4-*O*-Phosphoryl-L-threonine, a substrate of the *pdx*C(*ser*C) gene product involved in vitamin B_6 biosynthesis

Christel Drewke, Matthias Klein, Dorothee Clade, Ansgar Arenz, Rolf Müller, Eckhard Leistner*

Institut für Pharmazeutische Biologie, Rheinische Friedrich-Wilhelms-Universität Bonn, Nußallee 6, D-53115 Bonn, Germany

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Abstract **The *Escherichia coli pdx*C(*ser*C) gene codes for a transaminase (EC 2.6.1.52). The gene is involved in both pyridoxine (vitamin B_6) and serine biosynthesis and was over-expressed as a *Mal*E/*Pdx*C(*Ser*C) fusion protein. The fusion protein was purified by affinity chromatography on an amylose resin and hydrolyzed in the presence of protease factor Xa. Both the fusion protein and the *Pdx*C(*Ser*C) protein were characterized (K_M value, turnover number, optimum pH). Both enzymes used 4-*O*-phosphoryl-L-threonine rather than 4-hydroxy-L-threonine as a substrate indicating that the phosphorylated rather than the non-phosphorylated amino acid is involved in pyridoxine biosynthesis. Pyridoxal phosphate was shown to be the cofactor for both enzymes and therefore seems to be involved in its own biosynthesis.**

Key words: *Escherichia coli*; Vitamin B_6; Pyridoxal phosphate; 3-*O*-Phospho-L-serine:2-Oxoglutarate aminotransferase; 4-*O*-Phosphoryl-L-threonine:2-Oxoglutarate aminotransferase

1 Introduction

4-Hydroxy-L-threonine is a biosynthetic precursor of vitamin B_6. The amino acid supports growth of *E. coli* mutant strains with impaired vitamin B_6 biosynthesis [1]. ^{13}C-Labelled precursor amino acid is incorporated into carbons 5′, 5 and 6 of pyridoxine [1,2], a B_6 vitamin. A possible role in vitamin B_6 biosynthesis has also been attributed to 4-*O*-phosphoryl-L-threonine (Fig. 1, [3]). This compound is thought to be derived from 3-hydroxy-4-*O*-phosphoryl-2-oxobutyrate by transamination. The transaminase is encoded by *pdx*C(*ser*C), a gene believed to be involved in both serine and 4-hydroxy-L-threonine biosynthesis, or its phosphorylated derivatives [3]. Mutants lacking a functional allele of the gene fail to synthesize both serine and vitamin B_6 [4–6]. Whereas Dempsey and coworkers have detected phosphoserine aminotransferase activity with 3-*O*-phospho-L-serine as the substrate [5], evidence for 3-hydroxy-4-*O*-phosphoryl-2-oxobutyrate and 4-*O*-phosphoryl-L-threonine as intermediates in vitamin B_6 formation is circumstantial [3,7]. In both transamination reactions, the amino group of glutamic acid is transferred to the acceptor α-oxoacids, yielding the corresponding amino acids and α-oxoglutarate (Fig. 1). If the aminotransferase is a pyridoxal phosphate (PLP) dependent enzyme, it would mean that pyridoxal phosphate is involved in its own biosynthesis. This in turn would suggest that pyridoxal phosphate could act in an autocatalytic fashion, depending on the enzyme kinetics, able to stimulate its own production.

In this paper we describe experiments on the characterization of the *pdx*C(*ser*C) gene product and the role of 4-*O*-phosphoryl-L-threonine in vitamin B_6 biosynthesis.

2. Materials and methods

2.1. Materials

E. coli WG 25 [8] and *E. coli* WG 1145 [9] were obtained from Dr. I.D. Spenser, Hamilton, Ont., Canada. *E. coli* XL 1 blue (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F′ *pro*AB *lac*I^{q}ZDM15 Tn10 (Tet^r)] was from Stratagene, Heidelberg, Germany. *E. coli* Tir8 [10] was obtained from Dr. I. Saint-Girons (Pasteur Institut, Paris, France).

pMAL-c2 (Ap^r, *lac*I^q, P^{tac}/*mal*E-*lac*POZ′) was from NEB, Schwalbach, Germany. pBluescript (KS−) was from Stratagene, Heidelberg, Germany.

Taq polymerase was obtained from Stratagene, Heidelberg, Germany. 4-Hydroxy-L-threonine was a gift from Dr. H.G. Floss, Seattle, WA, USA. Hydroxypyruvate-dimethylketal, hydrolyzed according to the supplier's recommendation, and 3-*O*-phospho-L-serine were obtained from Sigma, Munich, Germany.

2.2. Methods

2.2.1. Growth of strains. For over-expression of *mal*E/*pdx*C(*ser*C) one liter of rich medium (10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose, 100 μg/ml ampicillin) was inoculated with a stationary preculture (1% inoculum) of *E. coli* XL 1 blue containing the recombinant plasmid (pCD10). Incubation was carried out at 37°C in an Infors shaker (180 rpm) until the OD_{600} reached a value of 0.5. IPTG (final concentration 0.3 mM) was added 3 h before harvest to induce the expression of *mal*E/*pdx*C(*ser*C). Cells were collected by centrifugation.

For growth of *E. coli* Tir8, 13 liters M9 medium (6.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 20 ml 20% glucose, 1 ml $MgSO_4$ (1 M), 1 ml $CaCl_2$ (0.1 M) in 1 l H_2O (pH 7.0)) containing 1 mg/ml thiamine-hydrochloride, were inoculated with a stationary preculture (1% inoculum). The cells were grown to the stationary phase and collected by centrifugation.

Growth of *E. coli* mutant WG 25 (*pdx*A−) was performed as described in the presence of pyridoxal phosphate [1].

*2.2.2. Construction of a mal*E/*pdx*C(*ser*C) *fusion gene (pCD10).* The *pdx*C(*ser*C) gene was generated by PCR using primers $pdx_{forward}$ (5′ GAGGGGAATTCGCTCAAATCTTCA 3′) and $pdx_{reverse}$ (5′ CACGGTTAATGTCGACATTTTGG 3′) deduced from the nucleotide sequence [11] and the genomic DNA of *E. coli* K 12 to prime an amplification product harboring the whole *pdx*C(*ser*C) coding region. The primers were constructed to yield a PCR product containing an *Eco*RI restriction site at the 5′ and a *Sal*I site at the 3′ non-coding region. Amplification was carried out in a Trio-Thermoblock (Biometra, Göttingen, Germany) using Taq polymerase according to the supplier's recommendation. Thirty cycles were carried out, each cycle consisting of denaturing at 95°C (30 s), annealing at 56°C (30 s) and extension at 72°C (65 s). Recombinant plasmids were constructed by standard techniques [12] using the vector's *Eco*RI and *Sal*I sites for cloning. For sequencing the cloned PCR product was subcloned into pBluescript (KS−) using the gene's flanking *Eco*RI and *Sal*I sites as well as the internal *Cla*I site. Sequencing was per-

*Corresponding author. Fax (49) (228) 733250.
E-mail unc80d@ibm.rhrz.uni-bonn.de

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formed using the standard pBluescript forward and reverse primers. In position 292 arginine [11] was found to be replaced by alanine as is the case with *Yersinia enterocolitica* [13] and *Salmonella gallinarium* [14]. We suspect that the sequence of Duncan and Coggins [11] contains a reading error, for none of the published *Pdx*C sequences [13–16] except that of Duncan and Coggins [11] was found to have an arginine in position 292.

2.2.3. Purification of MalE/PdxC(SerC). Bacterial cells (5 g wet weight) harboring *Mal*E/*Pdx*C(*Ser*C) were suspended in column buffer (5 ml, 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and frozen overnight at −20°C. Before ultrasonic treatment (Branson sonifier, Danbury, USA, 10 times, 20 s, 50% output at stage 5) cells were thawed in a cold water bath. After sedimentation of the cell debris (30 min, 10000×*g*, 4°C) the supernatant was diluted to about 2.5 mg protein/ml and passed over a column (2.5×10 cm, 1 ml/min) packed with 15 ml amylose resin (NEB, Schwalbach, Germany, #800-21 s) equilibrated with 5 column volumes of Tris-HCl buffer (specified above). The column was washed with 8 volumes of Tris-HCl buffer, and the fusion protein eluted with the same buffer containing 10 mM maltose. The yellow-colored fractions containing *Mal*E/*Pdx*C(*Ser*C) were combined and concentrated by ultrafiltration ('8050' cell, Amicon, Witten, Germany, using an 'Omega Filter 5K' filter, Filtron).

2.2.4. Cleavage of the MalE/PdxC(SerC) fusion protein. The *Mal*E/*Pdx*C(*Ser*C) fusion protein was cleaved using protease factor Xa (NEB) (10 μg/mg protein) for 24 h at 4°C. The solution containing the *Pdx*C(*Ser*C) enzyme was passed over the amylose column (see above) in order to remove the *Mal*E domain.

2.2.5. Preparation of MalE/PdxB and PdxB. The *Mal*E/*Pdx*B and *Pdx*B proteins were prepared as described for the *Mal*E/*Pdx*C(*Ser*C) and *Pdx*C(*Ser*C) proteins except that primers $pdx_{forward}$ (5′ CGTGGAATTCCTTGTTGATGAAA 3′) and $pdx_{reverse}$ (5′ GGCACGTTAATCTCTTCTAGATGC 3′) were employed.

2.2.6. Enrichment of homo-serine kinase. After harvest the cell sediment (43.5 g fresh weight) was resuspended in 43.5 ml buffer A (50 mM HEPES, pH 7.5, 0.1 M KCl, 1 mM L-threonine, 1 mM L-lysine, 0.1 mM pyridoxal phosphate, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM dithioerythritol) and frozen at −20°C. Before ultrasonic treatment (Branson sonifier, Danbury, USA, 10 times, 20 s, 50% output at stage 5) the cell suspension was thawed in a cold water bath. Enrichment of the enzyme was modified as described by Shames et al. [17]. This involved $(NH_4)_2SO_4$ precipitation (40% saturation), gel filtration on Sephadex G-25, heat treatment at 57°C and dye ligand chromatography on Affi gel blue (Serva, Heidelberg, Germany). The homo-serine kinase containing fractions were pooled and concentrated by ultrafiltration (specified above). Determination of homo-serine kinase activity was performed according to [10].

2.2.7. Enzyme assays. Transamination of 3-*O*-phosphoryl-pyruvate by *Mal*E/*Pdx*C(*Ser*C) and *Pdx*C(*Ser*C) was observed in a coupled assay using the glutamate dehydrogenase-catalyzed conversion of α-oxoglutarate to glutamate in the presence of ammonium ions as the indicator reaction. The oxidation of NADH was monitored in a spectrophotometer at 340 nm. The reaction was carried out in 0.1 M potassium phosphate buffer, pH 8.2, containing 4 μmol/ml KF, 0.25 μmol/ml ammonium acetate, 0.125 μmol/ml glutamic acid, 25 nmol/ml NADH, 250 μg/ml glutamate dehydrogenase (11 U), 3-*O*-phosphoryl-pyruvate and *Mal*E/*Pdx*C(*Ser*C) fusion protein or *Pdx*C(*Ser*C) protein, respectively.

The reverse reaction (4-*O*-phosphoryl-L-threonine or 3-*O*-phospho-L-serine to its respective 2-oxo acid) was carried out in 0.1 M potassium phosphate buffer, pH 8.2, 0.125 μmol/ml α-oxoglutarate, 4 μmol/ml KF, 250 μg/ml glutamate dehydrogenase, *Mal*E/*Pdx*C(*Ser*C) or *Pdx*C(*Ser*C) proteins, and 3-*O*-phospho-L-serine or 4-*O*-phosphoryl-L-threonine, together with 0.6 μmol/ml 3-acetylpyridine adenosine dinucleotide (APAD) [18]. The reduction of APAD was monitored at 365 nm.

Phosphorylation of 4-hydroxy-L-threonine was achieved with enriched homo-serine kinase fractions (see above) in the presence of 4-hydroxy-L-threonine and 6.1 mg/ml ATP, incubated for 30 min at 37°C. Phosphorylation was stopped by heating for 10 min at 100°C.

2.2.8. HPLC assays. B_6 vitamins were analyzed using a Merck-Hitachi L-6200A HPLC apparatus (Darmstadt, Germany), connected to a fluorescence detector (Shimadzu RF551 set to 330 nm (excitation) and 400 nm (emission)). The samples were separated on a Nucleosil 100 5SA column (250×4 mm ID) coupled to a Nucleosil 100 5SA guard column (30×4 mm ID) (Macherey-Nagel, Düren, Germany) with a flow rate of 1 ml/min. As eluants 0.01 M HCl (solvent A) and potassium phosphate buffer, 0.5 M pH 5 (solvent B) were used. Elution was started with solvent A (100%) decreasing to 90% A as component B increased from 0 to 10% over the first 6 min, followed by an increase to 20% B over the next 2 min. Subsequently B was increased to 100% over 7 min. After a plateau (100% B) for 13 min the column was reconditioned with 100% A for 15 min.

3. Results and discussion

3-*O*-phospho-L-serine:2-oxoglutarate aminotransferase (*Pdx*C(*Ser*C)), an enzyme involved in serine biosynthesis, is most likely also involved in the biosynthesis of 4-hydroxy-L-threonine, a precursor of the vitamin B_6 pyridine ring (Fig. 1). *pdx*C(*ser*C) mutants fail to synthesize both serine and pyridoxine. In order to examine the dual function of the enzyme, the corresponding gene, *pdx*C(*ser*C), was amplified by PCR and over-expressed as a *Mal*E/*Pdx*C(*Ser*C) fusion protein (see above). The recombinant plasmid encoding the *mal*E/*pdx*C(*ser*C) sequence complemented the *pdx*C(*ser*C) deficient *E. coli* mutant WG 1145, indicating that the fusion protein is active in vivo. The fusion protein was enriched on an amylose column as described above resulting in a single band at 79 kDa on a Coomassie blue stained SDS polyacrylamide gel. The *Mal*E/*Pdx*C(*Ser*C) fusion protein was cleaved by protease factor Xa yielding two bands on SDS PAGE; one of 43 kDa represented the *Mal*E domain and the other at 36 kDa had the predicted molecular weight for the *Pdx*C(*Ser*C) protein. Whereas the fusion protein was stable over several weeks, the cleaved protein showed a clear loss of activity within 2 weeks at 4°C.

Both proteins were examined for their PLP content. The UV spectrum of the intensely yellow fusion protein was superimposable on that of *Pdx*C(*Ser*C) alone. The shift of the maximum from 405 nm to 330 nm during treatment with $NaBH_4$ had also been observed for the corresponding transaminase from sheep brain [19]. Reduction with $NaBH_4$ coincided with loss of 3-*O*-phospho-L-serine transaminase activity. A control spectrum of *Pdx*B (4-*O*-phosphoerythronate dehy-

Table 1
Properties of *Mal*E/*Pdx*C(*Ser*C) and *Pdx*C(*Ser*C)

	K_M [μM]	V_{max} [μmol/s/mg]	Turnover number [s^{-1}]	Optimum pH
*Mal*E/*Pdx*C(*Ser*C)				
3-*O*-phosphoryl-pyruvate	10	1.6×10^{-2}	1.33	7.8
3-*O*-phospho-L-serine	37	7.6×10^{-3}	0.63	8.7
4-*O*-phosphoryl-L-threonine	110	1.0×10^{-3}	0.08	8.7
*Pdx*C(*Ser*C)				
3-*O*-phosphoryl-pyruvate	15	4.5×10^{-2}	1.75	8.0
3-*O*-phospho-L-serine	17	9.9×10^{-3}	0.39	8.8
4-*O*-phosphoryl-L-threonine	110	3.8×10^{-3}	0.15	8.3

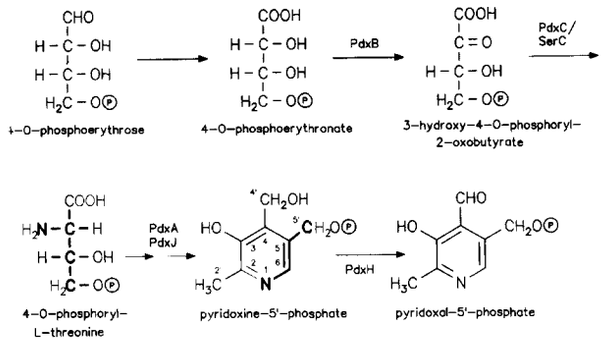

Fig. 1. Hypothetical scheme of pyridoxal phosphate biosynthesis showing the probable origin of atoms C-5, -5', -6 and N-1. *PdxB* = 4-*O*-phosphoerythronate dehydrogenase; *PdxC*(*SerC*) = 3-*O*-phospho-L-serine:2-oxoglutarate aminotransferase; *PdxH* = pyridoxine-5'-phosphate oxidase. *Pdx*A and *Pdx*J are of unknown function.

dehydrogenase), another enzyme of the vitamin B_6 pathway ([20], Fig. 1), was as expected devoid of maxima at 330 and 405 nm.

In another experiment the cofactor of *Pdx*C(*Ser*C) was investigated after treatment of the enzyme (8 mg) with TCA (final concentration 4.5%). After neutralization the supernatant was placed in wells of agar plates containing a suspension of the pyridoxine auxotrophic *E. coli* mutant WG 25 (see [1]). Whereas no growth zones were detected in controls with NaCl or supernatant from TCA precipitated *Mal*E/*Pdx*B fusion protein, a clear growth zone was present around the well containing the *Mal*E/*Pdx*C(*Ser*C) sample. The diameter of the growth zone compared favorably to an equimolar solution of pyridoxal phosphate. A third approach used to identify the cofactor requirements of the *Pdx*C(*Ser*C) transaminase was carried out by HPLC. After treatment of the TCA supernatant with phosphatase [21] peaks with retention times corresponding to those of authentic samples of pyridoxal (RT 20.6 min) and pyridoxamine (RT 25.9 min) appeared. In a control experiment again using the *Mal*E/*Pdx*B protein no vitamins could be detected. Thus, in agreement with former results [6], phosphoserine transaminase is a pyridoxal phosphate containing enzyme. It is striking that PLP appears to be involved in its own biosynthesis.

Both the properties of the fusion protein and the authentic *Pdx*C(*Ser*C) enzyme were characterized using multiply coupled assays. Since the substrate for 4-*O*-phosphoryl-L-threonine formation, 3-hydroxy-4-*O*-phosphoryl-2-oxobutyrate, is not yet available, the forward reaction could only be examined with the serine pathway-specific substrate 3-*O*-phosphoryl-pyruvate. Activity of *Pdx*C(*Ser*C) or *Mal*E/*Pdx*C(*Ser*C), respectively, was determined using glutamate dehydrogenase reduction of α-oxoglutarate to glutamate in the presence of ammonia with concomitant oxidation of NADH. The enzymatic properties of the proteins were also determined for the reverse reaction with either 3-*O*-phospho-L-serine or 4-*O*-phosphoryl-L-threonine as substrates and α-oxoglutarate as the amino group acceptor. The glutamate formed was determined quantitatively with glutamate dehydrogenase. The indicator reaction was monitored by reduction of APAD as cofactor which shifts the equilibrium of the indicator reaction in the direction of α-oxoglutarate [18]. Transamination depended on the presence of enzyme, substrate, cofactor, glutamate dehydrogenase and α-oxoglutarate or glutamate. Phosphorylation of 4-hydroxy-L-threonine (see above) was performed enzymatically with homo-serine kinase, an enzyme of the bacterial threonine pathway. The enzyme was enriched from *E. coli* Tir 8, a strain de-repressed for the enzymes of the threonine operon [10,17,22,23]. The amount of 4-*O*-phosphoryl-L-threonine in each assay was determined as described [10]. No activity of *Pdx*C(*Ser*C) or *Mal*E/*Pdx*C(*Ser*C) could be detected in controls with denatured homo-serine kinase or in the absence of either 4-hydroxy-L-threonine or ATP.

The properties of both *Mal*E/*Pdx*C(*Ser*C) and *Pdx*C(*Ser*C) determined with different substrates are listed in Table 1. The data for K_M, V_{max} and turnover number are in the same range for both the fusion protein and the authentic *Pdx*C(*Ser*C) enzyme. Comparison of the data for 3-*O*-phosphoryl-pyruvate with those for 3-*O*-phospho-L-serine and 4-*O*-phosphoryl-L-threonine shows that 3-*O*-phosphoryl-pyruvate is a better substrate for both enzymes. The turnover number for 4-*O*-phosphoryl-L-threonine is particularly low compared to those for 3-*O*-phosphoryl-pyruvate and 3-*O*-phospho-L-serine. This agrees with the K_M value for 4-*O*-phosphoryl-L-threonine, which is significantly higher than the data for the other substrates. In agreement with observations for pyridoxine-5'-phosphate oxidase, another enzyme of the PLP pathway (compare Fig. 1) [24], these findings again confirm the assumption of Dempsey [25] that the biosynthesis of B_6 vitamins in *E. coli* is catalyzed by a series of 'sluggish enzymes' with rather low turnover numbers and consequently rather high K_M values (because only small amounts of B_6 vitamin are required).

Interestingly, no activity of either enzyme could be observed with non-phosphorylated substrates, indicating that the phosphorylated rather than the non-phosphorylated form is a precursor of the vitamin B_6 pyridine ring. This agrees with recent mutant studies [7] and the assumption that phosphorylation occurs at an early step during biosynthesis of PLP (compare [26]) and with previous results, which show that non-phosphorylated B_6 vitamins (pyridoxine, pyridoxamine) are not oxidized by the *pdx*H gene product ([24]; compare Fig. 1) to form the final cofactor PLP.

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