Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate

Martin R. Boocock and John R. Coggins*

Department of Biochemistry, University of Glasgow, Glasgow, G12 8QQ, Scotland

Received 8 February 1983

The herbicide glyphosate (*N*-phosphonomethyl glycine) is a potent reversible inhibitor of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase activity of the purified *arom* multienzyme complex from *Neurospora crassa*. Inhibition of the EPSP synthase reaction by glyphosate is competitive with respect to phosphoenolpyruvate, with K_i 1.1 μM, and uncompetitive with respect to shikimate-3-phosphate. The kinetic patterns are consistent with a compulsory order sequential mechanism in which either PEP or glyphosate can bind to an enzyme:shikimate-3-phosphate complex.

Shikimate pathway *Glyphosate* *Fosfomycin* *5-Enolpyruvylshikimate-3-phosphate*
Neurospora crassa Arom *enzyme complex*

1. INTRODUCTION

Glyphosate (*N*-phosphonomethyl glycine, 'Roundup') is a successful, broad-spectrum, post-emergence herbicide. It is believed to disrupt aromatic amino acid biosynthesis in plants by reducing metabolic flux in the shikimate pathway (fig.1). In extracts of *Aerobacter aerogenes* and of mung beans EPSP synthase (EC 2.5.1.19) is highly sensitive to inhibition by glyphosate [1,2].

Enzymes catalyzing two earlier steps in the shikimate pathway, a DAHP synthase (EC 4.1.2.15) partially purified from mung beans [3] and DAHP and DHQ synthases (EC 4.6.1.3) present in bacterial extracts [4], can be inhibited by concentrations of glyphosate in the mM range. Many DAHP and DHQ synthases require transition metal cations for activity [5–7], and the inhibition may reflect non-specific metal-chelating properties of glyphosate [8].

The results of elegant mechanistic experiments on partially purified bacterial EPSP synthases [9,10] are consistent with the addition/elimination scheme originally proposed for this reaction [11]. Steady-state kinetics had not been used to examine the sequence of substrate binding steps for any EPSP synthase. We report here on some steady-state kinetic properties of a fungal EPSP synthase (the fifth activity of the pentafunctional *arom* enzyme complex purified to homogeneity from *Neurospora crassa* [12,13] and on its interaction with the herbicide glyphosate.

* To whom correspondence should be addressed

Abbreviations: Bis-tris, bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane; DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate; DHQ, 3-dehydroquinate; EPSP, 5-enolpyruvylshikimate-3-phosphate; FMN, flavin mononucleotide; G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); HK, hexokinase (EC 2.7.1.1); LDH, lactate dehydrogenase (EC 1.1.1.27); PEP, phosphoenolpyruvate; PK, pyruvate kinase (EC 2.7.1.40); UDPGlcNAc, uridine-5′-diphospho-*N*-acetyl-2-amino-2-deoxyglucose

2. MATERIALS AND METHODS

2.1. *Chemicals*

All reagents and coupling enzymes except those listed below were obtained from BDH Chemicals (Poole, Dorset) or from the Boehringer Corp. (London). *bis*–Tris, FMN, cyclohexylammonium

Published by Elsevier Science Publishers
00145793/83/0000–0000/$3.00

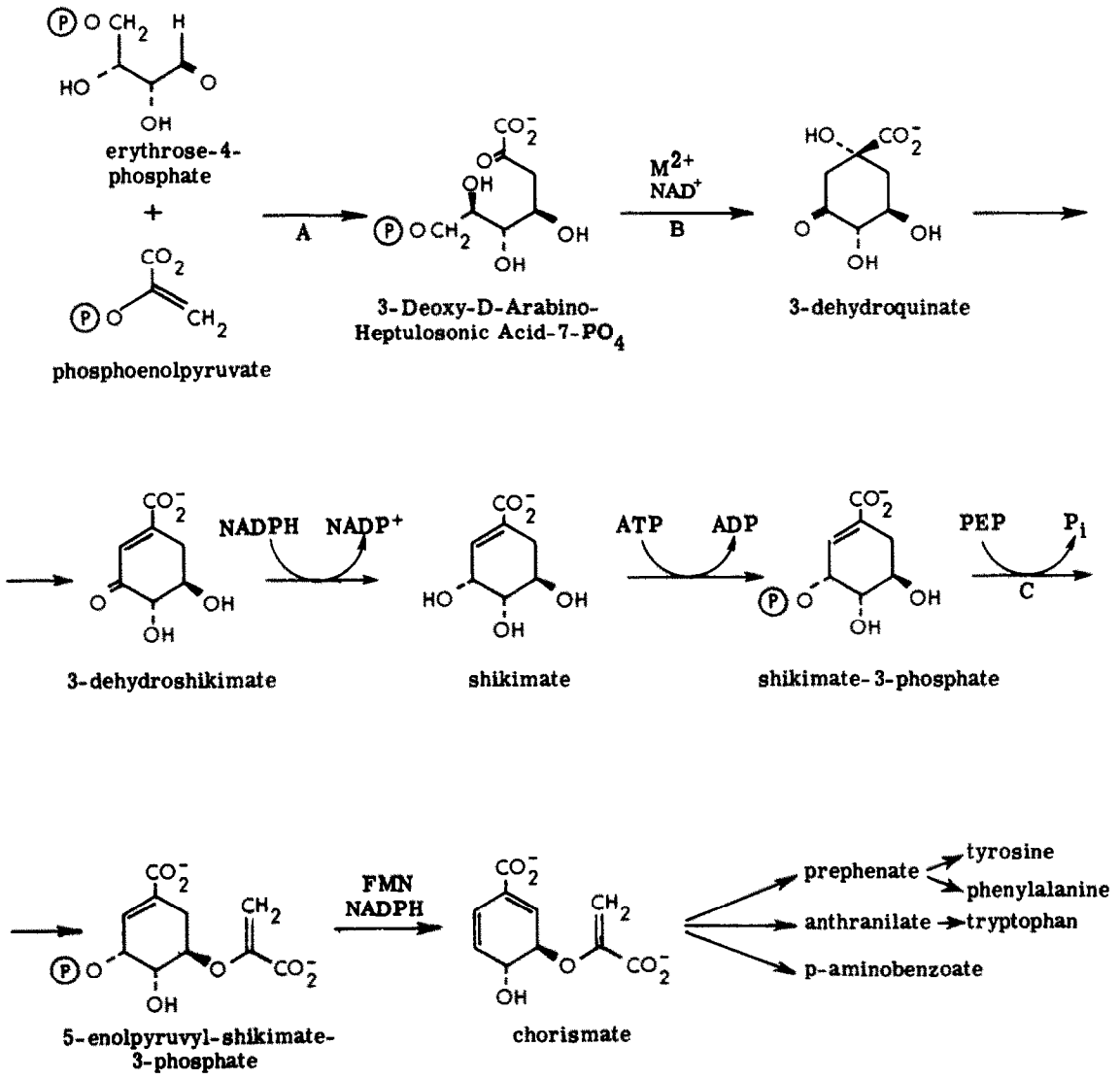

Fig.1. The shikimate pathway in plants and micro-organisms. The lettered steps are those catalysed by enzymes mentioned in the text: (A) DAHP synthase; (B) DHQ synthase; (C) EPSP synthase.

ADP and bovine serum albumin were from Sigma Chemical Co. (Poole, Dorset). Glyphosate was a gift from Dr S. Ridley (Imperial Chemical Industries).

Shikimate-3-phosphate was a gift from Dr G.A. Nimmo. EPSP was synthesized enzymatically using the *arom* complex, isolated as the barium salt [14], and converted to the potassium salt for use in enzyme assays. Chorismic acid was prepared as in [15].

EPSP and PEP solutions were standardized by conversion to lactate using *arom*, PK and LDH, and shikimate-3-phosphate by enzymatic conversion to chorismate ($\epsilon^{1\,cm}_{275\,nm} = 2630$) [15].

2.2. *Enzymes*

The *arom* enzyme complex was purified to homogeneity from *N. crassa*. The phosphocellulose step in our earlier procedure [12] was replaced by chromatography on blue dextran–Sepharose

[submitted]; 2-mercaptoethanol (1.4 mM) was substituted for dithiothreitol in all chromatography buffers, EDTA was omitted from the extraction buffer, the 37°C step was omitted altogether and zinc acetate (10 μM) was added to buffers for the final two stages of purification [in preparation]. The specific activities of the *arom* complex (at 25°C) were 7.04 U/mg (3-dehydroquinase, EC 4.2.1.10), 7.7 U/mg (EPSP synthase, method 3) and 3.4 U/mg (EPSP synthase reverse reaction, 10 mM potassium phosphate in buffer A) ($E^{1\%}_{280\ nm}$ = 11.0 for the *arom* complex [13]).

Chorismate synthase (EC 4.6.1.4) and anthranilate synthase (EC 4.1.3.27) were partially purified from *N. crassa*. The procedure for purification of *arom* was followed, except that the Tris–HCl buffer used for mycelial extraction, the first chromatography step, and for ammonium sulphate fractionation, was replaced by 100 mM potassium phosphate (pH 7.0) [16]. Chorismate synthase and anthranilate synthase were not bound to the blue dextran–Sepharose bed and were separated from each other by chromatography on phosphocellulose [17].

2.3. *Enzyme assays*

EPSP synthase activity was assayed by several methods:

(1) For routine purposes the reverse reaction was followed by coupling release of PEP to the PK and LDH reactions. Oxidation of NADH was monitored at 340 nm. The assay contained 50 μM EPSP (to initiate assay), 2.5 mM ADP, 100 μM NADH, 2.5 mM $MgCl_2$, 100 mM potassium phosphate (pH 7.0), PK (3 U/ml) and LDH (2.5 U/ml). For kinetic studies the reverse assays were conducted in buffer A which contained 2.5 mM $MgCl_2$, 50 mM KCl, 50 mM *bis*-Tris–KOH (pH 7.0). Assays contained 100 μM NADH, 1 mM ADP, substrates and coupling enzymes.

(2) The reverse reaction was also coupled to the PK, HK and G6PDH reactions. NADPH fluorescence was monitored at excitation/emission wavelengths of 340/460 nm using a chart recorder full scale deflection equivalent to 0.5 μM NADPH. The assays contained 2 mM glucose, 50 μM monocyclohexylammonium ADP, 200 μM NADP, substrates and 2 U PK/ml, 17 U HK/ml, 3 U G6PDH/ml in buffer A [18].

(3) For kinetic studies the forward reaction was coupled to chorismate and anthranilate synthases in a continuous fluorimetric assay [19]. The assay contained 10 μM FMN, 50 μM NADPH, 10 mM glutamine, substrates and inhibitors in buffer A, and ~8 mU chorismate synthase/ml and 5 mU anthranilate synthase/ml. Assays were normally initiated by adding PEP, A chart recorder full scale deflection equivalent to 250 nM anthranilate was routinely used, at excitation/emission wavelengths of 315–380 nm.

Chorismate synthase was assayed spectrophotometrically at 275 nm. The assay contained 50 μM EPSP, 20 μM NADPH, 10 μM FMN, 2.5 mM $MgCl_2$, 50 mM KCl, 50 mM triethanolamine·HCl–KOH (pH 7.0).

Anthranilate synthase was assayed fluorimetrically [20]. The assay contained 100 μM chorismate, 10 mM glutamine, 2.5 mM $MgCl_2$, 100 mM potassium phosphate (pH 7.0).

Dehydroquinase was assayed spectrophotometrically [21]. The assay contained 100 μM dehydroquinate in 100 mM potassium phosphate (pH 7.0).

The instruments used throughout were a Gilford-Unicam model 252 spectrophotometer (chart full scale 0.1*A* or 0.05*A*) and a Hitachi-Perkin Elmer model MPF-2A spectrofluorimeter. Fluorimetric assays (3 ml) and spectrophotometric assays (1 ml, 1 cm) were all conducted at 25°C. One unit of activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol substrate/min.

2.4. *Steady state kinetics*

Arom was diluted into buffer A containing 1 mg BSA/ml and 1 mM DTT and kept on ice to ensure stability during the experiments. Coupling enzyme suspensions were dialyzed into buffer A at 4°C and frozen for storage. Partially purified chorismate synthase and anthranilate synthase were stored (−20°C) and used as concentrated solutions in Tris-buffered 50% glycerol. The final glycerol concentration in fluorimetric assays was ~0.5% (v/v).

3. RESULTS AND DISCUSSION

Fig.2 shows the initial velocity patterns obtained when the concentrations of both substrates of EPSP synthase were varied. The primary and secondary plots were all linear within the chosen range of substrate concentrations, and the double-reciprocal plot (fig.2) clearly shows intersecting lines. These patterns are taken to indicate a sequential kinetic mechanism [23]. Limiting K_m-values of 2.7 μM for PEP and 0.36 μM for shikimate-3-phosphate were obtained from the secondary replots.

The kinetic patterns of glyphosate inhibition of the EPSP synthase reaction are shown in fig.3,4. Glyphosate is a linear competitive inhibitor with respect to PEP (fig.2). A K_i for glyphosate of 1.1 μM, and an app. K_m for PEP of 3.5 μM, were obtained under the specified conditions at fixed 177 μM shikimate-3-phosphate.

Glyphosate appears to be a linear uncompetitive inhibitor with respect to shikimate-3-phosphate

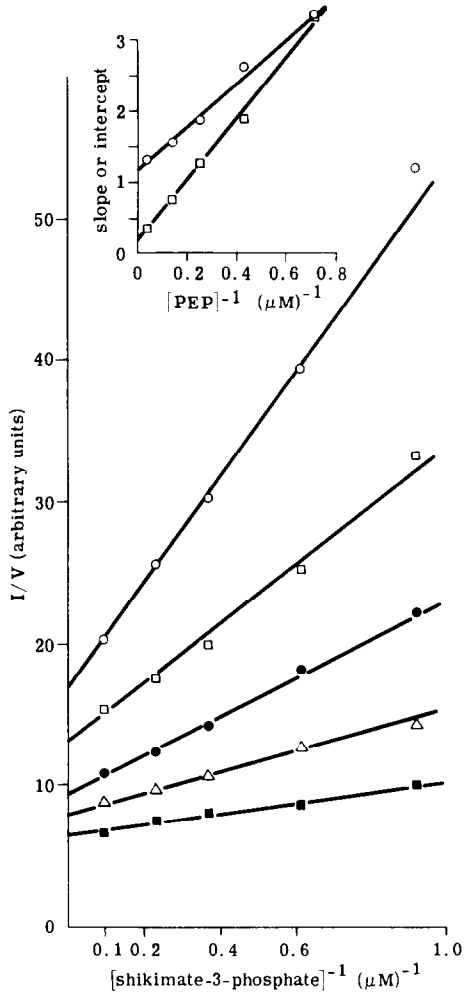

Fig.2. Double reciprocal plot of initial velocity *vs* [shikimate-3-phosphate] at a series of fixed PEP concentrations: (■) 28.7 μM; (△) 7.04 μM; (●) 4.03 μM; (□) 2.35 μM; (○) 1.41 μM. Inset: replot of slopes (□) and intercepts (○) as a function of $[PEP]^{-1}$.

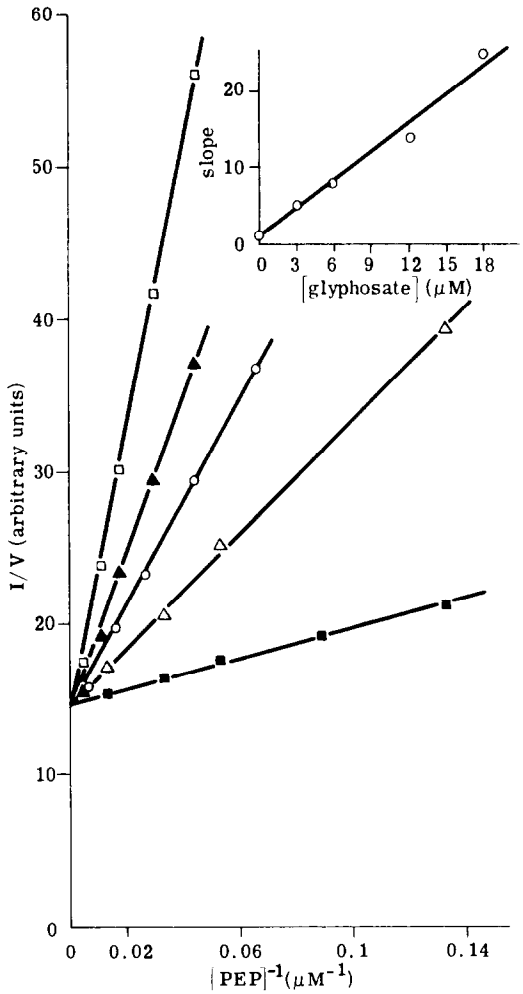

Fig.3. Effect of glyphosate on EPSP synthase: double reciprocal plot of initial velocity *vs* [PEP] at fixed 177 μM shikimate-3-phosphate and glyphosate at: (■) 0; (△) 3 μM; (○) 6 μM; (▲) 12 μM; (□) 18 μM. Inset: replot of slopes as a function of [glyphosate].

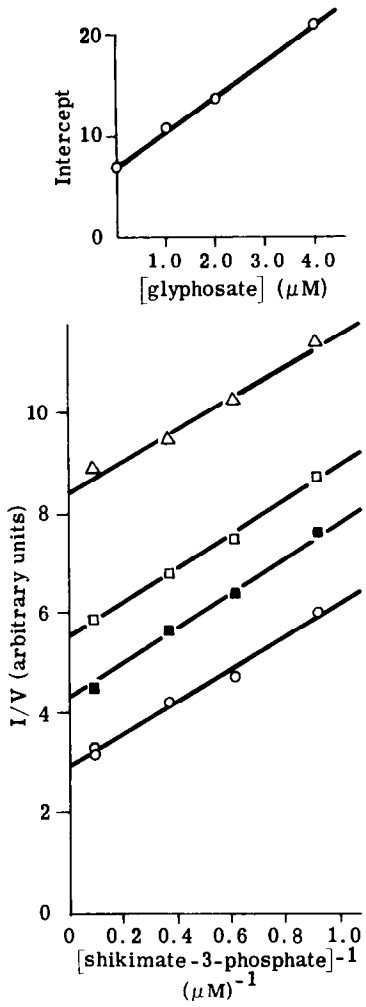

Fig.4. Effect of glyphosate on EPSP synthase: double reciprocal plot of initial velocity *vs* [shikimate-3-phosphate] at fixed 5.63 μM PEP and glyphosate at: (○) 0; (■) 1 μM; (□) 2 μM; (△) 4 μM. Inset: replot of intercepts as a function of [glyphosate].

(fig.4). This result indicates that glyphosate binds only to enzyme:substrate complexes downstream in the kinetic sequence from the point(s) where shikimate-3-phosphate binds to the enzyme. Since glyphosate must inhibit by binding to kinetic intermediates that can also bind PEP productively, it appears that PEP and glyphosate must compete for binding to an enzyme:shikimate-3-phosphate complex.

The inhibitory effects of glyphosate on the reverse reaction of EPSP synthase are shown in fig.5. Glyphosate is a non-competitive inhibitor [23] with respect to phosphate, but much higher concentrations of glyphosate are required for significant inhibitory effects on the reverse reaction. A K_i (slope) of 33 μM and a K_i (intercept) of 82 μM were estimated at a fixed 20 μM EPSP. Glyphosate is not a competitive inhibitor with respect to EPSP and >20 μM EPSP gave no significant relief of glyphosate inhibition (not shown). An app. K_m for EPSP of 0.25 μM was estimated for the reverse reaction at fixed 1.0 mM phosphate [not shown].

The kinetic properties of the reverse reaction are in accord with our proposal that glyphosate binds to an enzyme:shikimate-3-phosphate complex, and cannot bind to the free enzyme. The pronounced 'slope' component of glyphosate inhibition with respect to phosphate is readily explained

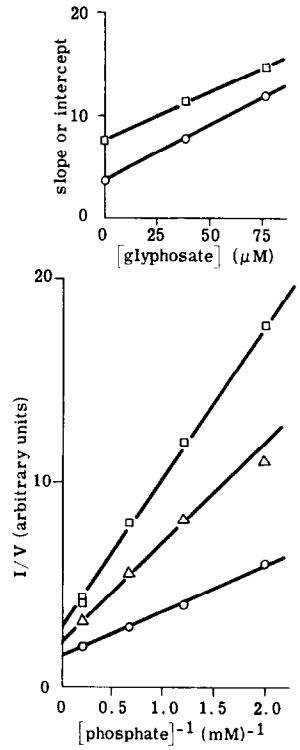

Fig.5. Effect of glyphosate on the reverse reaction of EPSP synthase: double reciprocal plot of initial velocity *vs* [phosphate] at fixed 20 μM EPSP and glyphosate fixed at: (○) 0; (△) 38.5 μM; (□) 77 μM. Inset: replot of primary slopes (○) and intercepts (□) as a function of [glyphosate].

if phosphate can bind to the enzyme:shikimate-3-phosphate complex in the reverse reaction. It is then not necessary to postulate binding of glyphosate to any other kinetic intermediate.

Our evidence indicates that the inhibitory effects of glyphosate on EPSP synthase are due to a specific and reversible interaction between glyphosate and an enzyme:shikimate-3-phosphate complex, leading to exclusion of productive binding of PEP. Glyphosate therefore shows the behaviour expected of a PEP analogue. This surprising finding is difficult to rationalize on structural grounds and it should be emphasised that glyphosate does not inhibit all PEP-utilizing enzymes. For example, competition between glyphosate and PEP has not been observed with rabbit muscle pyruvate kinase (EC 2.7.1.40) nor with the tryptophan-sensitive DAHP synthase [22] of *N. crassa* [unpublished].

The formation of a dead-end complex between glyphosate and the EPSP synthase:shikimate-3-phosphate complex suggests a rationale for the potent effects of glyphosate on plants. In particular, the accumulation of shikimate pathway intermediates would not be expected to relieve glyphosate inhibition of EPSP synthase.

The inability of glyphosate to bind to the free enzyme raises the possibility that binding of substrates also follows a compulsory order. The kinetic patterns discussed above are fully consistent with an ordered sequential mechanism in which binding of shikimate-3-phosphate precedes binding of PEP [23]. Preliminary results from product inhibition studies are consistent with this model; phospohate was found to be a non-competitive inhibitor with respect to PEP in the forward reaction, while shikimate-3-phosphate was found to be a competitive inhibitor with respect to EPSP in the reverse reaction [not shown]. However the kinetic patterns presented here do not rule out a random sequential mechanism. Further product inhibition studies and an analysis of the behaviour of arsenate as a pseudo-substrate for the reverse reaction are being conducted in an attempt to gain further evidence for ordered substrate binding.

UDPGlcNAc pyruvyl transferase (EC 2.5.1.7) catalyzes a reaction that is closely analogous to that of EPSP synthase [24–26], and is the target enzyme for the antibiotic fosfomycin. There is good evidence that in two species of bacteria the transferase follows an ordered sequential mechanism, and that UDPGlcNAc must bind to the enzyme before it can be inactivated by the presumed PEP-analogue fosfomycin [24,26]. This behaviour provides a striking parallel to the reversible inhibition of EPSP synthase by glyphosate.

The catalytic intermediates implicated in the UDPGlcNAc pyruvyl transferase reaction are quite different from those encountered in an addition/elimination mechanism of the type proposed for EPSP synthase [11]. Our study of the kinetic properties of the *N. crassa* EPSP synthase does not rule out a chemical mechanism of the type proposed for UDPGlcNAc pyruvyl transferase [25]. A closer examination of the extent of the resemblance between these two enzymes would therefore be of great interest.

ACKNOWLEDGEMENTS

This study was supported by the Science and Engineering Research Council (London). We are grateful to Drs H.G. and G.A. Nimmo for helpful discussions and advice and to Miss A.A. Coia for the preparation of some of the coupling enzymes.

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