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THE ANOMALOUS INHIBITION OF SHIKIMATE DEHYDROGENASE BY ANALOGUES OF DEHYDROSHIKIMATE

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SUMMARY

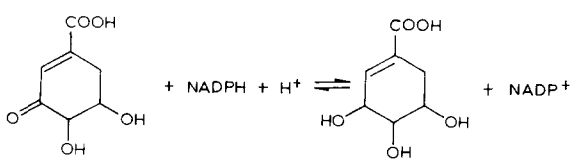
Shikimate dehydrogenase (shikimate:$NADP^+$ oxidoreductase, EC 1.1.1.25) has been extracted and partially purified from etiolated pea epicotyls. In order to explain anomalous inhibition results by dehydroshikimate analogues the enzyme kinetic mechanism has been studied in both directions.

The initial velocity pattern in the absence of products, and the product inhibition pattern, have been determined. These are consistent with an ordered mechanism which has a kinetically significant ternary complex, and in which $NADP^+$ or NADPH bind to the free enzyme.

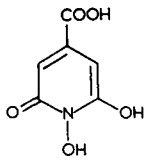
However, the dissociation constants determined for the coenzymes differ from estimates of the same constants obtained by studying the coenzymes as product inhibitors and some of the Dixon plots exhibit non-linearity. These effects may be related to the formation of unreactive ternary complexes. Such a scheme could explain the analogue inhibition data.

INTRODUCTION

Shikimate dehydrogenase (shikimate:$NADP^+$ oxidoreductase, EC 1.1.1.25) is a key enzyme in the synthesis of many aromatic compounds by plants and microorganisms. It catalyses the reduction of dehydroshikimate to shikimate:



There is no evidence for the existence of the enzyme in mammalian systems, and it has been isolated and partially purified from a number of plant sources[1,2]. It was thus considered to be a desirable site for the rational design of novel herbicides.

1,6-Dihydroxy-2-oxoisonicotinic acid 

, a novel dehydro-

shikimate analogue synthesized as a potential herbicide, proved to be a competitive inhibitor when the enzyme was assayed in the direction of dehydroshikimate formation (K_i 0.12 mM), but in the reverse direction showed no inhibition, even at very low dehydroshikimate concentrations (Fig. 1). This differential inhibition was particularly surprising as the structure resembles dehydroshikimate more than it does shikimate. Other compounds in the same series also exhibited this anomalous behaviour[3].

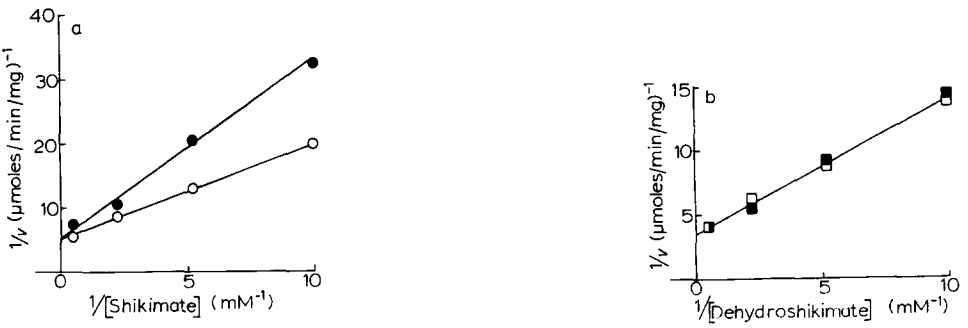


Fig. 1. Inhibition of shikimate dehydrogenase by 1,6-dihydroxy-2-oxoisonicotinic acid, at pH 8.0, 25 °C. (a) Assayed in the direction of dehydroshikimate formation. Inhibitor at zero (○) and 0.1 mM (●); $NADP^+$ at 0.167 mM. (b) Assayed in the direction of shikimate formation. Inhibitor at zero (□) and 0.1 mM (■); NADPH at 0.0835 mM.

A kinetic investigation of the reaction mechanism was therefore carried out to try to explain these results. Since this work was completed, some kinetic studies on the enzyme have been described[4] but under different conditions, so that differences in conclusions have emerged.

EXPERIMENTAL

Materials

These were obtained from the usual laboratory suppliers, except for dehydroshikimate which was synthesized[5] and the inhibitors which were devised by P. McCloskey and synthesized by A. C. Baillie at Chesterford Park.

Purification of enzyme

All manipulations were carried out at 2–4 °C; centrifugation was at 25 000 × *g* for 10 min.

500 g of etiolated pea epicotyls (*Pisum sativum* var Onwards) harvested 7–14 days after emergence were extracted in a Warburg Blendor with an equal volume of 0.1 M KH_2PO_4 adjusted to pH 7.0 with 2 M KOH. The extract was then purified using $(NH_4)_2SO_4$ precipitation, heat denaturation and ammonium citrate precipitation[6]. The final citrate precipitate was dissolved in 10 ml of 0.025 M KH_2PO_4 buffer adjusted to

pH 7.0 with 2 M KOH, and applied to a 75 cm × 2.5 cm column of Sephadex G-150 equilibrated with the same buffer. The proteins were eluted from the column by upward displacement using the buffer at a flow rate of 15–20 ml/h, and 10-ml fractions were collected.

Next day the most active fractions were pooled and applied to a 15 cm × 1.5 cm column of Whatman DE-52 cellulose equilibrated with the 0.025 M buffer. The enzyme was eluted from the column using 120 ml of pH 7.0 potassium phosphate buffer in a linear concentration gradient from 0.025–0.1 M at a flow rate of 50 ml/h. 5 ml fractions were collected and the most active were pooled and concentrated to 4 ml using a colloidon bag under suction from a water pump. The results of a typical purification are shown in Table I.

TABLE I

PURIFICATION OF SHIKIMATE DEHYDROGENASE FROM ETIOLATED PEA EPICOTYLS

One enzyme unit is the amount of enzyme converting 1 μmole shikimate·min^{-1} at 25 °C, assayed using 6 μmoles sodium shikimate and 0.5 μmole of $NADP^+$ in 3 ml 0.1 M Bicine buffer (pH 9.0).

Fraction	*Vol. (ml)*	*Activity (units·ml^{-1})*	*Total units*	*Protein (mg·ml^{-1})*	*Spec. act. (units·mg^{-1})*	*Recovery (%)*	*Purification (-fold)*
Original homogenate	900	0.0795	71.5	14.2	0.00559	100	1.0
35–55% satd $(NH_4)_2SO_4$	100	0.432	43.2	21.0	0.0205	60.4	3.7
Supernatant after 1 min at 55 °C	94	0.314	29.5	10.5	0.0299	41.3	5.3
30–50% (w/v) ammonium citrate	8	1.68	13.4	37.2	0.0451	18.8	8.1
Eluant from Sephadex G-150	83	0.165	13.7	0.76	0.217	19.2	38.8
Eluant from DE-52 cellulose	20	0.410	8.20	0.18	2.28	11.5	408
After concentration by vacuum dialysis	4	1.91	7.65	0.85	2.25	10.6	403

Unfortunately the final extract was very unstable (30% loss after 24 h at −15 °C) and for subsequent purifications the active fractions from the Sephadex column were pooled and concentrated to 20 ml by vacuum dialysis. This preparation, with a specific activity of between 0.2 and 0.4 units·mg^{-1} protein, was used for the kinetic studies. The instability of this enzyme preparation has been ascribed to the presence of proteolytic enzymes, which seem to be removed by chromatography on calcium phosphate gel[4].

Enzyme assay

The basic assay was an adaptation of the method described by Balinsky and Davies[6]. Into a quartz cuvette of 1-cm light path were pipetted 6 μmoles of sodium shikimate, 0.5 μmole of $NADP^+$ and sufficient 0.1 M *N*,*N*-bis-(2-hydroxyethyl)glycine (Bicine) buffer adjusted to pH 9.0 with 2 M NaOH to give a final volume of 3 ml. Enzyme solution (25–100 μl) was added to give ΔA of 0.01–0.1 min^{-1} and the increase in absorption at 340 nm was measured at 25 °C using an SP 800 spectrophotometer.

An enzyme unit is defined as the amount catalysing the conversion of 1 μmole shikimate·min^{-1} under the standard assay conditions.

Protein concentrations were determined by the method of Layne[7].

Kinetic data

For the comparison of inhibition in the forward and reverse directions by the dehydroshikimate analogues the enzyme assay was carried out at pH 8.0 in 0.1 M sodium pyrophosphate. At this pH approximately equal maximum velocities were obtained in either direction. Assays were carried out in duplicate, and amounts of enzyme were chosen so as to permit the accurate measurement of initial rates. K_i values were determined from double reciprocal plots[8].

The initial velocity pattern using four concentrations of each substrate and the product inhibition studies were also carried out at pH 8.0 in the same buffer. The lines of best fit and intersection points on double reciprocal[8] and Dixon plots[9] were determined by a computer programme kindly lent by J. A. Illingworth, Department of Biochemistry, Cambridge.

RESULTS

Initial velocity data

The initial rates in both directions produced a series of intersecting lines on double reciprocal plots. The kinetic constants determined from secondary plots[10–12] are shown in Table II.

TABLE II

K_m AND V VALUES

The enzyme was assayed at pH 8.0, 25 °C and the kinetic parameters determined from secondary plots[10–12]. The apparent dissociation constants only correspond to true dissociation constants in the case of the substrates which bind first (NADP$^+$ or NADPH). SK and DHS are abbreviations for shikimate and dehydroshikimate, respectively.

	K_m (mM)	V (μmoles·min^{-1}·mg^{-1})	Binary kinetic constant (mM^2)	Dissociation constant (mM)
In the direction SK→DHS				
SK	0.34	0.40	0.04	0.60 ± 0.08
NADP$^+$	0.070	0.38		0.11 ± 0.01
In the direction DHS→SK				
DHS	0.36	0.35	0.006	0.22 ± 0.03
NADPH	0.028	0.39		0.017 ± 0.003

The equilibrium constant, 6.35, obtained[11] from initial velocity data compared favourably with the value of 6.50 determined directly at pH 8.0.

Product inhibition data

The type of inhibition (Table III) was determined from double reciprocal plots[8] and the K_i values from Dixon plots[9].

TABLE III

PRODUCT INHIBITION DATA

The enzyme was assayed at pH 8.0 and 25 °C. The type of inhibition obtained was determined from double reciprocal plots[8], and the K_i values from Dixon plots[9]. Only one concentration of product was used for the unlike pair inhibition studies and therefore only the inhibition patterns and not the K_i values are shown. "Mixed inhibition" indicates an effect on both V and the apparent K_m. Limits in the table refer to the computed standard error and SK and DHS are abbreviations for shikimate and dehydroshikimate, respectively.

Product inhibitor	*Substrate*	*Type of inhibition*	*K_i (mM)*
DHS	SK	Competitive	0.25 ± 0.01
SK	DHS	Mixed	0.75 ± 0.06
NADPH	$NADP^+$	Competitive	0.033 ± 0.003
$NADP^+$	NADPH	Competitive	0.072 ± 0.004
DHS	$NADP^+$	Mixed	
NADPH	SK	Non-competitive	
SK	NADPH	Mixed	
$NADP^+$	DHS	Non-competitive	

All data produced linear plots except for the inhibition by dehydroshikimate with respect to shikimate when plotted by the method of Dixon (Fig. 2).

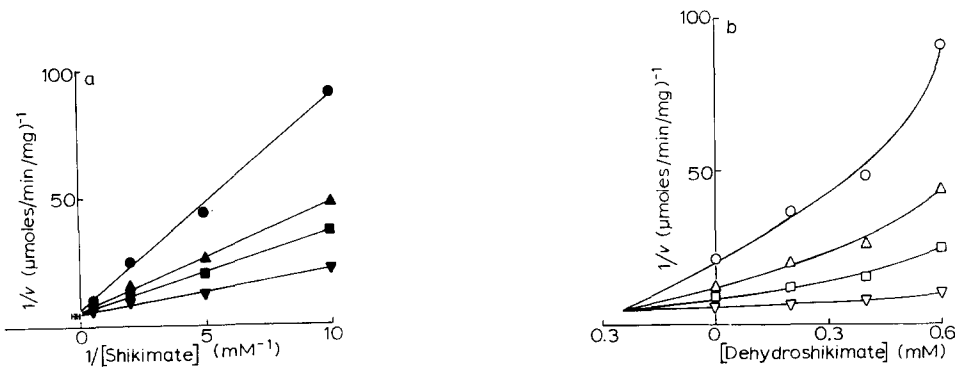


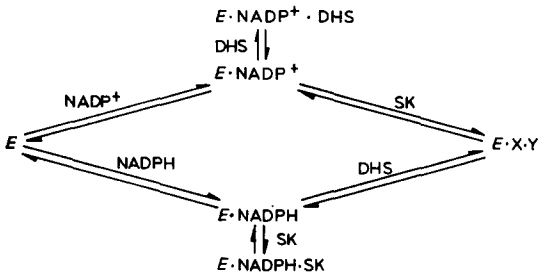
Fig. 2. Inhibition by dehydroshikimate with respect to shikimate at constant concentration of $NADP^+$ (0.167 mM). (a) Double reciprocal plot. Dehydroshikimate at zero (▼), 0.2 (■), 0.4 (▲) and 0.6 mM (●). (b) Dixon plot. Shikimate at 0.1 (○), 0.2 (△), 0.5 (□) and 2.0 mM (▽). v is expressed as μmoles of substrate converted·min^{-1}·mg^{-1} at 25 °C, pH 8.0.

DISCUSSION

The limiting Michaelis constants obtained by us for shikimate and dehydroshikimate are very similar to those published by Balinsky *et al.*[4]. However, our K_m values for $NADP^+$ and NADPH are somewhat higher than those reported (10 and 4 μM), possibly due to the different pH employed in the assay[4]. The initial velocity and product inhibition pattern are consistent with an ordered Bi–Bi mechanism with $NADP^+$ or NADPH binding to the enzyme first, as proposed by Balinsky *et al.*[4], except that dehydroshikimate is a competitive inhibitor with respect to shikimate. However the inhibition by dehydroshikimate (DHS) produces non-linear Dixon plots, particu-

larly at low shikimate (SK) concentrations, indicating dead end complex formation[13]. Such complexes are further indicated by the discrepancy between the true dissociation constants for $NADP^+$ and NADPH determined from the initial velocity data and the K_i values for product inhibition (Table III). Further, the relative value of these constants indicates that $E \cdot NADP^+ \cdot DHS$ dissociates less readily than $E \cdot NADPH \cdot SK$. Abortive ternary complexes have been shown to occur in a variety of dehydrogenase catalysed reactions[14].

We propose that a general mechanism of the following type best fits the available data:



E·X·Y is a ternary complex

The apparent K_i for the product form of the coenzyme is a function of the true K_S of the enzyme–coenzyme complex and the dissociation constant (K_S') of the ternary dead end complex. Since the inhibition produced by dehydroshikimate is purely competitive rather than mixed as expected in an ordered Bi–Bi mechanism[15], dehydroshikimate appears to bind much more strongly to $E \cdot NADP^+$ than to its "own" enzyme–coenzyme complex ($E \cdot NADPH$). It is therefore suggested that analogues of dehydroshikimate would be expected to behave similarly, that is bind preferentially to $E \cdot NADP^+$. Considerable inhibition by these compounds would thus be predicted when the enzyme is assayed in the direction of dehydroshikimate formation ($NADP^+$, and hence $E \cdot NADP^+$, being present), whereas little inhibition would be expected in the reverse direction (in the presence of NADPH only). This is exactly what is observed.

REFERENCES

1 B. A. Bohm, *Chem. Rev.*, 65 (1965) 435.
2 J. Udvardy and G. L. Farkas, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 3 (1968) 153.
3 A. C. Baillie, J. R. Corbett, J. R. Dowsett and P. McCloskey, *Pestic. Sci.*, 3 (1972) 113.
4 D. Balinsky, A. W. Dennis and W. W. Cleland, *Biochemistry*, 10 (1971) 1947.
5 R. Grewe and P. J. Jeschke, *Chem. Ber.*, 89 (1956) 2080.
6 D. Balinsky and D. D. Davies, *Biochem. J.*, 80 (1961) 292.
7 E. Layne, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 447.
8 H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658.
9 M. Dixon, *Biochem. J.*, 55 (1953) 170.
10 J. R. Florini and C. S. Vesterling, *Biochim. Biophys. Acta*, 25 (1957) 575.
11 K. Dalziel, *Acta Chem. Scand.*, 11 (1957) 1706.
12 C. Frieden, *J. Am. Chem. Soc.*, 79 (1957) 1894.
13 W. W. Cleland, *Biochim. Biophys. Acta*, 67 (1963) 188.
14 J. Everse, R. F. Barnett, C. J. R. Thorne and N. O. Kaplan, *Arch. Biochim. Biophys.*, 143 (1971) 444.
15 W. W. Cleland, *Biochim. Biophys. Acta*, 67 (1963) 104.