Cloning and characterization of the *thiDIJ* gene of *Escherichia coli* encoding a thiamin-synthesizing bifunctional enzyme, hydroxymethylpyrimidine kinase/phosphomethylpyrimidine kinase

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A 1.7 kb DNA fragment isolated from an *E. coli* genomic library was able to complement the thiamin requirement of strains carrying the *thiM*, *thiJ* and *thiD* mutations. The three genes encode hydroxyethylthiazole kinase, hydroxymethylpyrimidine (HMP) kinase and phosphomethylpyrimidine (HMP-P) kinase, respectively. Sequence analysis revealed that the 1.7 kb fragment contained two ORFs of 708 bp and 801 bp. The former ORF complemented the *thiM* mutation and the latter ORF both the *thiJ* and *thiD* mutations. The latter ORF was cloned into the expression vector pET3a, and the encoded protein was purified through three successive column chromatographies. The purified protein was able to convert HMP to its monophosphate and the monophosphate to its pyrophosphate. These results suggest that the two distinct enzyme activities, HMP kinase and HMP-P kinase, are indeed a bifunctional enzyme encoded by a single gene, designated *thiDIJ*.

Keywords: *Escherichia coli*, hydroxymethylpyrimidine kinase, thiamin biosynthesis, bifunctional enzyme

INTRODUCTION

In the *de novo* synthesis of thiamin pyrophosphate (thiamin-PP) in *Escherichia coli*, reactions proceed through the independent formation of two heterocyclic intermediates, hydroxymethylpyrimidine pyrophosphate (HMP-PP) and hydroxyethylthiazole monophosphate (HET-P) with their subsequent coupling; the condensation gives rise to thiamin monophosphate (thiamin-P), an immediate precursor of thiamin-PP (Fig.

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Abbreviations: HET, 4-methyl-5-(β -hydroxyethyl)-thiazole; HMP, 2-methyl-4-amino-5-(hydroxymethyl)-pyrimidine; IMAC, immobilized-metal affinity chromatography; PAL, pyridoxal; -P, monophosphate; -PP, pyrophosphate; PIN, pyridoxine.

The DDBJ accession numbers for the *thiD* and *thiM* genes reported in this paper are D84200 and D88442, respectively.

1). When hydroxymethylpyrimidine (HMP) and hydroxyethylthiazole (HET) are supplied exogenously, they are taken up by the cell, phosphorylated, and converted to thiamin-PP by the *de novo* synthetic pathway. HMP-PP is formed from HMP via the intermediate formation of hydroxymethylpyrimidine monophosphate (HMP-P). Two distinct enzymes, HMP kinase and HMP-P kinase have been reported to catalyse the stepwise phosphorylation of HMP (Kawasaki *et al.*, 1968). Our previous studies showed that mutations in the *thiJ*, *thiD* and *thiM* loci, leading to the inactivation of HMP kinase, HMP-P kinase and HET kinase, respectively, are located very close together on the chromosome at about 46 min (Mizote & Nakayama, 1989b; Mizote *et al.*, 1996).

In the course of these studies, we have found that pyridoxal (PAL) kinase (EC 2.7.1.35, *pdxL* gene) also catalyses the phosphorylation of HMP to HMP-P

Fig. 1. Phosphorylation of HMP and HET in the biosynthesis of thiamin-PP in *E. coli*. The *thiB* locus was originally assumed to define the structural gene for thiamin-P synthetase (Kawasaki *et al.*, 1968) but a strain carrying mutations in the locus was later shown to be complemented by clones carrying both *thiF* and *thiG* (Horn *et al.*, 1993). More recently, *thiE* was identified as the gene encoding thiamin-P synthetase and was shown to be contained in the main operon at 90 min (Backstrom *et al.*, 1995). Parentheses show approximate map positions (min) of each of the genes. Broken lines indicate salvage pathways.

(Mizote & Nakayama, 1989a) and that the inactivation of both the *pdxL* and *thiJ* genes in the same cell resulted in the complete loss of HMP kinase activity (Nakayama, 1990; Mizote *et al.*, 1996).

In this paper we have re-examined the properties of a *thiD* mutant, NI500, which was reported to lack HMP-P kinase activity (Imamura & Nakayama, 1981) and found that a *thiD pdxL* double mutant lacked not only HMP-P kinase but also HMP kinase activities. We also provide molecular-genetic evidence showing that an *E. coli* 1.7 kb chromosomal DNA fragment, which contains two ORFs, is able to complement the *thiJ*, *thiD* and *thiM* mutations. One ORF complements the *thiM* mutation, while the other complements both the *thiJ* and *thiD* mutations. A protein encoded by the latter ORF proved to catalyse two phosphorylation reactions: HMP to HMP-P and HMP-P to HMP-PP. These results suggest that HMP kinase and HMP-P kinase are encoded by a single gene previously designated either *thiJ* or *thiD*.

METHODS

Bacteria, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1. Minimal medium 56 modified by Low (1973) was used as a basal medium. For P1 transduction, thiamin, pyridoxal (PAL) and PIN were added at 20, 100 and 100 nM, respectively, as required. Luria broth (LB) was used for other experiments. Ampicillin, chloramphenicol and kanamycin were added to the medium at 100, 30 and 50 µg ml⁻¹, respectively.

Genetic manipulation. P1 transductions were carried out by the methods described by Lennox (1955) and Taylor & Trotter (1967). DNA manipulations were carried out by the procedures of Sambrook *et al.* (1989). Tn1722 (Ubben &

Schmit, 1986) loaded on pMT1282 was used for transposon mutagenesis as described previously (Tsuda & Iino, 1987). PCR was performed using the touch-down method as described by Don *et al.* (1991) with primers designed as follows: F1 (5' CATATGACCAATGATGTGGTGC 3') and R1 (5' GGATCCTCATGCCTCACCTCC 3') for ORF1 and F2 (5' CATATGAAACGAATTAACGCTCTGACG 3') and R2 (5' GGATCCTCACCACCAGGCGTGAAGTGG 3') for ORF2. Complementation of each *thi* gene was assessed by using three *thi* mutant strains, SN164L-1 (*thiM*), SN372L-3-9-10 (*thiJ*) and NI500 (*thiD*). Strains with various plasmids carrying the *thi* region were tested for growth at 37 °C on minimal medium containing the required amino acid supplements either with or without 20 nM each of HET or HMP.

Growth of *E. coli* and preparation of cell-free extracts. *E. coli* strain BL21(DE3)(pLysS) was transformed with pTRD63 carrying ORF2. A 40 ml portion from an overnight culture of the cells was inoculated into a 2 l culture of LB. When the cell count reached 3×10^7 cells ml⁻¹, IPTG was added to a final concentration of 0.08 mM and the culture was incubated for a further 2 h. The cells were harvested by centrifugation, washed with 20 mM Tris/HCl buffer, pH 7.5, and lysed by French pressing at a cell pressure of 16000 p.s.i. (110.4 MPa). Unbroken cells and cell debris were eliminated by centrifugation for 60 min at 20000 r.p.m. using a Sorvall SS-34 rotor and the cell-free extract (65.1 ml) was obtained.

Purification of the *thiD* product. The *thiD* product was purified from the cell-free extracts using the following three successive chromatography steps.

Step 1: Chromatography on HiTrap Q. The cell-free extract (21.7 ml) was loaded onto a HiTrap Q column (5 ml, Pharmacia) which had been equilibrated with 50 ml 20 mM Tris/HCl, pH 7.5. Then the column was washed with 10 ml starting buffer, and proteins were eluted with a 40 ml linear gradient of NaCl (0–0.4 M) in the same buffer at a flow rate of 4 ml min⁻¹. Fractions having the HMP-phosphorylating activity with an A_{280} over 0.1 were pooled and dialysed against 2.5 l 20 mM MOPS buffer containing 0.2 M NaCl, pH 7.0 (buffer A). The rest of the cell-free extract (43.4 ml) was similarly purified and combined.

Step 2: Immobilized-metal affinity chromatography (IMAC). The preparation obtained in step 1 was applied to a Ni-affinity column (1 ml, Pharmacia) which had been pre-equilibrated with buffer A. The column was washed with 2.5 ml buffer A and eluted with a 20 ml linear gradient of imidazole (0–50 mM) in buffer A at a flow rate of 1 ml min⁻¹ and the active fractions were collected. Active fractions were pooled and dialysed against 2 l 20 mM Tris/HCl buffer, pH 8.0.

Step 3: Mono Q chromatography. The preparation obtained in Step 2 was applied to a Mono-Q HR5/5 column (1 ml, Pharmacia) which had been pre-equilibrated with 20 mM Tris/HCl, pH 8.0, containing 0.5 mM DTT (buffer B). Prior to application, DTT was added to the sample to a final concentration of 0.5 mM. After application of the sample, the column was washed with 5 ml buffer B and then eluted with a 16 ml linear gradient of NaCl (0–0.4 M) at a flow rate of 1 ml min⁻¹. The active fractions were pooled and stored at –20 °C after the addition of 20% (v/v) glycerol.

Characterization of the purified enzyme. SDS-PAGE was carried out on a 10–20% gradient gel of Tris-Tricine (Novex) and the gel was stained with Coomassie blue. Isoelectric-focusing electrophoresis was carried out on a polyacrylamide gel (IEF-PAGE, pH 3–10; Novex) and run as recommended by

Table 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype*	Relevant phenotype†	Source‡
Bacterial strains			
JC1552	<i>argG6 metB1 his-1 leu-6 trp-31 rpsL104</i>		CGSC
SN372L-3-9-10	JC1552, <i>thiC pdxB20 pdxL2 thiJ1</i>	PAL ⁻ Thi ⁻	Mizote <i>et al.</i> (1996)
K3-9-101	SN372L-3-9-10, <i>pdxL⁺ nupC3146::Tn10kan</i>	PIN ⁻ Thi ⁻	Mizote <i>et al.</i> (1996)
SN372L-K1	SN372L-3-9-10, <i>nupC3146::Tn10kan</i>	PAL ⁻ Thi ⁻	SN372L-3-9-10 × p1 <i>kc</i> (CAG18565)
SN372L-3-T1	SN372L-3 <i>pdxL2 nupC3146::Tn10kan</i>	PIN ⁻ Thi ⁻	Mizote <i>et al.</i> (1996)
NI500	JC1552, <i>thiD1</i>	Thi ⁻	Imamura & Nakayama (1981)
NI500-T1	NI500 <i>pdxL2 nupC3146::Tn10kan</i>	Thi ⁻	NI500 × P1 <i>kc</i> (SN372L-K1)§
SN164L-1	JC1552 <i>thiA thiM1</i>	Thi ⁻	Mizote & Nakayama (1989b)
CAG18565	<i>nupC3146::Tn10kan</i>		NIG
DH5α	<i>recA endA1 gyrA96 thi-1 hsdR17 supE44 (lac)U169 (φ80 acdM15)</i>		BRL
HB101	<i>hsdS20 recA13 ars-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44</i>		Maniatis <i>et al.</i> (1982)
JM109	<i>e14⁻ (McrA⁻) recA endA1 gyrA96 thi-1 hsdR17 (r_k⁻ m_k⁺) supE44 relA1 (lac⁻ proAB) [F⁺traD36 proAB lacI^qZ M15]</i>		CGSC
BL21 (DE3)(pLysS)	<i>E. coli B F⁻ dem ompT hsdS (r_B⁻ m_B⁻) gal-1(DE3)[pLsS Cam^r]</i>		Studier & Moffatt (1986)
Plasmids			
pBR322	<i>E. coli</i> vector, Ap ^r Tc ^r		Bolivar <i>et al.</i> (1977)
pUC18	<i>E. coli</i> vector, Ap ^r		Yanisch-Perron <i>et al.</i> (1985)
pET3a	<i>E. coli</i> vector, Ap ^r		Rosenberg <i>et al.</i> (1987)
pMT1282	Tra ⁺ Tp ^r Su ^r Tc ^r , R388::Tn1722		Tsuda <i>et al.</i> (1989)
pAM410	A 6.9 kb <i>EcoRV</i> fragment in pBR322		This work

* Genetic nomenclature is as given by Berlyn *et al.* (1996).

† PIN⁻, strains requiring PIN or PAL at concentrations above 0.1 µM for their maximum growth; PAL⁻, strains capable of growing on PAL to the same extent as PIN⁻ strains, but incapable of growing on PIN below 0.1 µM and requiring PIN above 10 µM for maximum growth; Thi⁻, strains requiring thiamin for growth.

‡ CGSC, Coli Genetic Stock Center, Department of Biology 255 OML, Yale University, New Haven, CT, USA; NIG, National Institute of Genetics, Department of Microbiology, Mishima, Shizuoka, Japan.

§ To introduce the *pdxL* mutation (51.65 min) into NI500, we transduced *nupC::Tn10kan* (51.75 min) from strain CAG18565 into SN372L-3-9-10 (*pdxB pdxL thiJ*) by P1-mediated transduction. Tn10kan transductants were first screened on minimal agar plates containing thiamin, PAL and kanamycin, and then *pdxL* transductants were selected on plates containing thiamin and PIN. One of the transductants, SN372L-K1 was used as a donor to introduce the *pdxL* mutation into NI500 and transductants were isolated on plates containing thiamin and kanamycin. Several transductants were assayed for HMP kinase activity in crude extracts and NI500-T1 was isolated that had neither thiamin-repressible (ThiJ) nor thiamin non-repressible (PdxL) HMP kinase.

the manufacturer. Gel-filtration chromatography of the purified enzyme (50 µl) was performed using a column (29.5 × 1.2 cm) packed with Sepharose 6B. Chromatography was performed at a flow rate of 0.8 ml min⁻¹ in 20 mM Tris/HCl, pH 7.5, containing 0.2 M NaCl and 0.5 mM DTT. The molecular mass of the protein was calibrated using proteins of standard molecular mass (New England Biolabs). Protein concentrations were determined using the Bradford method (1976).

Enzyme assay. Enzyme activity with crude extracts was measured as described previously (Nakayama & Hayashi, 1972, 1979). When ThiD-overexpressing cells were used to purify ThiD, enzyme activity was measured with the reaction mixture in a final volume of 100 µl containing 0.1 M Tris/HCl, pH 7.0, 10 mM ATP, 1.0 mM MgCl₂, 1 µl protein preparation at a concentration of 1 mg protein ml⁻¹ and either 0.1 mM HMP or HMP-P. After completion of the reaction at 37 °C,

the reaction mixture was heated to 100 °C for 5 min and centrifuged. The supernatant fraction was used to quantify the reaction products.

Chemicals. HMP was a gift from Takeda Chemical Industries, Osaka, Japan. HMP-P and HMP-PP were prepared from bromomethylpyrimidine according to the method of Lewin & Brown (1961). PIN and alkaline phosphatase were purchased from Sigma.

Amino acid sequence analysis. The sequence of the first 10 residues of the purified ThiD protein was determined by an Applied Biosystems model 476A protein sequencer.

Nucleotide sequence analysis. The nucleotide sequences of the *thiD* and *thiM* genes have been submitted to DDBJ under the accession numbers D84200 and D88442, respectively. The DNASIS alignment program was used to align homologous *E. coli* and *Salmonella typhimurium* sequences.

RESULTS AND DISCUSSION

Characterization of a *thiD*-mutant strain, NI500

E. coli strain NI500 is defective in thiamin-PP biosynthesis because of a mutation in *thiD* encoding HMP-P kinase. NI500 had a wild-type level of HMP kinase activity, which could be due to HMP kinase (*thiJ* gene) and PAL kinase (*pdxL* gene) whereas SN372L-K1 (*pdxL thiJ*) had no HMP kinase activity (Table 2). To determine the role of ThiJ in NI500, we transduced *pdxL* into this strain to obtain a *thiD pdxL* double mutant, NI500-T1. The extract from NI500-T1 had no HMP kinase activity, irrespective of the growth conditions. We used two control strains, K3-9-101 (*pdxL⁺ thiJ*) and SN372L-3-T1 (*pdxL thiJ⁺*). K3-9-101 cells contained HMP kinase activity even when grown with excess thiamin (100 nM) due to the non-repressible *pdxL* gene product. On the other hand, SN372L-3-T1 cells had the repressible HMP kinase activity due to the *thiJ* gene product. The results showing that NI500-T1 had no HMP kinase activity appeared to be in conflict with our previous conclusion that NI500 was missing only HMP-P kinase, encoded by *thiD*. Consideration of the present results together with the previous ones have led to the assumption that the *thiD* mutation carried by NI500 might be responsible for the loss of activities of HMP-P kinase together with the inactivation of HMP kinase and that the measured HMP kinase activity in the previous study might virtually all be due to PdxL.

Cloning of a region containing *thiD*, *thiJ* and *thiM*

We have previously reported that the *thiD*, *thiJ* and *thiM* mutations are located around 46 min on the chromosome (Imamura & Nakayama, 1981; Mizote & Nakayama, 1989b; Mizote *et al.*, 1996) which was covered by 8F4 of an *E. coli* genomic library (Kohara *et al.*, 1989). A 6.9 kb *EcoRV* fragment on 8F4 was cloned into the *EcoRV* site of pBR322, and the resulting plasmid

Fig. 2. Restriction maps of plasmids carrying *thiJ*, *thiD* and *thiM* mutations, and complementation of the *thiJ*, *thiD* and *thiM* mutations. Coding sequences are indicated by open boxes. The numbers below the arrowheads in the pAM410::Tn1722 map show the insertion sites of Tn1722 in numerical order of their isolation. The restriction sites located within Tn1722 are indicated by asterisks. The complementation pattern of each of the subclones is shown on the right. + or - indicates the positive or negative growth response of transformants to supplementation of the following compounds in the minimal medium. When *thiJ* cells were used as the host, PAL and HMP were added to the medium; for *thiD* cells, no supplements were added; and HET was added for the growth of *thiM* cells. Phenotypes of the strains carrying each of the *thi* mutations are listed in Table 1. The leftward arrow indicates the direction of transcription. Abbreviations for the restriction sites employed are as follows: A, *Apa*I; E, *Eco*RI; C, *Cl*aI; Hc, *Hinc*II; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sp, *Sph*I; V, *Eco*RV.

Table 2. HMP kinase activity of NI500 and its *pdxL* derivatives

Strain (relevant genotype)	HMP-P formed†	
	Thiamin concn (nM) ...	
	2	100
SN372L-K1 (<i>pdxL thiJ</i>)	<0.01	<0.01
NI500 (<i>thiD pdxL⁺</i>)	7.12 ± 0.41	8.32 ± 0.27
NI500-T1 (<i>thiD pdxL</i>)	<0.01	<0.01
K3-9-101 (<i>pdxL⁺ thiJ</i>)‡	6.12 ± 0.21	7.56 ± 0.28
SN372L-3-T1 (<i>pdxL thiJ⁺</i>)‡	6.26 ± 0.46	<0.01

† nmol HMP-P formed (mg protein)⁻¹ h⁻¹. Extracts were prepared from cells grown on the indicated concentrations of thiamin. The reaction mixture contained 0.1 mM HMP and crude cell-free extract (6 mg protein ml⁻¹), and was incubated at 37 °C for 1 h. Results are shown as the mean ± SD of three independent experiments.

‡ Mizote *et al.* (1996).

pAM410 (Fig. 2) was formed to complement the three *thi* mutations. Since appropriate restriction sites were not available in the 6.9 kb fragment, transposon Tn1722, which contains *Eco*RI and *Sph*I sites, was randomly inserted into pAM410. Digestion of the resulting plasmids with *Eco*RI or *Sph*I and subsequent self-ligation generated pAM71, pAM301 and pAM392. Both pAM71 and pAM392 complemented the *thiJ*, *thiD* and *thiM* mutations, indicating that the common 1.7 kb fragment on the two plasmids contained *thiJ*, *thiD* and *thiM*. The plasmid, pAM410::Tn1722-39 was then digested with *Eco*RI and *Hinc*II and the resulting 1.7 kb fragment was blunt ended and subcloned into the *Hinc*II site of pUC18 to give pUA30. Detailed restriction analysis of the 1.7 kb fragment and comparison with the published physical map of the *E. coli* K-12 chromosome (Kohara *et al.*, 1989) revealed that the fragment matched to the 2195 kb coordinate on the Kohara map.

Nucleotide sequence analysis and cloning of *thiM*, *thiJ* and *thiD*

The nucleotide sequence of the 1.7 kb fragment has been deposited separately as *thiD* and *thiM* at DDBJ as D84200 and D88442, respectively. This fragment contains two ORFs, 708 bp ORF1 (235 amino acids) and 801 bp ORF2 (266 amino acids), encoding proteins with predicted molecular masses of 24378 and 28728, respectively. The start codon of ORF2 overlapped with the

Fig. 3. Overexpression of ThiD and its purification. Overexpression of ThiD was performed as described in the text. The samples were separated by SDS-PAGE on a 10–20% gradient Tris-Tricine gel and visualized by Coomassie blue staining. Lane 1, molecular mass markers; lane 2, crude extract from induced cells obtained by French pressing; lane 3, active fractions (5 µg protein) pooled after HiTrap Q column chromatography; lane 4, active fractions (5 µg protein) pooled after IMAC; lane 5, active preparation (5 µg protein) from Mono Q column chromatography. Molecular mass markers: maltose-binding protein-β-galactosidase (83.0 kDa), glutamic dehydrogenase (62.0 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin A (25.0 kDa), lysozyme (16.5 kDa), aprotinin (6.5 kDa).

stop codon of ORF1. Each ORF was separately amplified by PCR. Each PCR product was, after insertion into the *EcoRV* site of pBluescript KS(+), excised by digestion with *NdeI* and *BamHI* and cloned into *E. coli* expression vector pET3a to obtain pTRM110 and pTRD63 for ORF1 and ORF2, respectively. As shown in Fig. 2, pTRM110 complemented the *thiM* mutation, whereas pTRD63 complemented both the *thiJ* and *thiD* mutations.

The 1.7 kb *E. coli* fragment showed very similar properties to that of *S. typhimurium*, where the *thiM* and *thiD* genes are organized as an operon (Petersen & Downs, 1997). The two fragments exhibited high nucleotide-sequence identity (63%). The lengths of *E. coli* ORF1 and ORF2 were identical to those of *thiM* and *thiD*, respectively, of *S. typhimurium*, and the amino acid sequences of the ThiD proteins from the two organisms showed 90% identity. The Shine–Dalgarno sequence for *thiD* was found in the upstream gene, *thiM*, suggesting that a similar regulatory mechanism operates for expression of the two *thiMD* operons.

In *Saccharomyces cerevisiae*, HET kinase and thiamin-P synthetase activities are specified by a bifunctional enzyme having two similar ATP-binding motifs (Nosaka *et al.*, 1994). However, such an ATP-binding motif was not found in the sequence of ThiM in *E. coli*. Many ATP- and GTP-binding proteins contain a consensus sequence motif [AG]X₄GK[ST] (Walker *et al.*, 1984). A sequence similar to this motif, GIQLDLKT, is found in the ThiD sequence starting at position 19 from the N-terminus. Although the phosphorylation reaction of HMP appeared to occur by two steps, only one ATP-binding motif was found in ThiD. The two-step mechanism for phosphorylation is still unclear.

Purification and characterization of the *thiD/J* product

pTRD63, carrying *thiD/J*, was transformed into a host strain, *E. coli* BL21(DE3)(pLysS), and the *thiD/J* gene was overexpressed by incubation with IPTG for 2 h. The uninduced cells contained no *thiD/J* gene product. After preparation of the crude extracts by French pressing of the cells, the *thiD/J* product, ThiD, was purified from the crude extract by three chromatography steps and the enzyme activities recovered from each purification step were measured (Fig. 3 and Table 3). HMP/HMP-P kinase eluted from the IMAC column as a broad peak centred at 35 mM imidazole and from the Mono Q column as a sharp peak centred at 0.28 M NaCl. IMAC can be used to separate protein by means

Table 3. Enzyme activity recovered from each purification step

The reaction mixture containing 0.1 mM HMP and 10 µg protein ml⁻¹ was incubated at 37 °C for 30 min. Results are given in nmol phosphate ester formed (mg protein)⁻¹ min⁻¹ and are the mean ± SD of three independent experiments.

Purification step	Vol. (ml)	Total protein (mg)	Specific activity*		(a)/(b)	Yield (%)
			HMP kinase (a)	HMP-P kinase (b)		
Crude extract	65.1	71.0	289 ± 5	189 ± 2	1.53	100
HiTrap Q	15.8	30.8	264 ± 7	692 ± 8	0.38	84.5
IMAC	11.9	20.8	302 ± 6	798 ± 12	0.38	66.3
Mono Q	5.8	15.6	329 ± 2	843 ± 4	0.39	53.6

* nmol (mg protein)⁻¹ min⁻¹.

Fig. 4. Formation of HMP-P and HMP-PP from HMP (a) and HMP-PP from HMP-P (b) by purified ThiD. Results shown are the means $\pm$ SD of three independent experiments. The remaining amount of HMP in the former case and HMP-P in the latter case are also shown. The reaction mixture and the assay system are described in the text. HMP was not detected when HMP-P was used as the substrate. The amounts of HMP ($\square$), HMP-P ($\blacktriangle$) and HMP-PP ($\bullet$) in the reaction mixture are shown.

of an interaction between matrix-bound metal and surface histidine residues. This method is often used for purification in the protein fused with histidine. Successful purification of the non-fusion protein might be ascribed to the presence of three histidine residues at the C-terminus of ThiD (HHFHAWW). The SDS-PAGE analysis revealed that the purified ThiD has a molecular mass of 27.5 kDa (Fig. 3). IEF-PAGE of the purified protein gave a pI of 5.6 (data not shown), which was in good agreement with the value of 5.7 deduced from the protein sequence. Gel-filtration chromatography gave a molecular mass of 31 kDa, indicating that ThiD exists as a monomer in solution. The actual amino acid sequence that was determined agreed with that predicted from the nucleotide sequence.

Phosphorylation of HMP and HMP-P by ThiD

The time courses of the formation of HMP-P and HMP-PP from HMP and of HMP-PP from HMP-P were measured in the reaction mixture containing ATP (Fig. 4a). When HMP was used as a substrate, it decreased

rapidly with the concomitant formation of HMP-P and HMP-PP. The HMP-P concentration peaked 1 min after initiation of the reaction, and the HMP-P was then converted to HMP-PP. When HMP-P was used as a substrate, it was converted to HMP-PP and the formation of HMP could not be detected (Fig. 4b). Under the same experimental conditions, ThiD did not phosphorylate PIN (data not shown), which contrasted with the specificity of PdxL (Mizote & Nakayama, 1989a; Mizote *et al.*, 1996). The time-course study of the ThiD-mediated phosphate ester formation from HMP clearly indicated that the enzyme catalyses two successive phosphorylation reactions. Transient formation of HMP-P was followed by accumulation of HMP-PP, suggesting that HMP-P is a dissociable intermediate in the production of HMP-PP from HMP. Although PdxL phosphorylated both HMP and PAL derivatives (Mizote & Nakayama, 1989a; Mizote *et al.*, 1996), ThiD did not phosphorylate PAL derivatives (data not shown). These results lead us to conclude that the substrate recognition of this enzyme is more discriminating than that of PdxL. As the purified ThiD protein of *E. coli* phosphorylated HMP to form both HMP-P and HMP-PP, and also HMP-P to form HMP-PP, there is a possibility that ThiD of *S. typhimurium*, which has 90% identity with *E. coli* ThiD, might also be a bifunctional enzyme having the same properties.

The results presented here indicate that the *thiD* mutant, NI500, which had lost both HMP and HMP-P kinase activities, and that the *thiJ* mutant, SN372L-3-9-10, acquired mutations in the same, *thiD/J* gene. Further experiments will be needed to characterize the *thiD* and *thiJ* mutations at the nucleotide level.

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