Characterization of a glyphosate-insensitive 5-enolpyruvylshikimic acid-3-phosphate synthase

Dietrich Sost, Arno Schulz and Nikolaus Amrhein*

Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, D-4630 Bochum, FRG

Received 17 May 1984

A glyphosate (*N*-[phosphonomethyl]glycine)-insensitive 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase has been purified from a strain of *Klebsiella pneumoniae* which is resistant to this herbicide [(1984) Arch. Microbiol. 137, 121–123] and its properties compared with those of the glyphosate-sensitive EPSP synthase of the parent strain. The apparent K_m values of the insensitive enzyme for phospho*enol*pyruvate (PEP) and shikimate 3-phosphate (S-3-P) were increased 15.6- and 4.3-fold, respectively, as compared to those of the sensitive enzyme, and significant differences were found for the optimal pH and temperature, as well as the isoelectric points of the two enzymes. While PEP protected both enzymes against inactivation by *N*-ethylmaleimide, 3-bromopyruvate, and phenylglyoxal, glyphosate protected only the sensitive enzyme.

Glyphosate *5-Enolpyruvylshikimic acid-3-phosphate synthase* *Enzyme inhibition* *Resistance*

1. INTRODUCTION

The broad-spectrum, non-selective herbicide glyphosate (*N*-[phosphonomethyl]glycine) is a potent and specific inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase (3-phosphoshikimate-1-carboxyvinyltransferase, EC 2.5.1.19) of both plant and microbial origin [1–7]. Inhibition of EPSP synthase by glyphosate is reversible and competitive with respect to PEP [2–7], and it has been proposed that glyphosate may act as a transition-state analogue of PEP ([2,5]; Steinrücken and Amrhein, submitted). Resistance to glyphosate in bacteria and a plant tissue culture was found to be correlated with either an increased production of glyphosate-sensitive EPSP synthase [7–9] or the production of a glyphosate-insensitive EPSP synthase [10,11]. We showed in [11] that EPSP synthase activity in a crude extract from a glyphosate-resistant strain of *Klebsiella pneumoniae* (= *Aerobacter aerogenes*) was no longer subject to inhibition by glyphosate (50 mM) and had antigenic determinants similar to but not identical with those of the glyphosate-sensitive EPSP synthase of the wild type. We have now compared some physical and kinetic properties of the purified glyphosate-sensitive and -insensitive EPSP synthases.

* To whom correspondence should be addressed

Abbreviations: DEAE, diethylaminoethyl; EPSP, 5-enolpyruvylshikimate 3-phosphate; PEP, phospho*enol*pyruvate; S-3-P, shikimate 3-phosphate

2. MATERIALS AND METHODS

Sources and cultivation of the glyphosate-sensitive and -insensitive strains of *K. pneumoniae* (*A. aerogenes*) have been described [9,11]. The protocol in ([2]; Steinrücken and Amrhein, submitted) was followed for the extraction, purification and assay of EPSP synthases of both strains. To elute the glyphosate-insensitive enzyme from the DEAE–cellulose column, a 0–0.3 M KCl gradient, rather than the standard 0–0.2 M KCl gradient [2], had to be used. References to SDS-gel electrophoresis and isoelectric focusing are also given in ([2]; Steinrücken and Amrhein, submitted). Electrophoresis under non-denaturing conditions was performed in gel system no.5 of [12] in

Published by Elsevier Science Publishers B.V.

the presence of 5 mM β-mercaptoethanol, and EPSP synthase activity was located as in [13], modified as follows: gels were preincubated for 15 min at 37°C in 0.1 M Tris–HCl buffer (pH 7.0) containing 0.1 M $CaCl_2$ and 5 mM β-mercaptoethanol, and then incubated overnight in the same solution containing, in addition, 2 mM S-3-P and 4 mM PEP.

3. RESULTS

As previously described for the glyphosate-sensitive EPSP synthase of *K. pneumoniae* ([2]; Steinrücken and Amrhein, submitted), the glyphosate-insensitive EPSP synthase was purified to apparent homogeneity by ammonium sulfate fractionation, heat precipitation, consecutive chromatography on DEAE–cellulose, Sephadex G-75, and cellulose–phosphate, and finally by chromatofocusing. The homogenous preparation had a specific activity of 181 nkat·mg^{-1} protein corresponding to a 370-fold purification with an overall yield of 12%. In comparison, the wild-type enzyme had been purified over 3000-fold with a specific activity of 630 nkat·mg^{-1} protein and a 49% recovery [2]. The less successful purification of the glyphosate-insensitive enzyme indicates differences in the properties of the two enzymes and, in particular, a greater instability of the purified glyphosate-insensitive enzyme. This is clearly shown by fig.1, in which the enzymatic activities of the two preparations have been visualized after electrophoresis in polyacrylamide gels under non-denaturing conditions. Even though the same amount of enzymatic activity of each preparation had been applied to the gel, the stain for EPSP synthase activity is much more intense in the case of the glyphosate-sensitive enzyme (lane 4) as compared to the glyphosate-insensitive enzyme (lane 3). It is also evident from fig.1 that, in the presence of 10 mM glyphosate, the wild-type enzyme is completely inhibited (lane 2), while the intensity of the stain for EPSP synthase activity of the glyphosate-insensitive enzyme is not diminished (lane 1). Fig.1 yields the further information that the glyphosate-sensitive enzyme is more acidic than the glyphosate-insensitive enzyme, since the former has migrated farther towards the anode than the latter. This behaviour during electrophoresis is in agreement with the observation

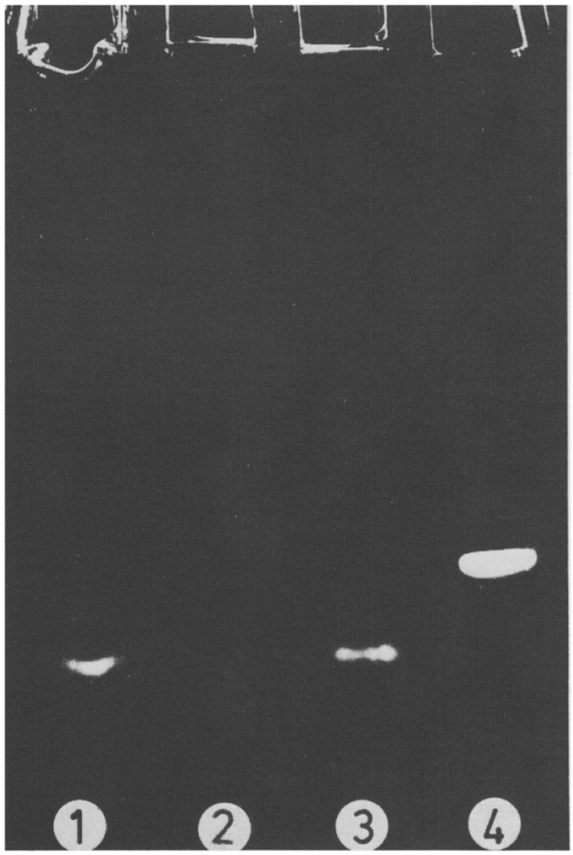


Fig.1. Detection of EPSP synthase activity, after electrophoresis on polyacrylamide gels under non-denaturing conditions, with the activity stain of [13]. Lanes: (1,3) glyphosate-insensitive enzyme; (2,4) glyphosate-sensitive enzyme. Lanes 1,2: staining solution contained 10 mM glyphosate. To each well, 0.5 nkat enzyme activity had been applied. Electrophoresis was at 125 V for 6 h at 4°C. Anode at bottom.

made during the purification of the two proteins that the glyphosate-sensitive enzyme was eluted by lower salt concentrations (17 mS conductivity of buffer at 4°C) from the DEAE–cellulose column than the glyphosate-insensitive enzyme (24 mS). Finally, the isoelectric points of the two proteins determined by isoelectric focusing were 4.6 for the glyphosate-sensitive and 4.1 for the glyphosate-insensitive enzyme, respectively (table 1). Table 1 lists further differences between the two enzymes:

The M_r of the glyphosate-sensitive enzyme had previously been determined by SDS gel electrophoresis to be 42900 ± 700 [2], and an M_r of

Table 1

Characteristics of glyphosate-sensitive and -insensitive EPSP synthases

Property	Glyphosate-sensitive enzyme	Glyphosate-insensitive enzyme
Apparent M_r (SDS-gel electrophoresis)	42500	43000
Isoelectric point (pH)	4.6	4.1
pH optimum in 50 mM Tris–maleate buffer	5.4 and 7.0[a]	6.0 and 7.3
Temperature maximum (°C)	60[a]	40
Apparent K_m values[b] (μM)		
PEP	9	140
S-3-P	45	193
Protection against inactivation by group-specific reagents provided by[c]		
PEP	yes	yes
Glyphosate	yes	no

[a] Data from [2]
[b] Data from fig.2.
[c] On the basis of data in table 2

42500 ± 200 was found here. For the glyphosate-insensitive enzyme, an M_r of 43000 ± 100 was determined (3 replicate runs). Even though M_r values varied slightly between individual gels, the higher apparent M_r of the glyphosate-insensitive enzyme was always observed when the two proteins were subjected to electrophoresis on the same gel. These differences in the apparent M_r of the two proteins were also persistently found when crude extracts of the glyphosate-sensitive and -resistant strains of *K. pneumoniae* were subjected to SDS gel-electrophoresis and EPSP synthases were located by immunological techniques after transfer to nitrocellulose (unpublished).

For the glyphosate-sensitive EPSP synthase two pH-dependent maxima of the enzymatic activity had been found: one at pH 5.4 and the second at pH 6.8 (forward reaction) [2]. Two pH-dependent maxima of activity were also found for the glyphosate-insensitive enzyme; they were, however, shifted to the more alkaline range (pH 6.0 and 7.3, respectively).

A very pronounced difference was found for the influence of temperature on the rate of the reaction of the two enzymes: the velocity of the reaction catalyzed by the glyphosate-sensitive enzyme increased up to 60°C [2], while the glyphosate-insensitive enzyme was rapidly inactivated at temperatures exceeding 40°C.

As previously found with the glyphosate-sensitive enzyme, double-reciprocal plots of initial velocities of the glyphosate-insensitive enzyme in the forward reaction with either of the substrates at constant concentration, and the other as variable substrate, yielded concave curves for concentrations exceeding 1 mM when the concentration of the constant substrate was fixed at 5 mM (not shown). In fig.2 the initial velocity data from the linear range of the double-reciprocal plots have been plotted according to [14]. Apparent K_m values for the two enzymes, determined as the intercepts of the resulting lines with the x-axis, are listed in table 1. It is evident that the glyphosate-insensitive enzyme has a reduced affinity for both of its substrates, the reduction of its affinity for PEP being, however, more pronounced.

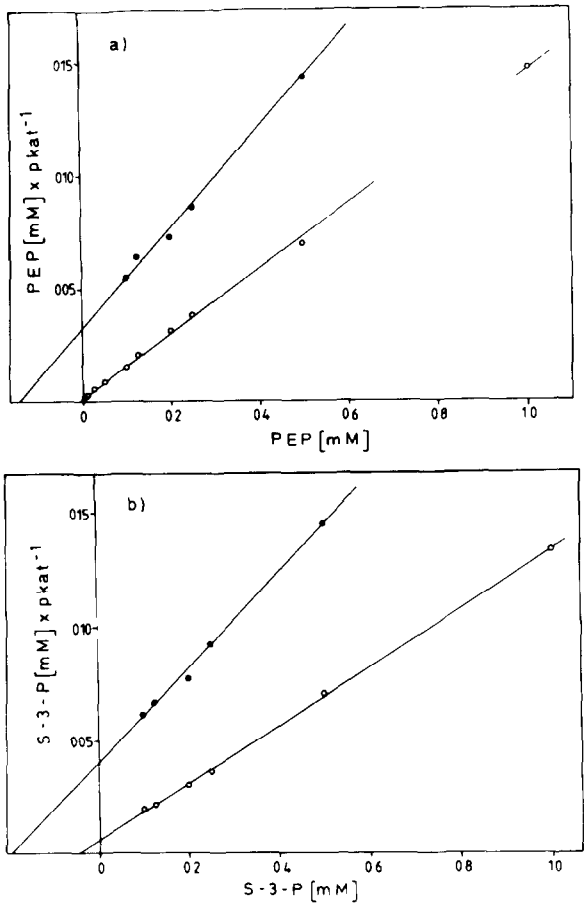
Contrary to the conclusions derived from kinetic studies with *Neurospora crassa* EPSP synthase [4], an affinity of *K. pneumoniae* EPSP synthase for glyphosate in the absence of S-3-P had previously been inferred from the protection which the inhibitor provided against the time-dependent inactivation of glyphosate-sensitive EPSP synthase by the group-specific reagents phenylglyoxal, 3-bromopyruvate and *N*-ethylmaleimide [2]. It was therefore of interest to determine whether the insensitivity of the variant enzyme to glyphosate was correlated with a loss of protection by glyphosate against the inactivating reagents. Table 2 shows that, indeed, the rate of inactivation of glyphosate-

Table 2

Time-dependent inactivation of glyphosate-sensitive and -insensitive EPSP synthases by group-specific reagents in the absence and presence of PEP or glyphosate[a]

Reagent	Substrate or inhibitor (1 mM)	$t_{1/2}$ (min) (time required for 50% inactivation)	
		Glyphosate-sensitive enzyme	Glyphosate-insensitive enzyme
Phenylglyoxal (2 mM)	None	32	26
	PEP	94	350
	Glyphosate	94	27
3-Bromopyruvate (1 mM)	None	3.4	3.5
	PEP	10.9	19.7
	Glyphosate	122	3.5
N-Ethylmaleimide (1 mM)	None	~3	1.3
	PEP	73	16.5
	Glyphosate	25	1.3

[a] For experimental details see ([2]; Steinrücken and Amrhein, submitted)



insensitive EPSP synthase by either of the 3 reagents was not reduced by glyphosate indicating that glyphosate does not bind to the site(s) of the enzyme which is (are) sensitive to the 3 reagents. PEP, on the other hand, protects both enzymes. The more efficient protection of the glyphosate-insensitive enzyme against phenylglyoxal inactivation by PEP remains unexplained considering the higher K_m value of this enzyme for PEP (table 1).

4. DISCUSSION

The glyphosate-insensitive EPSP synthase had been isolated from a *K. pneumoniae* strain capable of growth in the presence of 50 mM glyphosate [11]. Because of the probable nature of glyphosate as a transition state analogue of PEP in the reaction catalyzed by EPSP synthase ([2,4]; Steinrücken and Amrhein, submitted) one would expect that the glyphosate-insensitive enzyme has a

Fig.2. Hanes plots of initial velocities of EPSP synthases with fixed and variable substrates. (a) PEP concentration variable; (b) S-3-P concentration variable. Concentration of respective fixed substrate was 5 mM. (○—○) Glyphosate-sensitive enzyme; (●—●) glyphosate-insensitive enzyme.

reduced affinity for PEP, and the experimental data (fig.2, table 1) confirms this prediction : the apparent K_m value of the glyphosate-insensitive enzyme is increased 15.6-fold over that of the glyphosate-sensitive enzyme (S-3-P concentration fixed at 5 mM). A similar, but less pronounced effect is observed for the K_m value for S-3-P (4.3-fold increase; fig.2, table 1). In addition, the inactivation experiments (table 2) allow one to conclude that glyphosate does not bind to the site(s) of the glyphosate-insensitive enzyme which are modified by the group-specific reagents, or, presumably, that it does not bind at all to the enzyme. Further pronounced differences between the two enzymes were found for the isoelectric point, pH optima, temperature maximum, and stability during electrophoresis (table 1, fig.1). Thus, the introduction of a desired character into EPSP synthase (glyphosate-insensitivity) has reduced the general performance of the enzyme. The physical and kinetic properties of the only other presently known glyphosate-insensitive EPSP synthase (from *Salmonella typhimurium* [10]) have not been reported, but nucleotide sequence analysis of the cloned genes, both for the glyphosate-sensitive and -insensitive *S. typhimurium* EPSP synthases [15], has revealed that the difference between the two enzymes is due to a single amino acid substitution (Pro in position 101 of the putative reading frame of the gene encoding the wild-type enzyme exchanged for Ser in the gene encoding the mutant enzyme).

The more acidic nature of the *K. pneumoniae* glyphosate-insensitive EPSP synthase (table 1) might reflect at least 3 possible changes in the enzyme as compared to the glyphosate-sensitive EPSP synthase: (i) exposure of more acidic residues due to a change in the conformation; (ii) exchange of neutral or basic amino acid(s) for acidic amino acids; (iii) insertion of acidic amino acid(s). The latter possibility is supported by the increased apparent M_r of the glyphosate-insensitive EPSP synthase as revealed by SDS-gel electrophoresis (table 1). It cannot be excluded, however, that the different electrophoretic mobilities of the two proteins in SDS-gels are not related to changes in their M_r. While the acquisition of glyphosate resistance has resulted in the partial loss of some desirable properties of EPSP synthase (stability, affinity for substrates), our results nevertheless encourage efforts aimed at the generation of plants possessing a glyphosate-resistant EPSP synthase.

NOTE ADDED IN PROOF

Work cited in the text as Steinrücken and Amrhein, submitted has since been accepted for publication in Eur. J. Biochem.

ACKNOWLEDGEMENTS

Support of this work by the Deutsche Forschungsgemeinschaft, Bonn, and Monsanto Agricultural Products Co., St. Louis, MO, is gratefully acknowledged.

REFERENCES

[1] Steinrücken, H.C. and Amrhein, N. (1980) Biochem. Biophys. Res. Commun. 94, 1207–1212.
[2] Steinrücken, H.C. (1982) Doctoral Dissertation, Ruhr-Universität, Bochum.
[3] Amrhein, N., Holländer-Czytko, H., Leifeld, J., Schulz, A., Steinrücken, H.C. and Topp, H. (1982) Groupe Polyphenols. Journées Internationales d'Études et Assemblées Générales (Boudet, A.M. and Ranjeva, R. eds) Bulletin de Liason, vol.II, pp.21–30.
[4] Boocock, M.R. and Coggins, J.R. (1983) FEBS Lett. 154, 127–133.
[5] Anton, D.L., Hedstrom, L., Fish, S.M. and Abeles, R.H. (1983) Biochemistry 22, 5903–5908.
[6] Mousdale, D.M. and Coggins, J.R. (1984) Planta 160, 78–83.
[7] Duncan, K., Lewendon, A. and Coggins, J.R. (1984) FEBS Lett. 165, 121–127.
[8] Rogers, S.G., Brand, L.A., Holder, S.B., Sharp, E.S. and Brackin, M.J. (1983) Appl. Environ. Microbiol. 46, 37–43.
[9] Amrhein, N., Johänning, D., Schab, J. and Schulz, A. (1983) FEBS Lett. 157, 191–196.
[10] Comai, L., Sen, L.C. and Stalker, D.M. (1983) Science 221, 370–371.
[11] Schulz, A., Sost, D. and Amrhein, N. (1984) Arch. Microbiol. 137, 121–123.
[12] Maurer, H.R. (1968) Diselectrophorese, p.42, De Gruyter, Berlin.
[13] Nimmo, H.G. and Nimmo, G.A. (1982) Anal. Biochem. 121, 17–22.
[14] Hanes, C.S. (1932) Biochem. J. 1406–1421.
[15] Stalker, D., Hiatt, W. and Comai, L. (1984) Plant Molecular Biology, 11th Aharon Katzir-Katchalsky Conference, Jerusalem, January 8-12, 1984, Abstract D 10.