Plant Physiol. (1990) 93, 758–766
0032-0889/90/93/0758/09/$01.00/0

Received for publication November 15, 1989
and in revised form January 31, 1990

Shikimate Kinase from Spinach Chloroplasts[1]

Purification, Characterization, and Regulatory Function in Aromatic Amino Acid Biosynthesis

C. L. Schmidt, H.-J. Danneel[2], G. Schultz*, and B. B. Buchanan

Botanisches Institut der Tierärztlichen Hochschule Hannover, Bünteweg 17 D, D-3000 Hannover 71, Federal Republic of Germany (C.L.S., H.-J.D., G.S.), and Department of Plant Biology, University of California, Berkeley, California 94720 (B.B.B.)

ABSTRACT

Shikimate kinase was purified to near homogenity from spinach *Spinacia oleracea* L. chloroplasts and found to consist of a single 31 kilodalton polypeptide. The purified enzyme was unstable, but could be stabilized by a variety of added proteins, including oxidized and reduced thioredoxins. Whereas the isolated enzyme was stimulated by mono- and dithiol reagents, the enzyme in intact chloroplasts was unaffected by added thiols and showed only minor response to dark/light transitions. These results indicate that the previously reported stimulation of shikimate kinase activity by reduced thioredoxins is due to enzyme stabilization rather than to activation. In the current study, the purified enzyme was inhibited by added ADP and showed a strong response to energy charge. When intact chloroplasts were incubated in the dark in presence of shikimate, phosphoenolpyruvate and a source of ATP (dihydroxyacetone phosphate or ATP itself under appropriate conditions), aromatic amino acids were formed: phenylalanine and tyrosine. The data indicate that energy charge plays a role in regulating shikimate kinase, thereby controlling the shikimate pathway. An unidentified enzyme of the latter part of the pathway, leading from shikimate-3-phosphate to phenylalanine, appears to be activated by light.

The shikimate pathway is the major route of the *de novo* synthesis of aromatic compounds in plants and microorganisms. The enzymes of this pathway have been isolated and extensively studied from microorganisms (14). In the case of plants, a number of enzymes of the pathway have been isolated during the past few years (3, 5, 9, 11, 13, 23, 29–33), but there is still little understanding of their regulation. Effective feedback regulation, as present in microorganisms, was observed only for the post-chorismate part of the pathway (1, 5, 10, 12, 29–31). Regulation of the pre-chorismate portion is unclear (25, 26, 33).

It is now generally accepted that the shikimic acid pathway is present in chloroplasts, but it is still questionable whether there is a complete counterpart in the cytosol. In some cases, cytosolic isoenzymes have been described, *i.e.* DAHP[3] synthase (11) and chorismate mutase (31), whereas in others, *e.g.* shikimate oxidoreductase/dehydroquinate hydrolyase (9), the enzymes appear to be restricted to the chloroplast. In a previous report it was demonstrated that the synthesis of aromatic amino acids in spinach chloroplasts is controlled by light (19). In this article, we focus on shikimate kinase, a central enzyme of the pre-chorismate pathway and describe its purification and characterization from spinach chloroplasts. We also describe results on the regulation of shikimate kinase obtained both with the purified enzyme and with isolated intact chloroplasts, the only apparent source of the enzyme in spinach leaves (8).

MATERIALS AND METHODS

Biochemicals

[U-^{14}C]Shikimic acid with a specific activity of 0.81 GBq mmol^{-1} was purchased from NEN, Dreieich, FRG. Thioredoxin from *Escherichia coli* was obtained from IMCO, Stockholm, Sweden. All other chemicals were of analytical grade and were obtained from commercial sources.

Plant Material and Chloroplast Isolation

Spinach (*Spinacia oleracea* L.) cv Butterfly was field or greenhouse grown. Intact chloroplasts were isolated by method B described in reference 28. Chloroplast stroma was isolated as described in reference 26. For experiments with dark treated chloroplasts, leaves were harvested and stored in the dark for at least 12 h before isolation, which was conducted under reduced light.

Measurements of Shikimate Kinase Activity

Shikimate kinase activity was measured following the incorporation of [U-^{14}C]shikimate into shikimate-3-P. The as-

[1] Supported by the Deutsche Forschungsgemeinschaft, Bonn, F. R. G., and the U.S. National Science Foundation and by a grant from the Verband der Chemischen Industrie, Frankfurt, F.R.G.

[2] Present address: Institut für Mikrobiologie, Universität Göttingen, Grisebachstrasse 8, D-3400 Göttingen, F.R.G.

[3] Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosouate 7-phosphate; DHAP, dihydroxyacetone phosphate; FMN, flavine mononucleotide; FPLC, fast protein liquid chromatography; glyphosate, *N*-(phosphonomethyl)glycine; MDH, malate dehydrogenase; MESH, β-mercaptoethanol; PEP, phospho*enol*pyruvate; PMSF, phenylmethylsulfonyl fluoride.

Table I. *Purification of Shikimate Kinase from Spinach Chloroplast Stroma*

The procedure was carried out with the stromal fraction of chloroplasts isolated from 1.5 kg spinach leaves.

Purification Step	Total Activity	Total Protein	Specific Activity	Percent Recovery	Purification Factor
	nkat	*mg*	*nkat mg*$^{-1}$	%	
Chloroplast stroma	26.9	166.2	0.162	100	1
Sephadex G-75	24.0	1.15	20.87	89	129
Q Sepharose	14.0	0.10	140.0	52	864
Dextran blue agarose	2.8	ND[a]	ND	10.4	ND

[a] Not detected; the protein concentration was too low to be measured accurately. The total amount of protein was approximately 10 μg. The chromatography on dextran blue agarose resulted in an at least tenfold purification as indicated by SDS-PAGE.

say was routinely carried out for 10 to 60 min at 30°C in a solution containing (in 60 μL final volume) 100 mM glycine-NaOH buffer (pH 10.0) (20°C); 10 mM $MgCl_2$; 5 mM DTT; 4 mM ATP, and 1 mM shikimate. The reaction was terminated by adding 10 μL of 10.5% (w/v), TCA. The precipitate was removed by centrifugation (10 min, 15,000*g*) and 50 μL of the supernatant fraction were transferred to a column containing 83 mg Dowex 1-X8 (Cl^- form). Shikimate was eluted with 0.3 mM acetic acid (7 × 200 μL) and the shikimate-3-P formed was then eluted with 1 M NaCl (5 × 200 μL). Eluates were collected directly in scintillation vials; 5 mL of scintillation cocktail (Lumagel from J. T. Baker, Deventer, The Netherlands) were added and the samples were counted.

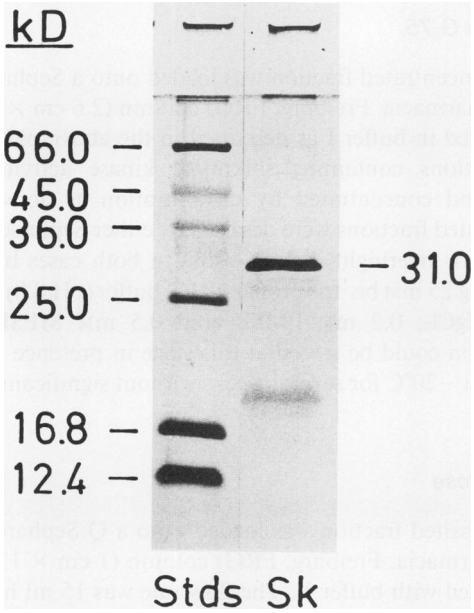

Figure 1. SDS-PAGE of shikimate kinase purified from spinach chloroplasts. Stds (protein standards) lane: BSA, 66 kD (0.25 μg); chicken egg ovalbumin, 45 kD (0.75 μg); rabbit muscle glyceraldehyde 3-P dehydrogenase, 36 kD (0.63 μg); bovine pancreas α-chymotrypsinogen A, 25 kD (0.5 μg); equine skeletal muscle myoglobin, 16.8 kD (0.26 μg); and horse heart cytochrome *c*, 12.4 kD (0.15 μg). Sk (shikimate kinase) lane: the enzyme purified from spinach chloroplasts, *ca* 2 μg.

The effect of proteins or specific compounds on shikimate kinase was determined by preincubating the enzyme (15 min, 30°C) in a solution containing 25 mM Tris-HCl, pH 8.0 (20°C), 1.25 mM $MgCl_2$, and the traditional component as indicated. The assay was started by adding ATP and shikimate in a buffer yielding final concentrations of 63 mM Tris-HCl (pH 8.0) (20°C); 4 mM $MgCl_2$; 1 mM ATP; and 1 mM shikimate.

Shikimate kinase activity was measured in intact chloroplasts in the same manner, except that the incubation medium was modified to contain 165 to 330 mM sorbitol as indicated; 2 mM $NaNO_3$; 2 mM EDTA; 4 mM ascorbic acid; 1 mM $MnCl_2$; 2 mM $MgCl_2$; 0.5 mM KH_2PO_4; 20 mM NaCl; 10 mM Tris; and 50 mM Hepes. The medium was adjusted to pH 7.6 with 1 M KOH. Substrates were added as indicated in the legends. Incubation temperature was 25 ± 2°C. In these experiments, interference of the shikimate kinase activity contributed by lysed chloroplasts present in the preparation was estimated by centrifuging the chloroplast suspension (3 min, 1500*g*) and incubating an aliquot of the supernatant fraction under the same conditions used for the chloroplast suspension. Activities shown with intact chloroplasts were corrected for this value. As indicated, the incubation mixtures were illuminated with 2250 W m^{-2} white light. The same conditions were used to investigate the synthesis of aromatic amino acids from added precursors.

Miscellaneous Techniques

Amino acids were purified from reaction mixtures according to reference 18 with the following modifications: (a) 0.01 M HCl was used to extract the amino acids from the reaction mixture; (b) Serdolit CS 2 (Serva, Heidelberg, FRG) was used as cation-exchange resin; (c) amino acids were collectively eluted from the ion exchange columns with an aqueous solution of 4% ammonia and the solvent was evaporated under reduced pressure; and (d) after dissolving in 0.5 mL methanol pro analysis, the residue was either transferred to scintillation vials and counted or analyzed by TLC as described in reference 19. Tyrosine, phenylalanine, and tryptophan present in this fraction were separated by a second TLC using RP-18/UV thin-layer plates (Machery and Nagel, Düren, FRG) and 10 mM potassium phosphate buffer pH 4.0 (20°C) as solvent. Alkaline phosphatase activity was assayed by the method described in reference 2. ATPase activity was

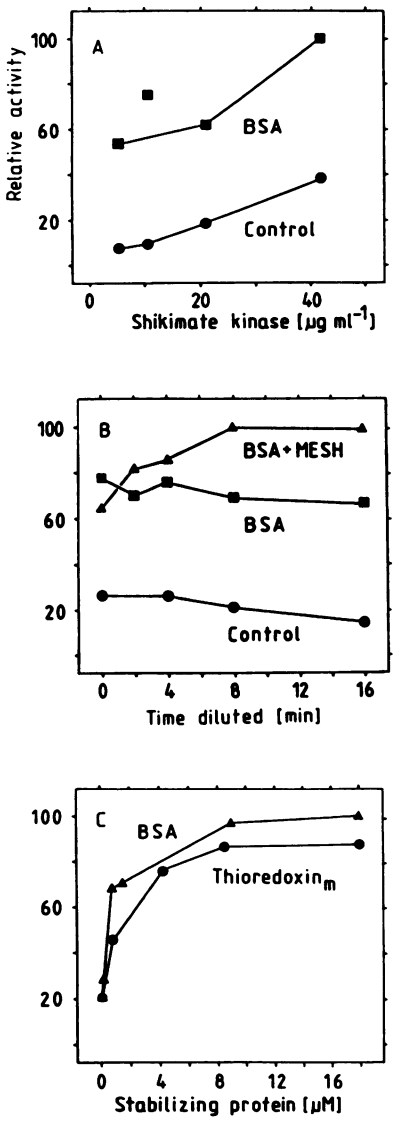

Figure 2. Effects of enzyme concentration (A), dilution time (B), and stabilizing proteins (C) on shikimate kinase from spinach chloroplasts. Pre-incubations were carried out for 15 min as described in "Materials and Methods" except in (B) when time was varied as indicated. (A) BSA was added to 0.6 mg mL^{-1} of the preincubation mixture; 100% activity is equivalent to 2.13 nkat mg^{-1}. (B) BSA was added to the preincubation mixture to 1.2 mg mL^{-1} and MESH to 10 mM; 100% activity is equivalent to 6.419 nkat mg^{-1}. (C) The indicated concentration of spinach chloroplast thioredoxin$_m$ and BSA were added to the preincubation mixture. MESH was added to 10 mM; 100% activity is equivalent to 0.816 nkat mg^{-1}.

measured by following ADP formation in a pyruvate kinase/lactate dehydrogenase system. The assay mixture contained (final volume 0.6 mL): 100 mM (hydroxymethyl)methylamino-propane-HCl (bis-tris propane-HCl buffer), pH 8.0 (20°C); 10 mM $MgCl_2$; 10 mM KCl; 1 mM ATP; 1 mM PEP; 0.5 mM NADH; 58 nkat pyruvate kinase (Boehringer, Mannheim, FRG); and 50 nkat lactate dehydrogenase (Boehringer Mannheim, FRG). Thioredoxin activity was assayed by the NADP-MDH assay as in Crawford *et al.* (7) and Jacquot and Decottignies (21). Protein was determined by the method of Bradford (4) using BSA as standard. SDS-PAGE was carried out as described in reference 9 using an acrylamide concentration of 4% for the stacking gel and 15% for the resolving gel. Silver staining of the gels was accomplished by using the method described in reference 17 with slight modifications. Molecular weight of the native shikimate kinase was determined by HPLC using a Spherogel TSK 3000 PWHR column (7.8 mm × 30 cm; Beckman, München, FRG) with a guard column (6.0 mm × 4 cm) packed with the same material. The column was equilibrated with buffer I containing: 25 mM Tris-HCl (pH 8.5) (20°C); 50 mM KCl; 2 mM $MgCl_2$; 1 mM EDTA; 0.2 mM PMSF; and 0.5 mM MESH. The flow rate was 1 mL min^{-1}. The following proteins were used to calibrate the column: BSA, 66.0 kD; chicken egg albumin, 45.0 kD; bovine pancreas α-chymotrypsinogen A, 25.0 kD; and equine skeletal muscle myoglobin, 16.8 kD.

Purification Procedure of Shikimate Kinase

All steps were performed at 4°C.

Isolation of Chloroplast Stroma

Chloroplast stroma, isolated as described above, was concentrated on a BM-100 ultrafiltration membrane (Berghof, Eningen, FRG) to a volume of 15 mL.

Sephadex G-75

The concentrated fraction was loaded onto a Sephadex G-75-50 (Pharmacia, Freiburg, FRG) column (2.6 cm × 90 cm) equilibrated in buffer I as described in the above paragraph. The fractions containing shikimate kinase activity were pooled and concentrated by ultrafiltration as above. The concentrated fractions were desalted by either Sephadex G 25 filtration or overnight dialysis using in both cases buffer II containing 25 mM bis-tris propane-HCl buffer (pH 8.0) (20°C); 5 mM $MgCl_2$; 0.2 mM PMSF; and 0.5 mM MESH. The preparation could be stored at this stage in presence of 15% glycerol at −20°C for several weeks without significant loss of activity.

Q-Sepharose

The desalted fraction was loaded onto a Q Sepharose fast flow (Pharmacia, Freiburg, FRG) column (1 cm × 13.5 cm) equilibrated with buffer II. The flow rate was 15 ml h^{-1}. The column was washed with 50 mL buffer II at a flow rate of 30 mL h^{-1} and eluted with a linear gradient from 0 to 700 mM KCl in 80 mL buffer II at a flow rate of 10 mL h^{-1}. Alternatively, this step was replaced by a FPLC treatment using a Pharmacia system with Mono Q column (HR 5/5). The flow rate was 1 mL min^{-1}; 1 mL fractions were collected. The fractions containing shikimate kinase activity were pooled and subjected to the following final purification step.

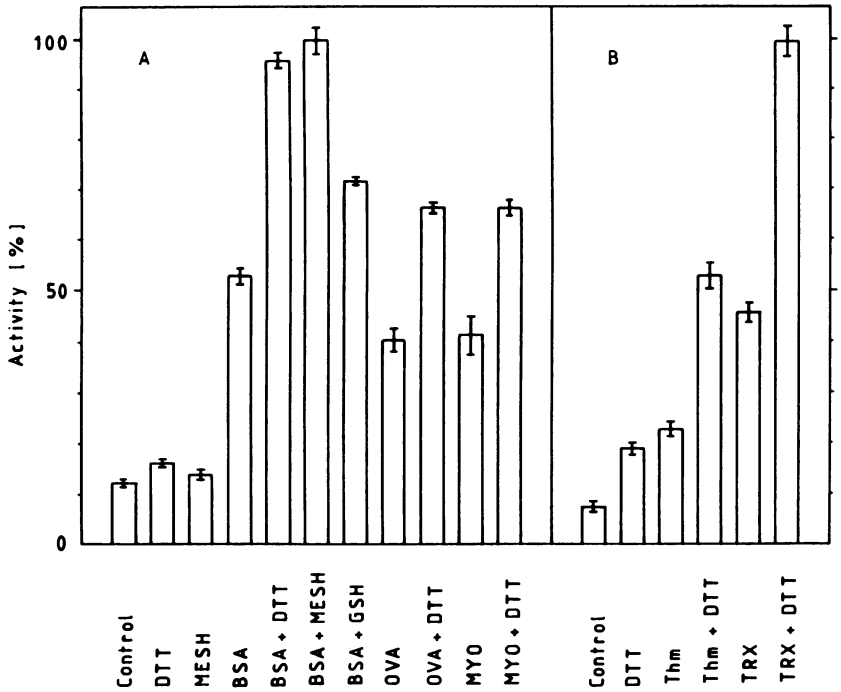

Figure 3. Effect of different non-thioredoxin (A) and thioredoxin (B) proteins and thiol reagents on shikimate kinase from spinach chloroplasts. Preincubation was carried out as described in Materials and Methods. (A) Additional compounds were added at the following concentrations: DTT, 2 mM; MESH and GSH, 4 mM; BSA, ovalbumin (OVA) and myoglobin (MYO), 2.4 mg mL^{-1} each; 100% activity is equivalent to 6.073 nkat mg^{-1}. (B) Comparison of the effects of spinach thioredoxin$_m$ and *E. coli* thioredoxin (TRX). The concentration of each protein was 25 μg mL^{-1}. DTT was applied at 2 mM; 100% activity is equivalent to 3.803 nkat mg^{-1}.

Dextran Blue Agarose

The fractions from the Q Sepharose column were loaded onto a Dextran Blue Agarose (Sigma, Deisenhofen, FRG) column (1 cm × 6.3 cm) equilibrated with buffer II. The column was washed with 20 mL buffer II and then 10 mL buffer II containing 10 mM ATP. Shikimate kinase was eluted with a linear gradient from 0 to 750 mM KCl in a total volume of 40 mL buffer II. The fractions containing shikimate kinase activity were pooled, concentrated by ultrafiltration as above and stored at 4°C.

Table II. *Influence of Different Cations on the Activity of the Shikimate Kinase*

Spinach chloroplast stroma was dialyzed overnight against buffer containing 50 mM glycine-NaOH (pH 9.0) and 4 mM DTT. Enzyme activity was assayed in a reaction mixture containing 100 mM glycine-NaOH (pH 10.0), 5 mM DTT, 4 mM ATP, 1 mM shikimate, and the indicated concentrations of cations. All tested cations were added in the chloride form.

Cation	Concentration	Activity
	mM	%
None		8
Mg^{2+}	1.0	65
	10.0	100[a]
Mn^{2+}	0.1	14
	1.0	51
Ca^{2+}	0.1	10
	1.0	18
Co^{2+}	0.1	8
	1.0	8

[a] One hundred percent is equivalent to 0.875 nkat mL^{-1}.

RESULTS

Purification

Table I summarizes the results of the procedure used for purifying shikimate kinase. The enzyme was purified at least 850-fold with a yield of 10%. As seen below, the final dextran blue agarose fraction was at least 70% pure as determined by SDS-PAGE.

Several comments should be made about the purification process. First, Sephadex G-75 chromatography proved to be an excellent step, giving >100-fold purification and a good enzyme recovery. The Q Sepharose column step was also effective, and could be further improved by reducing the slope of the elution gradient; however, this alteration reduced both the recovery and stability of the enzyme. Replacing the Q Sepharose with FPLC Mono Q column step generally resulted in less purification but increased the recovery and stability of the enzyme. For maximum purity, the Q Sepharose column was used routinely. The dextran blue agarose column proved to be the most critical step, resulting in at least a ten-fold purification as estimated by SDS-PAGE (data not shown). However, the recovery of activity from the column was poor probably because of the unstable nature of the enzyme when highly purified. In most cases all activity was lost from the Dextran Blue Agarose fractions within 1 to 3 days. Attempts to stabilize these fractions through addition of BSA, glycerol, dextran, dextran sulfate, DEAE-dextran, MESH, DTT, PMSF, benzamidine, EDTA, and storage at either 4°C or −20°C were successful. As a consequence, shikimate kinase was generally purified only through the first two purification steps of Table I (Sephadex G-75 and Q Sepharose). This 850-fold purified preparation, which was free of thioredoxins, ATPase, and phosphatase activity, was suitable for most ex-

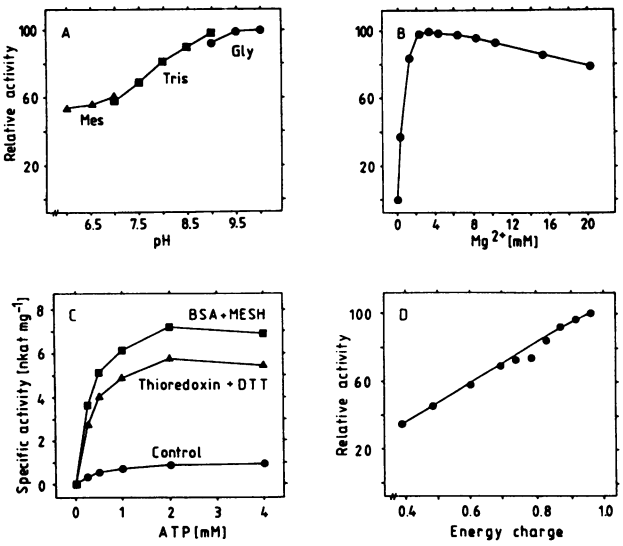

Figure 4. Spinach chloroplast shikimate kinase. Effect of pH (A), Mg^{2+} concentration (B), stabilizing proteins, thiols (C), and energy charge (D). (A) the pH of the Mes and the glycine (Gly) buffers was adjusted with NaOH; HCl was used for Tris buffer. The assay mixture contained buffer as indicated, 100 mM; ATP, 3 mM; shikimate, 1 mM; and $MgCl_2$, 3.3 mM. The enzyme was preincubated as described in "Materials and Methods" in the presence of 2 mg mL^{-1} BSA and 20 mM MESH; 100% activity is equivalent to 12.053 nkat mg^{-1}. (B) Tris-HCl (pH 8.0) was used as buffer. Other conditions were the same as described for Figure 4A; 100% activity is equivalent to 13.750 nkat mg^{-1}. (C) Preincubation was carried out at 30°C for 15 min. The concentration of stabilizing agents was: BSA, 1 mg mL^{-1}; MESH, 6 mM; a mixture of spinach thioredoxins *m* and *f*, 28 μg mL^{-1} each and DTT, 3 mM. The assay mixture also contained Tris-HCl buffer, pH 8.0, 63 mM; $MgCl_2$, 3.7 mM; shikimate, 1 mM; and ATP as indicated. (D) Preincubation was carried out as described for Figure 4A. The energy charge was adjusted as described in (26) using myokinase from chicken muscle. The final assay mixture contained: Tris-HCl buffer, (pH 8.0), 63 mM; $MgCl_2$ 3.7 mM; and shikimate, 1 mM. The final concentration of adenosine nucleotides was 1 mM. Each assay contained 1.67 nkat myokinase; 100% activity is equivalent to 8.186 nkat mg^{-1}.

periments. The enzyme could be stored at this stage at −20°C in presence of 15% glycerol for up to 4 weeks without loss of significant activity. It should be noted that attempts to replace dextran blue agarose by blue Sepharose or ATP agarose gels were not successful. Similarly, ammonium sulfate fractionation, a treatment often used for purification of other enzymes, could not be used with shikimate kinase because it led to nearly complete and irreversible inactivation. Finally, it should be noted that no additional peaks of shikimate kinase activity were detected when the starting material was leaf extract rather than chloroplasts. This finding is supporting earlier results (8) indicating that in spinach leaves, shikimate kinase resides mainly if not exclusively in chloroplasts.

Molecular Mass Determination

Figure 1 shows a SDS-PAGE gel of shikimate kinase purified through the dextran blue agarose step. The major band, with a molecular mass of 31 kD, is considered to be that of the enzyme because, unlike the minor (19 kD) band, it was enriched during purification and was detected in all preparations. Furthermore, the native enzyme showed a molecular mass of 28 kD on a HPLC gel filtration column (data not shown), a value in agreement with both the SDS-PAGE determination and the earlier published value of 27 kD determined by Sephadex G-100 filtration (26). The data suggest that native shikimate kinase consists of a single polypeptide with a molecular mass of approximately 30 kD.

Effects of Added Proteins and Thiol Reagents

The results in Figure 2 show that shikimate kinase purified through the Q Sepharose step is inactivated on dilution This inactivation, which was reflected by the nonproportionality of activity to enzyme concentration (Fig. 2A), took place within minutes (Fig. 2B) and was irreversible. Protease inhibitors had no effect on inactivation (data not shown). The addition of proteins, BSA, and thioredoxins (Fig 2, B and C) was, however, found to protect in a manner that was enhanced by thiol reagents. The added thiols exerted their stabilizing effect in a time dependent manner (Fig. 2B). Maximum effects were achieved at DTT concentrations of 2 mM and higher, or equivalent concentrations of MESH (data not shown). Figure 3 compares the effect of several proteins and thiol reagents on the protection of shikimate kinase against inactivation by dilution. Of the proteins tested, BSA was most effective in stabilizing activity, but similar effects were observed for other proteins including spinach chloroplast and *E. coli* thioredoxins. DTT and MESH added at equal concentrations supported similar protection when added in the presence of BSA; GSH was less effective (Fig. 3A). The stabilizing effect of BSA and spinach $thioredoxin_m$ were similar when compared on a molar basis in the presence of MESH (Fig. 2C).

Oxidized compounds had little effect on the enzyme. When added at 0.5 to 2 mM cystamine (2,2′-dithio bis[ethylamine] × 2 HCl), diamide (azodicarboxylic acid bis[dimethylamide]) and GSSG were without significant effect and dehydroascorbic acid showed a slight inhibition, *N*-ethylmaleimide added at 1 mM effected a weak stabilization (data not shown).

With respect to thioredoxins, we observed that when added in either the absence or presence of DTT, *E. coli* thioredoxin was more effective in stabilizing the enzyme than was spinach chloroplast $thioredoxin_m$ (Fig. 3B). $Thioredoxin_m$ was slightly more effective than $thioredoxin_f$ (data not shown). The finding that oxidized proteins (*e.g.* thioredoxins) were effective in protecting shikimate kinase against dilution inactivation (Fig. 3B) suggests that protein-linked sulfhydryl groups are not essential for stabilization of shikimate kinase. Furthermore, the observation that catalytically inert proteins (the BSA preparation used in these experiments was free of thioredoxin activity as determined by the NADH-MDH assay) and monothiols are effective in stabilization suggest that the earlier reported apparent activation of shikimate kinase by reduced thioredoxins (27) was due to enzyme stabilization rather than to enzyme activation.

Once this effect became clear, we assayed purified shikimate kinase in the current work only when the reaction mixture

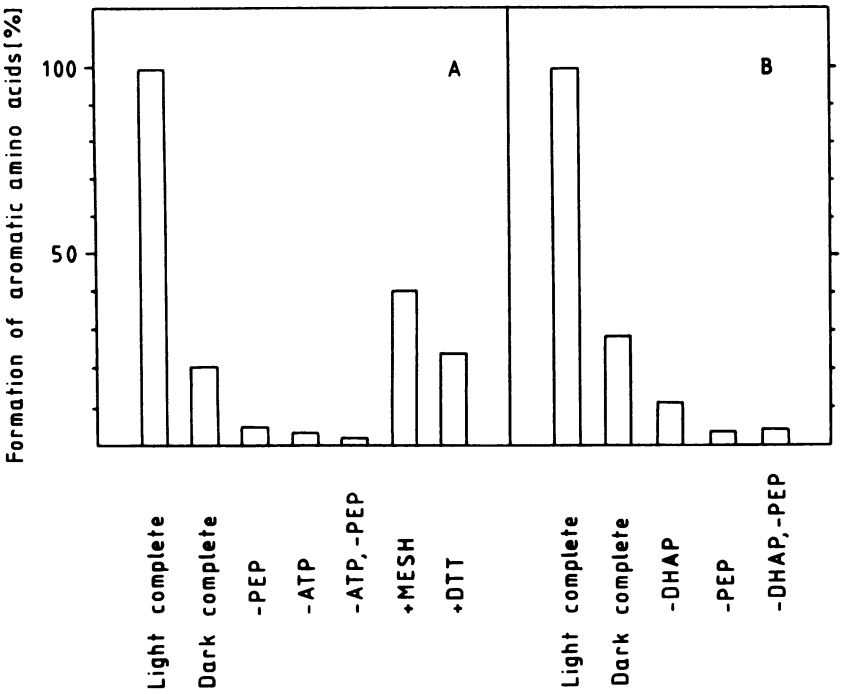

Figure 5. Formation of ^{14}C-aromatic amino acid (phenylalanine and tyrosine) from [U-^{14}C]shikimate appied to isolated chloroplasts in the dark. (A) Chloroplasts were incubated under hypotonic conditions. The complete reaction mixture contained the medium described under "Materials and Methods," and additionally the following: $MgCl_2$, 7 mM; L-tryptophan, 0.1 mM; L-glutamate, 0.05 mM; [U-^{14}C]shikimate, 05 mM; PEP, 2 mM; and ATP, 5 mM. The concentration of sorbitol was 200 mM. Additionally 20 mM MESH and 10 mM DTT were added as indicated. The final Chl concentration was 0.104 mg mL^{-1}. Incubation time was 45 min. The formation of 126.1 nmol amino acids mg^{-1} h^{-1} is equivalent to 100%. (B) The chloroplasts were incubated in an isotonic medium containing the compounds described under "Materials and Methods" with the following changes: The concentration of sorbitol was 330 mM; 0.05 mM L-glutamate, 2 mM PEP; 5 mM DHAP and 0.5 mM [U-^{14}C]shikimate were added to the complete reaction mixture. The final Chl concentration was 0.253 mg mL^{-1}. Incubation time was 45 min. The formation of 74.4 nmol amino acids mg^{-1}Chl h^{-1} is equivalent to 100%.

contained BSA and a thiol reagent. It may be that these conditions (*i.e.* high protein concentration and reducing compounds) simulate the environment of the enzyme in intact chloroplasts. This assumption was evidenced by experiments with isolated chloroplasts. Here, a constant rate of shikimate 3-P formation was observed for at least 30 min when ATP, shikimate, and glyphosate (to inhibit 5-enoylpyruvylshikimate 3-P synthesis [32]) were applied to isolated chloroplasts incubated in a hypotonic incubation mixture in the dark (134 nmol shikimate 3-P formed per mg Chl after 30 min). No effects of DTT or MESH were detected under these conditions, even after the enzyme had been released from chloroplasts and measured in the supernatant fraction (data not shown).

Enzyme Characterization

Shikimate kinase is known to require a divalent cation for activity (8). As shown in Table II, Mg^{2+} was the most effective cation tested, followed by Mn^{2+}; Co^{2+} was without effect. In parallel experiments we found that ATP and 2'-deoxy ATP could serve as substrate, whereas xanthosine triphosphate, ITP, GTP, TTP, CTP, and UTP could not (data not shown). Of the carbon substrates tested—shikimate, 3-dehydroquinate, 3-dehydroshikimate—only shikimate was phosphorylated by the enzyme (*cf.* ref. 26). These results demonstrated that shikimate kinase is highly substrate specific.

Figure 4, A to C, summarizes additional properties of shikimate kinase, the effect of pH, Mg^{2+} concentration, stabilizing proteins, and sulfhydryl compounds. Activity of the enzyme showed only moderate response to change in pH (from pH 6.5 to 9.5) with an optimum in the alkaline region (about pH 9.5) (Fig. 4A). More than 50% of maximal activity was retained at pH 6.0. The optimal concentration of Mg^{2+} was between 2 and 4 mM (Fig. 4B), the apparent K_m for shikimate did not change on addition of stabilizing proteins and thiols (data not shown). On the other hand, the affinity for ATP showed a considerable increase on addition of stabilizing proteins and thiol compounds (Fig. 4C). As reported earlier (27), the maximal apparent K_m value in the absence of thiols and stabilizing proteins was 1.8 mM. The addition of thioredoxin and DTT or BSA and MESH resulted in a decrease to an apparent K_m value of 0.3 mM (plots not shown).

Regulatory Properties

The activity of shikimate kinase was unaffected by 1 mM concentrations of end products or intermediates of the shikimate pathway, *i.e.*, DHAP, 3-phosphoglycerate, PEP, 3-dehydroshikimate, 3-dehydroquinate, or quinate. Previous studies demonstrated that the latter three metabolites, as well as the three aromatic amino acids, anthranilate, chorismate, and prephenate, had no effect on enzyme activity (26). Inorganic phosphate and AMP were also found to be without effect in the current study. Of the metabolites tested, only ADP effected an inhibition of activity and even this was weak (12% inhibition at 0.5 mM). However, inhibition by ADP was magnified (up to 75%) when energy charge was adjusted (Fig. 4D). Such strong dependence on energy charge is likely to result from a combination of ADP inhibition and the relatively high K_m of the enzyme for ATP.

Chloroplast Studies

The rate of shikimate-3-P formation by isolated chloroplasts under the above conditions was comparable to the rates of aromatic amino acid synthesis found previously (1, 19).

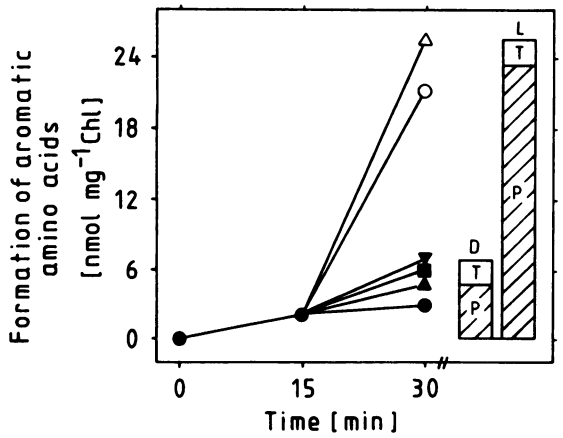

Figure 6. Effect of light and biochemical agents on the synthesis of aromatic amino acids (phenylalanine and tyrosine) in isolated spinach chloroplasts. Chloroplasts were incubated in the medium described under "Materials and Methods" with the following modifications: the pH was adjusted to 8.0; the concentration of sorbitol and $MgCl_2$ were 165 and 7 mM, respectively; PEP, 2 mM; L-tryptophan 0.05 mM; and L-glutamate 0.05 mM were added. After 15 min incubation in the dark, the following additions were made: ● none, ▲ 5 mM ATP, ■ 5 mM DHAP, ▼ 5 mM ATP, and 40 mM MESH. Incubation was continued for additional 15 min in the dark. Alternatively an aliquot of the reaction mixture was transferred to the light in the absence, ○ or presence △ of 0.6 mM ATP and 1.4 mM ADP. The latter ratio was used to maintain the physiological concentration of these nucleotides within the chloroplasts, which had been diluted by the hypotonic treatment. The final Chl concentration was 0.2 mg mL^{-1}. The columns at the right indicate the composition of aromatic amino acids formed in the dark in presence of ATP and MESH (D) and in the light in presence of ATP and ADP (L). The hatched area represents synthesized phenylalanine (P), the open area tyrosine (T).

When chloroplasts were incubated in a hypotonic medium in the dark with [^{14}C]shikimate, PEP, and either ATP or DHAP, which serves to regenerate ATP (16), ^{14}C-aromatic amino acids (phenylalanine and tyrosine) were formed at a considerable rate (Fig. 5, A and B). Formation of tryptophan was not detected under these conditions probably for two reasons; (a) tryptophan is synthesized only at minor rates as compared to phenylalanine and tyrosine (1); and (b) tryptophan, added at 50 μM to the incubation medium to maintain chorismate mutase activity (31), would, at this concentration, almost completely inhibit its own synthesis (1).

The addition of MESH to chloroplast incubation mixtures resulted in a twofold stimulation of aromatic amino acid synthesis: however, DTT showed only minor effect. The absence of DTT effect may be due to its inability to be taken up rapidly by whole chloroplasts (6). Added cofactors of chorismate synthase, NADPH and FMN (22), did not stimulate the synthesis of aromatic amino acids in these studies (data not shown).

Figure 6 shows the time course of the formation of ^{14}C-aromatic amino acids from [^{14}C]shikimate under hypotonic conditions. For the first 15 min, when only [^{14}C]shikimate and PEP were added to the incubation mixture, the sole source of ATP available to phosphorylate shikimate would have been endogenous. Accordingly, if no ATP was added, a decrease in the rate of aromatic amino acid formation was detected on additional incubation. The addition after 15 min of ATP, alone or in combination with MESH or DHAP, led to a significant stimulation in the rate of synthesis during the next 15 min in the dark (Fig. 6). An even more pronounced increase in the rate of synthesis was achieved by illumination either without further additions or in combination with an ATP/ADP mixture The latter was applied to a total concentration of 2 mM and in a nucleotide ratio of 0.3 as was reported to occur in the chloroplast in the dark (15). Significantly, the formation of aromatic amino acids was also observed when isolated intact chloroplasts were incubated in an isotonic medium in the dark in the presence of shikimate, PEP, and DHAP (Fig. 5B).

An unexpected change in the ratio of tyrosine/phenylalanine formed by chloroplasts was found to accompany illumination; the ratio decreased from 0.44 for chloroplasts in the dark in the presence of ATP and MESH to 0.09 for chloroplasts subjected to light (Fig. 6). Such a change indicates that the total amount of tyrosine was nearly constant in the dark or the light, whereas the amount of phenylalanine synthesized increased dramatically upon illumination. In addition to indicating an activating role of light in the synthesis of aromatic amino acids from shikimate, these results demonstrate that chloroplasts maintained in a hypertonic medium retain their enzyme content and synthetic capabilities for at least 30 min.

In contrast to the increase in phenylalanine, the formation of shikimate-3-P increased only slightly on illumination (Fig.

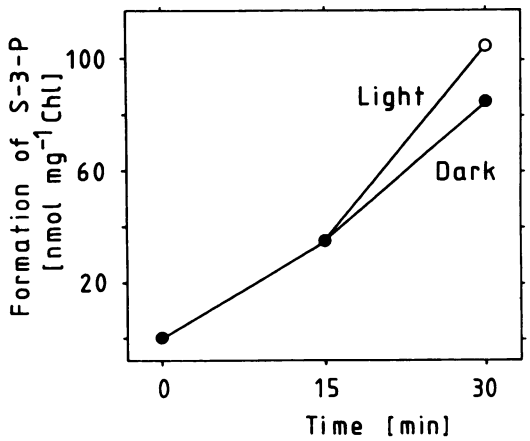

Figure 7. Effect of light on the activity of the shikimate kinase in isolated spinach chloroplasts. The experimental conditions were as described under "Materias and Methods" with the following modifications: The concentrations of sorbitol and $MgCl_2$ were 165 and 7 mM, respectively; ATP was added at a final concentration of 5 mM; [U-^{14}C]shikimate, 0.5 mM; and glyphosate, 2 mM. The reaction was carried out in the dark for the first 15 min. The reaction mixture was then divided and for the next 15 min separate aliquots were incubated in the light and in the dark. Corrections to eliminate the enzyme activity originating from the portion of lysed chlorplasts in the suspension were carried out as described in "Materials and Methods." The final Chl concentration was 0.207 mg mL^{-1}.

7). This observation, together with the results presented above, indicates that contrary to an earlier suggestion (27), the activity of the shikimate kinase is not influenced by light, other than by possible light-induced changes in energy charge.

DISCUSSION

The above results demonstrate that when purified, the shikimate kinase of spinach chloroplasts requires added proteins and sulfhydryl compounds for stabilization. In the absence of these agents, the purified enzyme is rapidly and apparently irreversibly inactivated by dilution. Apropos this point is the situation in fungi and bacteria in which shikimate kinase is part of a multienzyme complex (20). We found no evidence for such a complex throughout our studies with the chloroplast enzyme.

Contrary to an earlier indication of modulation by thiols (27), evidence obtained in the current study indicates that shikimate kinase is controlled primarily by energy charge. Shikimate kinase appears to be active independently of light *per se*, a feature that accounts for its function in chloroplasts maintained in the dark and in spinach roots, where, according to preliminary evidence, it occurs together with other shikimate pathway enzymes (CL Schmidt, unpublished data). Light, nevertheless, appears to play a regulatory role in the overall synthesis of aromatic amino acids by chloroplasts. When chloroplasts are supplied with shikimate, ATP (or a source for ATP), and PEP, light stimulates the synthesis of phenylalanine, but has little effect on the synthesis of tyrosine. Thus, while there is no question that the shikimate pathway, including the branch leading to phenylalanine, is operative at a basal level in the dark, light enhances its overall activity. The identification of a potential target enzyme that responds to light thus presents an interesting problem for the future. At present, a possible candidate in this regard is arogenate dehydrogenase—an enzyme recently shown to be nearly inactive in the absence of thiol reagents (29).

A related problem concerns the nature of the regulation of the early part of the shikimate pathway, *i.e.* the reactions leading to shikimate-3-P formation. While the present results indicate that energy charge modulates this part of the pathway via shikimate kinase, other factors may function as well. As there are currently contradictory reports on feedback regulation (25, 26, 33), the documentation of the relative importance of the various regulatory agents will require further work. On the basis of a recent report (24), differential gene expression may also be involved.

LITERATURE CITED

1. **Bagge P, Larsson C** (1986) Biosynthesis of aromatic amino acids by highly purified spinach chloroplasts—compartmentation and regulation of the reactions. Physiol Plant **68:** 641–647
2. Biochemica Information I (1973) Boehringer Mannheim GmbH, Mannheim F.R.G.
3. **Boven JR, Kosuge T** (1979) In vivo activity, purification and characterization of shikimate kinase from *Sorghum.* Plant Physiol **64:** 382–385
4. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem **72:** 248–254
5. **Connelly JA, Conn EE** (1986) Tyrosine biosynthesis in *Sorghum bicolor*: Isolation and regulatory properties of arogenate dehydrogenase. Z Naturforsch **41c:** 69–78
6. **Crawford NA, Droux M, Kosower NS, Buchanan BB** (1989) Evidence for function of the ferredoxin/thioredoxin system in the reductive activation of target enzymes of isolated intact chloroplasts. Arch Biochem Biophys **271:** 223–239
7. **Crawford NA, Yee BC, Hutchesson SW, Wolosiuk RA, Buchanan BB** (1986) Enzyme regulation in C_4 photosynthesis: purification, properties, and activities of thioredoxins from C_4 and C_3 plants. Arch Biochem Biophys **244:** 1–15
8. **Fiedler E, Schmidt CL, Gross C, Schultz G** (1985) Chloroplasts of higher plants are the site of synthesis of primary aromatic compounds: Compartmentation and partial characterization of crucial enzymes of the shikimate pathway. *In* L Farkas, M Gabor and F Kalley, eds, Flavonoids and Bioflavonoids. Proceedings of the 7th Hungarian Bioflavonoid Symposium, Szeged (Hungary). Elsevier, Amsterdam, pp 195–212
9. **Fiedler E, Schultz G** (1985) Localization, purification, and characterization of shikimate oxidoreductase-dehydroquinate hydrolyase from stroma of spinach chloroplasts. Plant Physiol **79:** 212–218
10. **Gaines CG, Byng GS, Whitaker RJ, Jensen RA** (1982) L-Tyrosine regulation and biosynthesis via the arogenate dehydrogenase in suspension-cultured cells of *Nicotiana silvestris* Speg. et Comes. Planta **156:** 233–240
11. **Ganson RJ, D'Amato TA, Jensen RA** (1986) The two-isozyme system of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in *Nicotiana silvestris* and other higher plants. Plant Physiol **82:** 203–210
12. **Goers SK, Jensen RA** (1984) The differential allosteric regulation of two chorismate-mutase isoenzymes of *Nicotiana silvestris.* Planta **162:** 117–124
13. **Graziana A, Boudet A, Boudet AM** (1980) Association of the quinate:NAD^+ oxidoreductase with one dehydroquinate hydrolyase isoenzyme in corn seedlings. Plant Cell Physiol **21:** 1163–1174
14. **Haslam E** (1974) The Shikimate Pathway. Butterworths, London
15. **Heber U, Takahama U, Neimanis S, Shimizu-Takahama M** (1982) Transport as basis of the KOK effect. Levels of some photosynthetic intermediates and activation of light-regulated enzymes during photosynthesis of chloroplasts and green leaf protoplasts. Biochim Biophys Acta **679:** 287–299
16. **Heldt HW** (1976) Metabolite transport in intact spinach chloroplasts. In J Barber, ed, The Intact Chloroplast, Vol 1. Elsevier, Amsterdam, pp 215–234
17. **Heukeshoven J, Dernick R** (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6:** 103–112
18. **Homeyer U, Schulze-Siebert D, Schultz G** (1985) Control of pyruvate metabolism in spinach chloroplasts by exogenously added products and coenzymes. J Plant Physiol **119:** 87–91
19. **Homeyer U, Schultz G** (1988) Acivation by light of plastidic shikimate pathway in spinach. Plant Physiol Biochem **26:** 365–370
20. **Huang L, Montoya AL, Nester EW** (1975) Purification and characterization of shikimate kinase enzyme activity in *Bacillus subtilis.* J Biol Chem **250:** 7675–7681
21. **Jacquot J-P, Decottignies P** (1986) Further evidence for a role of sulfhydryls in the thioredoxin dependent activation of corn NADP-malate dehydrogenase. FEBS Let **209:** 87–91
22. **Morell H, Clark MJ, Knowles PF, Sprinson DB** (1967) The enzymic synthesis of chorismic and prephenic acids from 3-enoylpyruvylshikimic acid 5-phosphate. J Biol Chem **242:** 82–90
23. **Mousdale DM, Coggins JR** (1984) Purification and properties of 5-enoylpyruvylshikimate 3-phosphate synthase from seedlings of *Pisum sativum* L. Planta **160:** 78–83
24. **Pinto JEBP, Dyer WE, Weller SC, Herrmann KM** (1988) Glyphosate induces 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in potato (*Solanum tuberosum* L.) cells grown in suspension culture. Plant Physiol **87:** 891–893
25. **Rubin JL, Jensen RA** (1985) Differentially regulated isozymes of

3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from seedlings of *Vigna radiata* (L.) Wilczek. Plant Physiol **79:** 711–718

26. **Schmidt CL, Gross C, Hennig H, Homeyer U, Fiedler E, Schultz G** (1987) The introductory enzymes of shikimate pathway in spinach (*Spinacia oleracea* L.): general features of enzymes and possible mode of regulation of aromatic compound synthesis in eukaryotic cells. Life Sci Adv **6:** 35–42
27. **Schmidt CL, Schultz G** (1987) Stimulation by thioredoxins of shikimate kinase from spinach chloroplasts. Physiol Plant **70:** 65–67
28. **Schulze-Siebert D, Heintze A, Schultz G** (1987) Substrate flow from photosynthetic carbon metabolism to chloroplast isoprenoid synthesis in spinach. Evidence for a plastidic phosphoglycerate mutase. Z Naturforsch **42c:** 570–580
29. **Siehl DL, Conn EE** (1988) Kinetic and regulatory properties of arogenate dehydratase in seedlings of *Sorghum bicolor* (L.) Moench. Arch Biochem Biophys **260:** 822–829
30. **Singh BK, Connelly JA, Conn EE** (1985) Chorismate isoenzymes from *Sorghum bicolor*: purification and properties. Arch Biochem Biophys **243:** 374–384
31. **Singh BK, Lonergan SG, Conn EE** (1986) Chorismate mutase isoenzymes from selected plants and their immunological comparison with the isoenzymes from *Sorghum bicolor*, Plant Physiol **81:** 717–722
32. **Steinrücken HC, Schulz A, Amrhein N, Porter CA, Fraley RT** (1986) Overproduction of 5-enoylpyruvylshikimate 3-phosphate synthase in a glyphosate-tolerant *Petunia hybrida* cell line. Arch Biochem Biophys **244:** 169–178
33. **Suzich JA, Dean JFD, Hermann KM** (1985) 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase from carrot roots is a hysteretic enzyme. Plant Physiol **79:** 765–770