assay of enzymes I–III and for 30 min in assay of enzyme IV), thiamine formed was determined by the thiochrome fluorescence method. Enzyme activities are expressed as millimicromoles of thiamine formed per milligram of protein per hour. Rate of thiamine formation catalyzed by enzyme IV was proportional to the amount of the extract of the control (*E. coli* W, thiazoleless strain) up to 2.0 mg of protein.

[b] Since activities of enzymes I–III in the extract of the control could be determined under the conditions described, depending upon amounts of the extract used in which an excess of enzyme IV was contained, these activities (enzymes I–III) in the extract of KG1673 could not be detected unless an extract (5 mg of protein) of a thiamine regulatory mutant (PT-R1) of *E. coli* K-12 was added. Strain PT-R1 contained completely repressed levels of HMP kinase and phospho-HMP kinase, a partially derepressed level of thiazole kinase (0.5 mμmole per mg per hr), and a derepressed level of thiamine phosphate pyrophosphorylase (20 mμmoles per mg per hr).

[c] This activity was also measured in the presence of the extract of PT-R1, but thiazole kinase activity derived from the extract of PT-R1 was subtracted from the total activity detected. Addition of the PT-R1 extract to the reaction mixtures for assay of enzymes I–III in the control did not alter the rate of thiamine formation catalyzed by each of these enzymes.

TABLE 2. *Linkage of thi with arg and met in the cross KG1673 × AB14501*[a]

| Recombinants | *thi* | *arg* | *met* | *ilv* | *str* | *his* | No. of recombinants |
|---|---|---|---|---|---|---|---|
| *arg⁺ str-r* | 0 | 1 | 0 | 0 | 0 | 0 | 20 |
| | 1 | 1 | 0 | 0 | 0 | 0 | 18 |
| | 1 | 1 | 1 | 0 | 0 | 0 | 44 |
| | 0 | 1 | 1 | 0 | 0 | 0 | 16 |
| | 1 | 1 | 0 | 1 | 0 | 0 | 2 |
| Total inheriting Hfr allele | 64 | 100 | 60 | 2 | 0 | 0 | 100 |
| *met⁺ str-r* | 0 | 0 | 1 | 0 | 0 | 0 | 28 |
| | 1 | 0 | 1 | 0 | 0 | 0 | 7 |
| | 1 | 1 | 1 | 0 | 0 | 0 | 36 |
| | 0 | 1 | 1 | 0 | 0 | 0 | 17 |
| | 1 | 1 | 1 | 1 | 0 | 0 | 7 |
| | 0 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 0 | 0 | 1 | 1 | 0 | 0 | 4 |
| Total inheriting Hfr allele | 50 | 61 | 100 | 12 | 0 | 0 | 100 |
| *ilv⁺ str-r* | 0 | 0 | 0 | 1 | 0 | 0 | 61 |
| | 1 | 1 | 1 | 1 | 0 | 0 | 25 |
| | 0 | 0 | 1 | 1 | 0 | 0 | 5 |
| | 1 | 0 | 0 | 1 | 0 | 0 | 4 |
| | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| | 0 | 1 | 1 | 1 | 0 | 0 | 2 |
| | 1 | 0 | 1 | 1 | 0 | 0 | 1 |
| | 1 | 1 | 0 | 1 | 0 | 0 | 1 |
| Total inheriting Hfr allele | 31 | 29 | 33 | 100 | 0 | 0 | 100 |

[a] KG1673: Hfr *thi⁻ arg⁺ met⁺ ilv⁺ str-s his⁺*, 111111; AB14501: F⁻ *thi⁺ arg⁻ met⁻ ilv⁻ str-r his⁻*, 000000. The alleles of markers derived from the Hfr parent are represented by 1, and those derived from the F⁻ parent, by 0. Only relevant markers are shown.

To locate the *thi* locus of KG1673 more precisely, AB14501 (F⁻ *thi⁺ arg⁻ met⁻ ilv⁻ his⁻ str-r*) was used in a cross with KG1673. Strain AB14501 was selected as a thiamine-nonrequiring revertant (like the thiazole revertant described above) of AB1450. Loci represented as *arg* and *met* in AB1450 are *argA*, *C*, *F*, *H* and *metB*, *F*, respectively (Y. Hirota, *personal communication*). The recombinants *arg⁺ str-r*, *met⁺ str-r*, and *ilv⁺ str-r* were selected on minimal agar supplemented with streptomycin (100 μg/ml), 0.01 μM thiamine, and the appropriate amino acids (50 μg/ml), and

were then purified twice on the same agar medium. Overnight cultures of the recombinants in minimal liquid medium supplemented with appropriate amino acids plus 0.01 $\mu$M thiamine were streaked on minimal agar with appropriate amino acids in the presence or absence of thiamine. After overnight incubation at 37 C, unselected markers were scored (Table 2). The *thi* gene corresponding to the mutation in KG1673 was found to be very closely linked to the *arg* and *met* genes, which are located just adjacent to the *thi* gene already known, but not to the *ilv* gene. This result indicates that the structural gene for thiamine phosphate pyrophosphorylase is linked to the *thi* gene and is probably involved in a small region including the *thi* gene already mapped on the chromosome of *E. coli*.

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