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THE KINETIC MECHANISM OF 5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE FROM A GRAM-POSITIVE PATHOGEN *STREPTOCOCCUS PNEUMONIAE*

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The *Streptococcus pneumoniae* 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase is a potential novel antibacterial target. The enzyme catalyzes a reversible transfer of an enolpyruvyl group from phospho(enol)pyruvate (PEP) to shikimate 3-phosphate (S3P) to give EPSP with the release of inorganic phosphate (Pi). Understanding the kinetic mechanism of this enzyme is crucial to the design of novel inhibitors of this enzyme that may have potential as antibacterial agents. Steady-state kinetic studies of product inhibition and inhibition by glyphosate (GLP) have demonstrated diverse inhibition patterns of the enzyme. In the forward reaction, GLP is a competitive inhibitor with respect to PEP, but an uncompetitive inhibitor relative to S3P. Product inhibition shows that EPSP is a competitive inhibitor versus both PEP and S3P, suggesting that the forward reaction follows a random sequential mechanism. In the reverse reaction, GLP is an uncompetitive inhibitor versus EPSP, but a noncompetitive inhibitor versus Pi. This indicates that a non-productive quaternary complex might be formed between the enzyme, EPSP, GLP and Pi. Product inhibition in the reverse reaction has also been investigated. The inhibition patterns of the *S. pneumoniae* EPSP synthase are not entirely consistent with those of EPSP synthases from other species, indicating that EPSP synthases from different organisms may adopt unique mechanisms to catalyze the same reactions.

Keywords: Shikimate pathway; EPSP synthase; Glyphosate; Steady-state kinetics; Product inhibition

Abbreviations: EPSP, 5-enolpyruvylshikimate-3-phosphate; PEP, phospho(enol)pyruvate; S3P, shikimate 3-phosphate; GLP, glyphosate; HEPES, *N*-[2-hydroxyethyl]piperazine]-N′-[2-ethanesulfonic acid].

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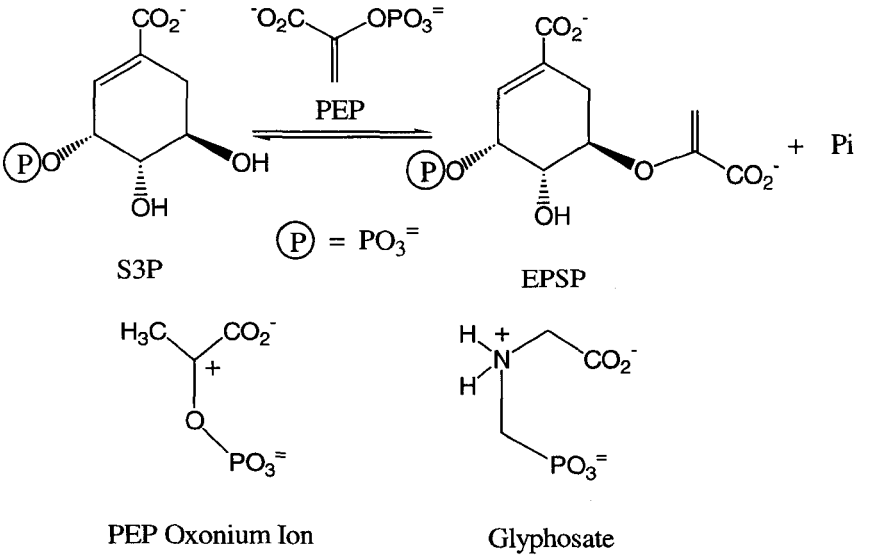
INTRODUCTION

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) is the sixth enzyme in the shikimate pathway that leads to the production of chorismate, the precursor of a series of essential aromatic metabolites in plants, fungi and bacteria.[1] The enzyme catalyzes a reversible transfer of an enolpyruvyl group from phosphoenol pyruvate (PEP) to shikimate 3-phosphate (S3P) to yield 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Pi) (Scheme 1). Early interest in EPSP synthase started with the discovery that the broad-spectrum, post-emergence herbicide glyphosate (*N*-phosphonomethylglycine, GLP) inhibits this enzyme[2,3] by functioning as a transition state analog of PEP[4] (Scheme 1).

The resurgence in antibiotic resistant bacteria has driven a quest for novel antibacterial targets to combat the emergence of multiply resistant Gram-positive pathogens.[5] EPSP synthase has been proposed to be a potential novel antibacterial target because it is essential for the survival of bacteria and it is absent in mammalian cells.[6,7] Consequently, we recently identified and sequenced the EPSP synthase from the Gram-positive pathogen *Streptococcus pneumoniae*.[8] Since EPSP synthase catalyzes a bi-substrate



SCHEME 1 EPSP synthase catalyzed reaction and comparison of the structures of transiently formed PEP oxonium ion and its analog, GLP.

reaction, the order of binding of the two substrates is crucial for our understanding of the mechanism of this enzyme.

In this work, we describe the first detailed kinetic evaluation of an EPSP synthase from a Gram-positive pathogen. This work will not only enable the configuration of optimal assays to screen for novel EPSP synthase inhibitors but also provide strategies for the *de novo* design of inhibitors of this enzyme. Such inhibitors may have potential as novel antibacterial agents for the treatment of multiple resistant Gram-positive pathogens.

MATERIALS AND METHODS

Chemicals and Enzymes

Biochemical reagents were from Sigma. S3P, EPSP and *S. pneumoniae* EPSP synthase were prepared as described previously.[8]

Kinetic Assays

EPSP synthase was assayed in HEPES buffer (0.1 M, pH 7.0) at 25 °C following the procedures described previously[8] unless otherwise indicated. The forward reaction rates were measured by following the release of Pi in buffer containing 1 mM NH_4Cl and 100 mM KCl. The reverse reaction was assayed by following the production of PEP. The inhibition constants were determined with the non-varied substrate maintained at saturating concentration.

Data Analysis

Steady-state kinetic constants were obtained by fitting the initial rate data to the following velocity equations in GraFit (ν4.09, Erithacus Software Limited). Equations (1), (2), and (3) describe competitive, uncompetitive and noncompetitive inhibition models, respectively.

$$\nu = VA/[K_a(1 + I/K_i) + A] \quad (1)$$

$$\nu = VA/[K_a + A(1 + I/K_i)] \quad (2)$$

$$\nu = VA/[(K_a(1 + I/K_i) + A(1 + I/K_i)] \quad (3)$$

The individual terms define ν, the velocity; V, the maximal velocity; K_a, the apparent Michaelis constant; K_i, the apparent inhibitory constant; A, the variable substrate concentration ; and I, the inhibitor concentration. The mode of inhibition was determined by the associated error and the reduced χ^2 of fitting the data into the equations.

RESULTS AND DISCUSSION

The Michaelis-Menten constants for both the forward and reverse reactions of the *S. pneumoniae* EPSP synthase were determined previously.[8] In the determination of K_m for PEP and S3P in the forward reaction, the lines in the double reciprocal plots of initial rates versus a range of substrate concentrations intercepted at one point. This indicates that binding of the two substrates (S3P and PEP) must have followed a sequential but not ping-pong mechanism.[9] Consequently, the two substrates must bind with the enzyme one after another to form a ternary complex before any product can be released. However, the order of binding of the two substrates was not determined. In this report, we have analyzed the kinetic mechanisms for both forward and reverse reactions using steady-state kinetics. First we studied the inhibition patterns of GLP with the enzyme because the compound is a known inhibitor of EPSP synthase. The kinetic mechanism was also examined via a product inhibition approach.

Inhibition by GLP in the Forward Reaction

The kinetic patterns of the inhibition of *S. pneumoniae* EPSP synthase by GLP in the forward reaction are shown in Figure 1. The initial rates determined at varied PEP and GLP but fixed S3P (1 mM) fitted well to the competitive inhibition model with a K_i of 2.8±0.3 μM (Figure 1A). At fixed PEP (1 mM), GLP appeared to be an uncompetitive inhibitor with respect to S3P with a K_i of 65.6±2.7 μM (Figure 1B). These results suggest that GLP binds to a productive enzyme form downstream from the point where S3P binds, and PEP binds to a kinetic intermediate that GLP binds (thus the competitive inhibition). Based on these arguments, a compulsory ordered mechanism is likely in which S3P binds to the enzyme first, followed by binding of PEP. However, a random sequential mechanism cannot be entirely excluded.[10]

Similar inhibition patterns were also observed with the *Escherichia coli* EPSP synthase,[10,11] and the forward reaction had been proposed to

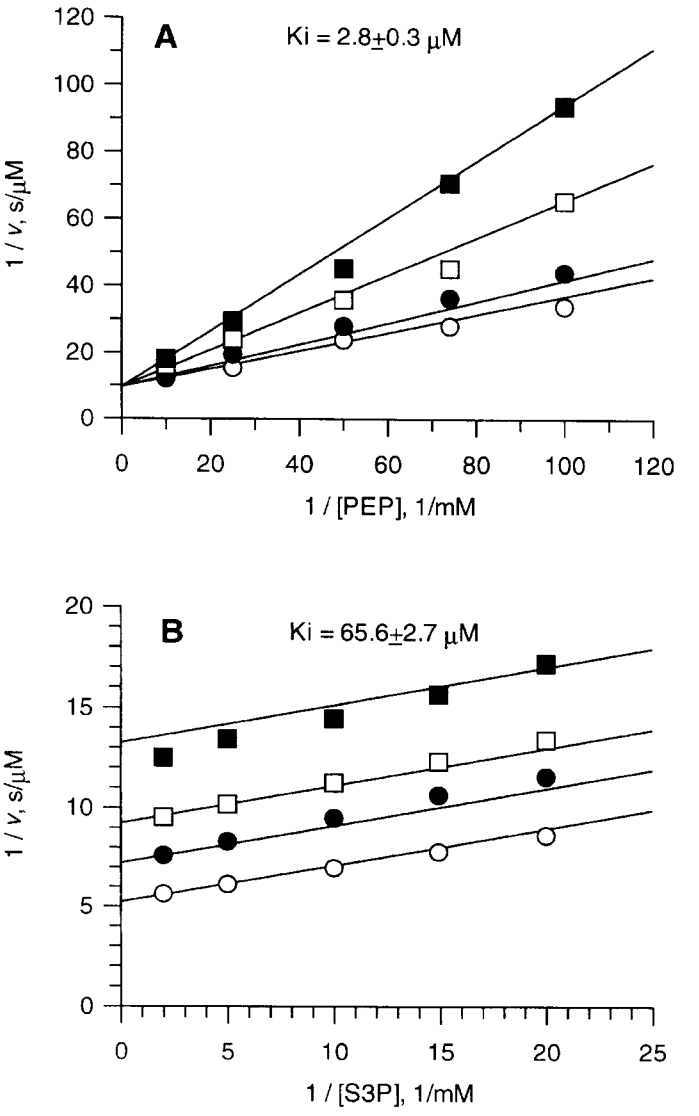


FIGURE 1 Inhibition of EPSP synthase by GLP in the forward reaction. (A) Competitive inhibition versus PEP determined at fixed S3P (1 mM); [GLP] = 0 (○), 0.5 μM (●), 3 μM (□), and 6 μM (■). (B) Uncompetitive inhibition versus S3P determined at fixed PEP (1 mM); [GLP] = 0 (○), 25 μM (●), 50 μM (□), and 100 μM (■).

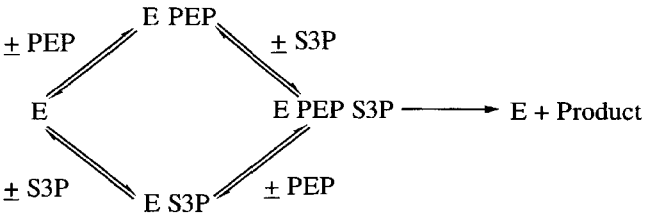
follow an ordered mechanism in which binding of S3P preceeds binding of PEP.[10-12] However, more recent studies have demonstrated that PEP[13] and

PEP analogue inhibitors[14] can bind with fairly good affinity to the free enzyme. These observations obviously contradict the compulsory ordered binding sequence which prevents PEP from binding in the absence of S3P. Interestingly, our isothermal titration calorimetry studies have shown that there exists a strong binding synergy between GLP and S3P (unpublished results), and this may help to explain the uncompetitive inhibition of GLP versus S3P as suggested by Gruys *et al.*[15]

Instead of consuming a large quantity of *S. pneumoniae* EPSP synthase to evaluate whether PEP can bind directly to the free enzyme as reported by Ream *et al.*,[13] we used product inhibition to analyze the binding sequence of the substrates.

Inhibition by EPSP in the Forward Reaction

Since the forward reaction assay is based on following the release of Pi, only EPSP can be used as the product inhibitor. At saturating concentration of the non-varied substrate (1 mM), EPSP was a competitive inhibitor with respect to both PEP ($K_i = 329 \pm 26\ \mu M$, Figure 2A) and S3P ($K_i = 44.0 \pm 2.4\ \mu M$, Figure 2B). This is a classical product inhibition pattern from a bireactant reaction that follows a random sequential mechanism.[16,17] Therefore, the forward reaction of the *S. pneumoniae* EPSP synthase is most likely to follow a random sequential mechanism in which either PEP or S3P binds to the enzyme first to form a binary complex (E•PEP or E•S3P), followed by binding of the other substrate to form a ternary complex (E•PEP•S3P) before any product can be released (Scheme 2). The more than 7-fold difference in the K_i values indicates that EPSP preferably binds to the S3P binding site but does not compete well for the PEP binding site. This is most likely to be due to the structural similarity between EPSP and S3P. The forward reaction mechanism is similar to that of the *E. coli* enzyme as proposed by Ream *et al.* based on their direct binding studies.[13]



SCHEME 2 Proposed mechanism for the forward reaction of *S. pneumoniae* EPSP synthase.

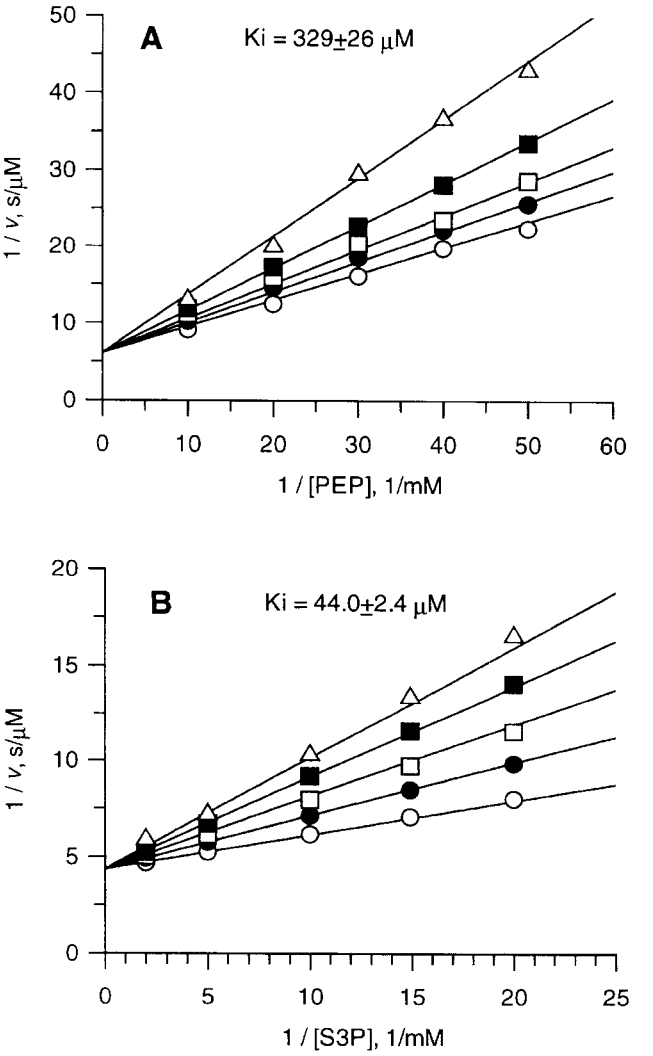


FIGURE 2 Inhibition of EPSP synthase by EPSP in the forward reaction. (A) Competitive inhibition versus PEP determined at fixed S3P (1 mM); [EPSP] = 0 (○), 50 μM (●), 100 μM (□), 200 μM (■), and 400 μM (△). (B) Competitive inhibition versus S3P determined at fixed PEP (1 mM); [EPSP] = 0 (○), 25 μM (●), 50 μM (□), 75 μM (■), and 100 μM (△).

Inhibition by GLP in the Reverse Reaction

The kinetic mechanism of the reverse reaction of *S. pneumoniae* EPSP synthase was analyzed in a similar fashion. GLP was found to be an

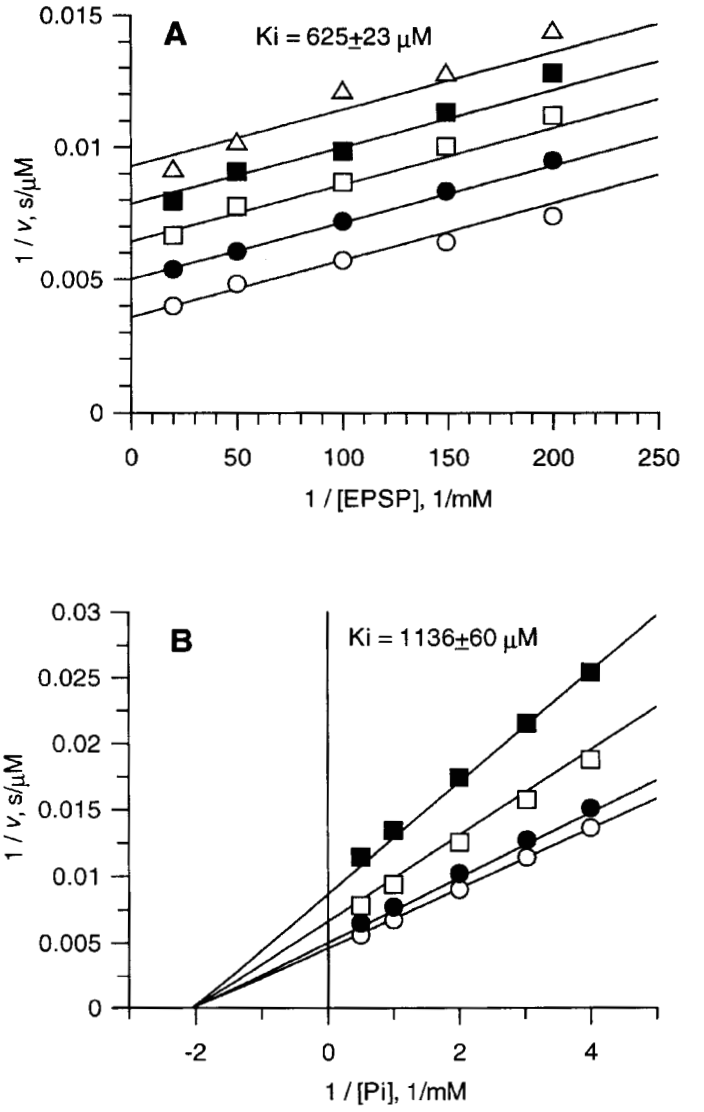


FIGURE 3 Inhibition of EPSP synthase by GLP in the reverse reaction. (A) Uncompetitive inhibition versus EPSP determined at fixed Pi (10 mM); [GLP] = 0 (○), 0.25 mM (●), 0.5 mM (□), 0.75 mM (■), and 1 mM (△). (B) Noncompetitive inhibition versus Pi determined at fixed EPSP (0.5 mM); [GLP] = 0 (○), 0.1 mM (●), 0.5 mM (□), and 1 mM (■).

uncompetitive inhibitor versus EPSP (Figure 3A; Pi at 10 mM) and a noncompetitive inhibitor versus Pi (Figure 3B; EPSP at 0.5 mM). These

results suggest that GLP binds to the enzyme after EPSP binds to form a ternary complex E•EPSP•GLP, but binding of GLP does not preclude Pi from binding, and vice versa. Therefore, a non-productive quaternary complex (E•EPSP•GLP•Pi) may exist as suggested by Sammons *et al.*[18]

Inhibition by S3P in the Reverse Reaction

Product inhibition in the reverse reaction showed that S3P was a competitive inhibitor versus EPSP (Figure 4A, Pi at 10 mM) and an uncompetitive inhibitor versus Pi (Figure 4B, EPSP at 0.5 mM). The competitive inhibition of S3P versus EPSP may be attributed to the structual similarities between these two compounds and it is also consistent with our observation that in the forward reaction, EPSP is a competitive inhibitor versus S3P.

The product inhibition and GLP inhibition patterns in the reverse reaction of *the S. pneumoniae* EPSP synthase are not entirely consistent with those of the *E. coli* enzyme[19] and EPSP synthases from other species. Therefore, although EPSP synthases from different organisms are overall highly homologous.[8] they may adopt unique mechanisms to catalyze the same reactions. Presumably these differences in kinetic mechanism for different EPSP synthases is a result of subtle differences in key amino acid residues at and around the substrate binding site.

Conclusion

We have performed a comprehensive steady-state kinetic study of EPSP synthase from a serious Gram-positive pathogen *S. pneumoniae*. Our data (Table I) support a random sequential mechanism in the forward reaction that is similar to the kinetic mechanism of the *E. coli* enzyme. This detailed

TABLE I Inhibition patterns and kinetic constants of *S. pneumoniae* EPSP synthase

Direction of Reaction	*Inhibitor versus Substrate*	*Mode of Inhibition*[a]	K_i, μM
Forward	GLP vs. PEP	Competitive	2.8±0.3
	GLP vs. S3P	Uncompetitive	65.6±2.7
	EPSP vs. PEP	Competitive	329±26
	EPSP vs. S3P	Competitive	44.0±2.4
Reverse	GLP vs. EPSP	Uncompetitive	625±23
	GLP vs. Pi	Noncompetitive	1136±60
	S3P vs. EPSP	Competitive	12.8±2.1
	S3P vs. Pi	Uncompetitive	4936±158

[a] The mode of inhibition was determined by the associated error and the reduced χ^2 of fitting the data into Equations (1–3). The inhibition patterns represent the best fit of the data to the respective models.

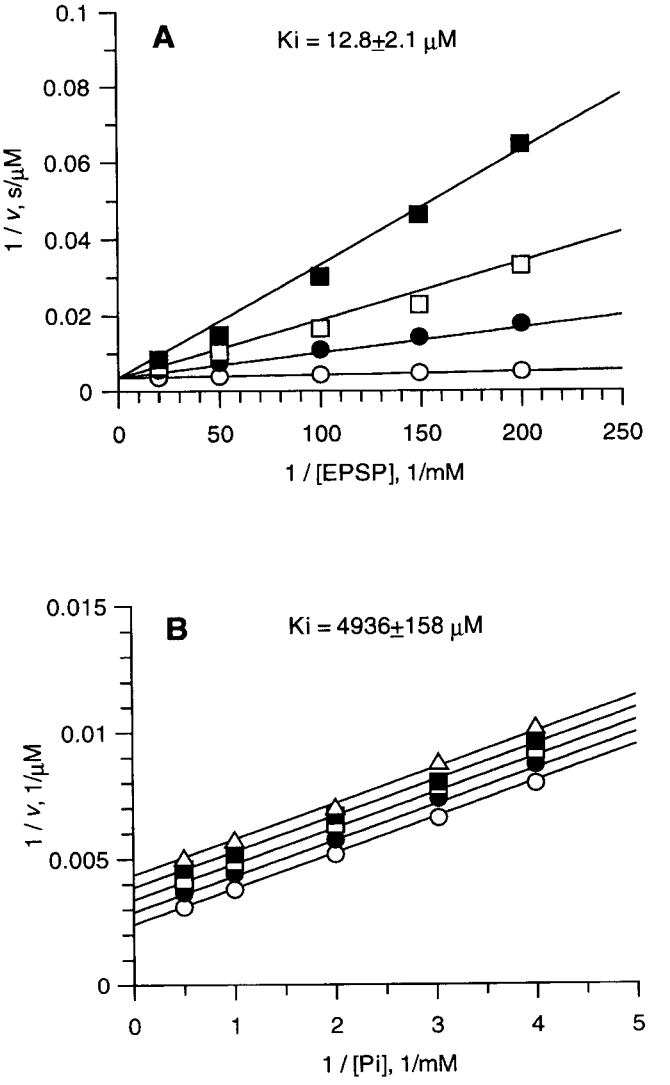


FIGURE 4 Inhibition of EPSP synthase by S3P in the reverse reaction. (A) Competitive inhibition versus EPSP at fixed Pi (10 mM); [S3P] = 0 (○), 0.1 mM (●), 0.25 mM (□), and 0.5 mM (■). (B) Uncompetitive inhibition versus Pi at fixed EPSP (0.5 mM); [S3P] = 0 (○), 1 mM (●), 2 mM (□), 3 mM (■), and 4 mM (△).

analysis of the *S. pneumoniae* EPSP synthase will facilitate the discovery and design of novel inhibitors of EPSP synthase which may have activity in the treatment of multiple resistant Gram-positive pathogens.

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