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Bioorganic & Medicinal Chemistry Letters 13 (2003) 737–739

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

# Functional Expression and Characterization of EryA, the Erythritol Kinase of *Brucella Abortus*, and Enzymatic Synthesis of L-Erythritol-4-phosphate

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Received 11 September 2002; accepted 30 October 2002

**Abstract**—The *eryA* gene of the bacterial pathogen *Brucella abortus* has been functionally expressed in *Escherichia coli*. The resultant EryA was shown to catalyze the ATP-dependent conversion of erythritol to L-erythritol-4-phosphate (L-E4P). The steady state kinetic parameters of this reaction were determined and the enzyme was used to prepare L-E4P which was shown to be a weak inhibitor of 2-*C*-methyl-D-erythritol-4-phosphate cytidyltransferase (YgbP).


The gram negative pathogenic bacterium *Brucella abortus* (*Brucella melitensis biovar abortus*) is the causative agent of the abortion-causing disease of cattle known as brucellosis.[1] The closely related pathogen *Brucella melitensis* causes abortion in goats and sheep as well as Malta fever in humans.[2] Members of the genus *Brucella* utilize erythritol in preference to glucose.[3] García-Lobo and coworkers have recently identified a cluster of four genes in *B. abortus* 2308, designated the *ery* operon, that was implicated in erythritol metabolism.[1] One 1.56-kb open reading frame (ORF), *eryA*, encoded a putative erythritol kinase of 519 amino acids, whose function was assigned on the basis of sequence similarity to several bacterial xylulose and glycerol kinases.[1]

To confirm the imputed role of the *eryA* gene product, we have carried out the functional expression of EryA, established that the enzyme catalyzes the phosphorylation of erythritol to L-erythritol-4-phosphate (L-E4P), and determined the steady state kinetic parameters of the ATP-dependent reaction. We have also tested the derived L-E4P as a potential inhibitor of 2-*C*-methyl-D-erythritol-4-phosphate cytidyltransferase (YgbP),[4] the enzyme that catalyzes the second committed step in the mevalonate-independent biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).[5]

Plasmid pSU6004,[1] a derivative of the vector pBluescript II SK(+) harboring the *ery* operon, was used as a template for PCR amplification of *eryA*.[6] The amplified DNA was ligated into the expression vector pHIS8,[7] the integrity of the PCR insert was verified by sequencing,[8] and the resultant plasmid, pHIS8-*eryA*, was used to transform the expression host *Escherichia coli* BL21(DE3).[9] The resulting EryA protein was then purified from the cell free extract via nickel affinity chromatography (Fig. 1).[10]

EryA (1.36 mg, 24.9 nmol) was incubated with *meso*-erythritol (**1**) (1 mmol) and ATP (1 mmol) in the presence of $MgCl_2$ (10 μmol) and sodium iodoacetate (50 μmol) in 1.5 mL of Tris–HCl buffer (50 mM, pH 8) at room temperature for 24 h (Scheme 1). Activated charcoal (3 g) was then added to the incubation mixture which was allowed to stand for 20 min before removal by filtration and washing with deionized water (15 mL). The filtrate was applied in two equal portions to an anion exchange column packed with DOWEX 1-X8 resin (2.5×16 cm, formate form). The column was washed with deionized water (100 mL) and eluted with a solution of 1 M ammonium formate and 1 M formic

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doi:10.1016/S0960-894X(02)01032-6

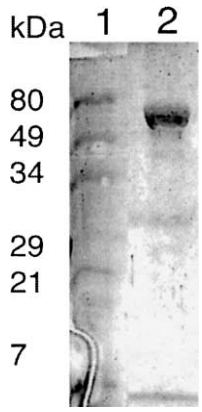
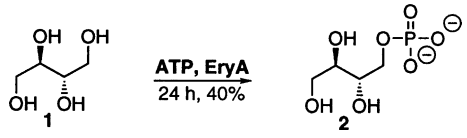


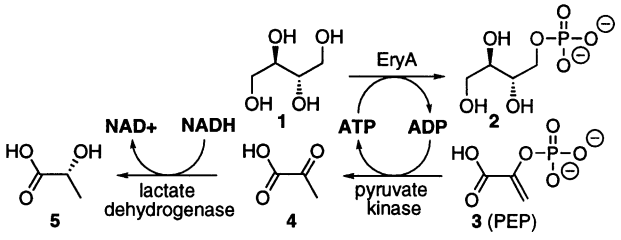

**Figure 1.** SDS-PAGE of EryA purification: lane 1, size markers; lane 2, purified EryA.




**Scheme 1.** Synthesis of L-E4P (**2**) from *meso*-erythritol (**1**) catalyzed by EryA.

acid (100 mL). The total eluate was subjected to multiple rounds of lyophilization to remove the volatile salts, yielding L-erythritol-4-phosphate (**2**)[11,12] (78 mg, 40% yield, >95% pure by NMR). $^1$H NMR ($D_2O$) δ 4.06 (m, 1H), 3.98 (m, 1H), 3.82 (dd, $J$ = 17.21, 4.72 Hz, 1H), 3.80 (m, 1H), 3.75 (m, 1H), 3.65 (dd, $J$ = 17.21, 5.7 Hz, 1H); $^{13}$C NMR ($D_2O$) δ 71.71 (s), 70.98 (d, $J$ = 5.0 Hz), 70.98 (s), 67.10 (d, $J$ = 7.6 Hz); $^{31}$P NMR ($D_2O$) δ 1.3 (s); MS (ESI) $m/z$ [M-H]$^-$ calcd = 201.02, obs. = 201.23; $[\alpha]_D^{20}$ ($c$ 20.0, water) acid form = −2.6°, $[\alpha]_D^{20}$ (c 26.9, water) cyclohexylammonium salt = +2.1°.[13] The formation of L-erythritol-4-phosphate (**2**) (D-erythritol-1-phosphate) confirms the proposed erythritol kinase activity of the EryA protein and is consistent with the known intermediacy of D-erythrulose-1-phosphate in the catabolism of erythritol by *Brucella* species.[1,2,14]

EryA activity was measured using a coupled spectrophotometric assay to monitor the consumption of NADH as outlined in Scheme 2, separately varying the concentrations of erythritol and ATP.[15] The conditions of the assay were such that less than 10% of the substrate was turned over in 5 min.[16] The steady state kinetic parameters (Table 1) were obtained by direct fitting of the data to the Michaelis–Menten equation by nonlinear least squares regression. The observed $K_m$ values are similar to those reported for the erythritol




**Scheme 2.** Coupled enzymatic assay of EryA.

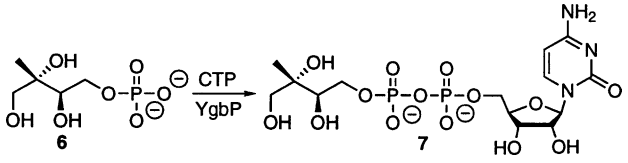
**Table 1.** Steady-state parameters determined for EryA

| Substrate | $K_M$ (mM) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|
| *meso*-Erythritol | 0.26±0.04 | 230±70 |
| ATP | 9.8±2.8 | 230±70 |

kinase from *Propionibacterium pentosaceum*, $K_m$ (erythritol) = 0.88 mM and $K_m$ (ATP) = 4.8 mM.[12c]

Erythritol-4-phosphate is the desmethyl analogue of 2-*C*-methyl-D-erythritol-4-phosphate (MEP, **6**), an intermediate in the mevalonate-independent pathway of isoprenoid biosynthesis in bacteria and the chloroplasts of plants.[5] E4P, of unspecified absolute configuration, has in fact previously been found to inhibit 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) synthase (YgbP, IspD), which catalyzes the condensation of CTP and MEP (**6**) to form CDP-ME (**7**) (Scheme 3) with an $IC_{50}$ of 1.36 mM.[4] E4P was also reported to be turned over by YgbP at a reduced rate compared to the natural substrate.[4] We therefore examined the inhibition of YgbP by L-erythritol-4-phosphate (**2**), using varying inhibitor (**2**) and substrate (**6**) concentrations.[17] L-erythritol-4-phosphate (**2**) was found to be a weak competitive inhibitor of YgbP with respect to MEP, with a calculated $K_i$ of 240±17 mM (Fig. 2). Although one cannot directly compare $K_i$ and $IC_{50}$ values, the nearly 200-fold difference strongly suggests that E4P used by Wungsintaweekul[4] was in fact D-E4P, which is a much more potent inhibitor, consistent with the configuration of the natural D-MEP substrate **6**.

In summary, the erythritol kinase EryA has been functionally expressed and characterized and then used to prepare L-erythritol-4-phosphate (**2**) in a single step in 40% purified yield from *meso*-erythritol. Lastly, **2** has been shown to be a weak competitive inhibitor of the enzyme YgbP.




**Scheme 3.** YgbP catalyzed condensation of CTP and MEP (**6**) to form CDP-ME (**7**).

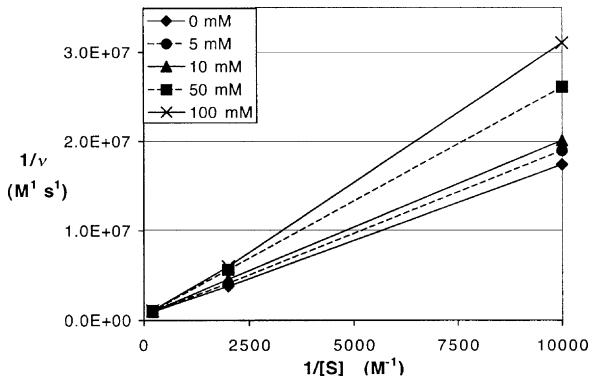



**Figure 2.** Lineweaver–Burke plot of the inhibition of YgbP with **2**.

## Acknowledgements

This work was supported by NIH grant GM30301 to DEC. We thank Dr. Juan M. García-Lobo of the Universidad de Cantabria for helpful comments on the manuscript. The authors would also like to thank David Wilbur (Tufts University, Medford, MA, USA) for his generous assistance in acquiring spectropolarimetric data.

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6. The plasmid pSU6004 was used to transform competent *E. coli* XL-10 Gold cells (Stratagene). Transformants were inoculated on LB agar plates containing ampicillin (100 μg/mL) and then grown in LB liquid media. pSU6004 was then extracted from the culture and used for PCR amplification(*Pfu* Turbo PCR kit, Stratagene) of the eryA gene. The primers (Integrated DNA Technologies) utilized for PCR were designed to introduce *Bam*H I and *Sal* I restriction sites at the 5′ and 3′ termini of the amplified genes (primer 1 = aataatggatccgtgtcagccatgcgtgaaaaaggtg, primer 2 = actaatgtcgacttatttgccggaagcgagtttgtccc). The PCR amplification mixture contained pSU6004 (72 ng), primer 1 (10 nmol), primer 2 (10 nmol), DMSO (7.5% v/v), dNTP's (0.6 mM each), *Pfu* buffer (1:10 diluted stock), and *Pfu* polymerase (0.03 U) in 20 μL total volume. The temperature program consisted of the following: 10 min at 95 °C, and then 40 cycles of amplification (0.5 min at 63 °C, 4 min at 72 °C, 1.5 min at 95 °C).
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8. Sequencing one of the *eryA* inserts indicated several discrepancies with respect to the published sequence of *B. abortus eryA* (trEMBL: Q9ZB32, Accession # AAD11519). In order to rule out mutations due to infidelity during the PCR amplification, the *eryA* portion of pSU6004 was resequenced. The corrected sequence of *B. abortus eryA* is, in fact, identical to that of the *eryA* gene of the biovar *B. melitensis* (Accession # AAL53672) with three exceptions: nt 300 in *B. abortus eryA* is A instead of G, nt 478 is G in place of A, and nt 1155 is C in place of T. (Numbering is from the GTG start codon of *B. abortus eryA*.) The corrected *B. abortus eryA* sequence has been deposited in the EMBL database.
9. The PCR product of *eryA* amplification and vector pHIS8 (a derivative of vector pET-28a(+) from Prof. J. Noel, Salk Institute, CA) were both digested with *Bam*H I and *Sal* I (Promega). The PCR product was treated additionally with *EcoR* I (Promega) in order to digest possible contaminants. Upon purification from the restriction enzymes the two solutions of digested DNA (~50 μg/mL) were combined and incubated at 4 °C in the presence of T4 DNA ligase (Gibco) for 12 h. The ligation mixture was used without further treatment for transformation of competent *E. coli* XL-10 Gold cells. Transformants were grown on LB agar plates containing kanamycin (3 μg/mL) and then in LB liquid media. The pHIS8-*eryA* plasmid was then extracted and used to transform *E. coli* BL21(DE3) cells (Stratagene).
10. *E. coli* BL21(DE3)/pHIS8-EryA was grown at 37 °C in LB media (1 L) supplemented with kanamycin (3 μg/mL) to an $OD_{600}$ of 0.5. The culture was then induced with IPTG (0.5 mM final concn) and allowed an induction time of 3.5 h at 37 °C. The resulting cell pellet was resuspended in lysis buffer [Tris–HCl (28 mM), $MgCl_2$ (2.8 mM), NaF (0.1 mM), β-mercaptoethanol (2.7 mM), pH 8], disrupted by sonication (5, 3 min bursts at 40 MHz with a 50% duty cycle at 0 °C) and centrifuged (20,000*g*, 20 min). SDS-PAGE of the lysate showed that a protein of $M_r$ ~55,000 was present in the soluble and insoluble fractions. The resulting soluble portion was applied to a nickel-chelating affinity column (Hi-trap, Phamacia) and eluted with a linear gradient of 10 to 500 mM imidazole in Tris–HCl (50 mM), $MgCl_2$ (5 mM), glycerol (10% v/v), NaCl (500 mM), and β-mercaptoethanol (27 mM) at pH 8. Fractions determined to contain the highest purity of eryA were collected and concentrated using a 10,000 MW cutoff filter.
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12. (a) L-Erythritol-4-phosphate: Ballou, C. E.; Fischer, H. O. L.; MacDonald, D. L. *J. Am. Chem. Soc.* **1955**, *77*, 5967. (b) Shetter, J. K. *J. Am. Chem. Soc.* **1956**, *78*, 3722. (c) Holten, D.; Fromm, H. J. *J. Biol. Chem.* **1961**, *236*, 2581.
13. D-E4P:$[\alpha]_D^{25}$ +2.6° (*c* 1.5, free acid in water)[11] and $[\alpha]_D^{25}$ −2.3° (*c* 3, cyclohexylammonium salt in water).[11] L-E4P: $[\alpha]_D^{25}$ +2.3° (*c* 5,cyclohexylammonium salt in water).[11]
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16. Assay mixtures contained: Tris–HCl (120 mM), $MgCl_2$ (12 mM), KCl (240 mM), $MgSO_4$ (80 mM), NADH (0.18 mM), phosphoenol pyruvate (0.35 mM), L-lactic acid dehydrogenase (from bovine heart, 4.3 U/mL, Sigma), pyruvate kinase (from rabbit muscle, 2 U/mL, Sigma) and sodium iodoacetate (2 mM) at a pH of 8. The assay was conducted at room temperature using a HP8452 diode array spectrophotometer and data was acquired and processed by the software package UV-Visible Chemstation (Rev. A.08.03[71])(Agilent Technologies). When the concentration of **1** was varied (0.1–25 mM) the concentration of ATP was 10 mM. When the concentration of ATP was varied (0.2–50 mM) the concentration of *meso*-erythritol was 10 mM. Stock solutions of ATP were pre-treated with PEP, NADH, pyruvate kinase, and L-lactic acid dehydrogenase to remove ADP contamination (~0.7%). The reaction was followed by observing the decrease in absorbance at 340 nm corresponding to the consumption of NADH over the course of 5 min.
17. Assay mixtures contained: Tris–HCl (100 mM), $MgCl_2$ (10 mM), CTP (5 mM), inorganic pyrophosphatase (2U), and YgbP (0.218 μg/mL) at pH 8. Three concentrations of **6** (0.1, 0.5, and 5 mM) and seven concentrations of **2** (0, 0.5, 1, 5, 10, 50, and 100 mM) were used to give a total of 21 combinations of conditions. The assay was carried out at room temperature in a total volume of 6 μL with $^{14}C$ labeled **6**. Aliquots of 2 μL were removed at 2, 6, and 12 min and quenched by application onto silica TLC plates pretreated with acid. TLC plates were developed (eluent: *n*-isopropanol/water/EtOAc 7:2:1) and data acquired by phosphorimaging (GS-363 Molecular Imaging System, BioRad). Steady state kinetic parameters, $k_{cat}$, $K_M$, and $K_i$ were obtained by direct non-linear least squares fitting of the data to the relevant form of the Michaelis–Menten equation for competitive inhibition.