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Localization, Purification, and Characterization of Shikimate Oxidoreductase-Dehydroquinate Hydrolyase from Stroma of Spinach Chloroplasts[1]

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ABSTRACT

The stroma of chloroplasts is probably the sole site of the shikimate pathway enzymes shikimate oxidoreductase/dehydroquinate hydrolyase (SORase/DHQase) in spinach leaves. (a) The chromatographic behavior of the bifunctional protein SORase/DHQase on several separation materials with extracts from stroma compared with leaf extracts showed only one peak of enzymic activity originating from the stroma. (b) Polyacrylamide gel electrophoresis (PAGE) of these extracts followed by specific staining resulted in the same pattern without a band of extraplastidic enzyme. (c) In protoplast fractionation experiments it was shown that SORase/DHQase was present only in the soluble chloroplast protein fraction.

An improved purification procedure for SORase/DHQase from stroma of chloroplasts, yield 40%, 1600 times as pure, gave essentially one protein band on sodium dodecyl sulfate-PAGE. Our results demonstrate that both enzyme functions are carried out by a single polypeptide. Nondenaturing PAGE exhibited a pattern of four bands with SORase/DHQase showing that they differ in charge but not in their molecular weight. Molecular weight was determined to be 67 kilodaltons (gel filtration) and 59 kilodaltons (PAGE) for all four forms. It was proven they were not due to artifacts. The four forms show similar kinetic properties, their K_m and pH optima differing only very slightly. Response to some metabolites is reported.

It is accepted that the shikimate pathway, the primary route leading to aromatic amino acids, prenylquinones, lignins, and flavonoids, is active in the soluble protein fraction of plant tissues. Former research focused on the pathway itself and not its localization (17, 20). Even where a step in the biosynthesis within the chloroplast was studied the possibility of cross contamination with other organelles and cytoplasm have not been ruled out (8). Extraplastidic enzymes of SORase[2] in pea leaves were proposed due to multibanded pattern on polyacrylamide gels (8) and differences of enzymic activities measured in plastidic and leaf extracts (22). The existence of cytoplasmic isozymes was also suggested because multiple forms of SORase in bean seedlings have been detected (17). The incorporation of $^{14}CO_2$ and [^{14}C] shikimate into tyrosine, phenylalanine, and tryptophan (2, 4, 5) as well as the incorporation of L-[ring-3,5-^{3}H]-4-hydroxyphenylpyruvate into tyrosine (11) and the synthesis of [^{14}C]phylloquinone arising from [^{14}C]shikimate (24) by spinach chloroplast fractions suggests that its complete shikimate pathway exists in chloroplasts (Fig. 1).

We have used an essentially different approach to provide evidence that—in spinach leaves—two crucial enzymic activities SORase and DHQase of the shikimate pathway are restricted to the soluble chloroplast extract. It was proposed that the activities of SORase and DHQase in higher plants were on a single polypeptide (20). This bifunctional protein catalyzes the conversion of dehydroquinate into dehydroshikimate (DHQase activity) and sequentially the synthesis of shikimate from dehydroshikimate (SORase activity). The two enzymic activities were found exclusively in the stroma of spinach chloroplasts as demonstrated in this paper and the preceding abstract (13). To understand the structural/functional relationships of the multiple forms requires the ability to isolate them in a purified state.

We have improved the previously reported purification procedure (12) to achieve the apparent homogeneity and stability which was necessary for the characterization of SORase/DHQase. The purification procedure of a shikimate pathway enzyme from the chloroplast stroma is described here for the first time. Furthermore we investigated the regulatory properties of these enzymes.

MATERIALS AND METHODS

Chemicals. Sepharose CL-6B, DEAE-Sepharose CL-6B, Sephacryl S 300 and Sephadex G 100 (superfine) were obtained from Pharmacia, hydroxylapatite Bio-Gel HPT from Bio Rad, cellulase Onozuka R 10 and macerase Maceroenzyme R 10 from Serva (Heidelberg, F.R.G.), and glyphosate (*N*-phosphonomethylglycine) from Ehrenstorfer (Augsburg, F.R.G.). Blue Sepharose CL-6B was prepared by the procedure of Böhme *et al.* (3). Dehydroquinate and dehydroshikimate were synthesized according to Grewe and Jeschke (14). The materials used for gelelectrophoresis and isoelectric focusing were purchased from Serva (Heidelberg, F.R.G.). All other chemicals were of an analytical grade.

Plant Material. Spinach plants (*Spinacia oleracea*) were greenhouse grown.

Preparation of Total Leaf Extract. Washed deribbed leaves were homogenized (1/1 w/v) by a Waring Blendor in buffer I (50 mM Tris/HCl [pH 7.5], 1 mM EDTA, 1.2 mM PMSF, 0.4

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[2] Abbreviations: SORase, shikimate oxidoreductase (EC 1.1.1.25); DHQase, dehydroquinate hydrolyase (EC 4.2.1.10); PMSF, phenylmethylsulfonylfluoride; GAP, glyceraldehyde 3-phosphate; U, μmol min^{-1}; DAHP, deoxy-*D*-arabino-heptulosonate-7-phosphate.

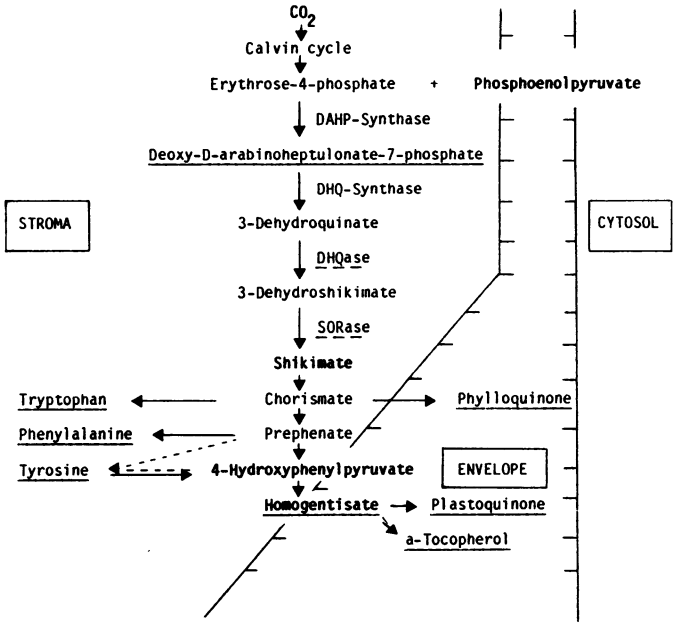

FIG. 1. Compartmentation of shikimate pathway and prenylquinone-synthesis in spinach chloroplasts. The labeled substrates are in bold print, the isolated labeled products are underlined. Enzymes now proven to be present in the stroma fraction of chloroplasts are indicated by dashed lines.

mM DTT) or 50 mM Tris/HCl (pH 7.5) with the additions described in "Results". Following filtration and subsequent centrifugation (105,000*g* for 30 min) the supernatant was used as total leaf extract. The serine protease inhibitor PMSF was added from a stock solution (6 mg/ml ethanol).

Isolation of Chloroplasts and Chloroplast Stroma. Intact chloroplasts were isolated as described previously (11) and freed from other organelles and most cytosolic enzymes by centrifugation through a linear Percoll density gradient (11). The soluble contents of the stroma were released by osmotic shock in hypotonic solution of buffer I, plastidic membranes were centrifuged down (105,000*g* for 10 min) and discarded.

Isolation of Protoplasts, Cytoplasm, and Chloroplast Stroma. Protoplasts were prepared by a method modified from Edwards *et al.* (7). They were purified on a discontinuous gradient and then ruptured mechanically (7). Intact chloroplasts were completely separated from cytoplasm by repeated centrifugation (1000*g*, 2 min, three times) and the stroma was obtained as described above. Marker enzymes of cytoplasmic and stroma fractions were measured to determine cross-contaminations.

Separation of Chloroplast Subfractions. Envelopes, thylakoids, and the soluble chloroplast fraction were prepared from purified chloroplasts by the method of Douce and Joyard (6), except the media contained all the additions described for buffer I.

Criteria of Purification. For criteria of the purity of chloroplasts and their subfractions see Douce and Joyard (6), Fiedler *et al.* (11), Schulze-Siebert *et al.* (23), and "Results".

Enzyme Assays. DHQase was assayed spectrophotometrically by measuring the formation of 3-dehydroshikimate at 234 nm. The assay mixture contained 0.1 M Tris/HCl (pH 8.0) and 0.2 mM 3-dehydroquinate in a final volume of 0.5 ml. SORase was routinely assayed in the reverse direction by measuring the increase in absorbance at 340 nm during the oxidation of shikimate to 3-dehydroshikimate (NADPH-formation) in 0.1 M glycine/NaOH buffer (pH 9.5), 2 mM shikimate, 0.5 mM NADP. The purified SORase was also assayed in the foreward direction by following changes in *A* at 340 nm as a result of NADPH oxidation in an assay mixture containing 0.1 M glycine/NaOH buffer (pH 9.5), 0.2 mM dehydroshikimate, and 0.5 mM NADPH in a final volume of 0.5 ml. The enzyme activity of SORase and DHQase was traceable to less than 0.1 nmol ml^{-1} min^{-1} and 0.05 nmol ml^{-1} min^{-1}, respectively. The reversible NADP-GAP dehydrogenase (EC 1.2.1.13) (a marker enzyme for the chloroplast stroma) and the nonreversible NADP-GAP dehydrogenase (EC 1.2.1.9) (a marker enzyme for the cytoplasm) were measured as described in Kelly and Gibbs (16) and Stitt *et al.* (28).

Gelelectrophoresis and Staining for Enzyme Activity. Analytical disc-electrophoresis was performed with 7.5% and 10% polyacrylamide gels (19). The stacking gels were polymerized with the aid of riboflavin. Gels were held at 4°C during electrophoresis. They were either stained for protein using Coomassie brilliant blue R 250 or stained for SORase activity with 10 ml of a solution consisting of 100 mM glycine/NaOH buffer (pH 9.5), 0.8 mM NADP, 3 mM shikimate, 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, and 0.35 mM phenazine methosulfate. The SORase bands could be recognized within 5 min after incubation at room temperature. The gels were alternatively stained or assayed. For the latter slab gels were cut into 1-mm slices at the identical R_F values as visualized for SORase activity by specific staining of both margins. These slices were extracted overnight in 0.2 ml buffer I.

Two-dimensional PAGE was carried out in the following way. The multiple forms of SORase/DHQase were separated during the first dimension by rod gels, 7.5% in acrylamide (diameter 2 mm), and then subjected to another PAGE on slab gels (7.5% acrylamide) in the second dimension. A solution of 1% (w/v) agarose (Sigma, ultralow gelling temperature: 15°C) was used to get the rod gel contact with the slab one.

SDS-gelelectrophoresis was conducted as described by Laemmli (18).

Isoelectric Focusing. For isoelectric focusing experiments a procedure described by Radola (21) was followed.

Other Methods. CO_2 fixation rate, Chl as well as protein contents were determined as described in Buchholz *et al.* (5).

Mol Wt and Characterization of the Different SORase/DHQase Forms. Four essential different attempts were made to study the mol wt, as well as the relationships, of the four distinct forms of SORase/DHQase.

(a) HPLC. A Bio-Sil TSK-250 column (300 × 7.5 mm) equilibrated with 0.1 M Na_2SO_4, 20 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0) containing 1 mM EDTA, 1.2 mM PMSF, and 0.4 mM DTT was used to chromatograph the enzyme at a flow rate of 0.3 ml per min. Fractions were collected and assayed for both *A* at 254 nm and enzyme activity. Thyroglobulin (670,000), IgG (150,000), ovalbumin (44,000), and myoglobin (16,800) were used as mol wt markers.

(b) Gelfiltration. The mol wt was also estimated from gel filtration chromatography performed according to Andrews (1). In these experiments a column of Sephadex G-100 superfine (1 × 34.5 cm) was employed equilibrated with buffer I additionally containing 100 mM KCl. The enzyme sample, Blue Dextran and the protein standards (creatine kinase [81,000], BSA [66,500], ovalbumin [44,500], and myoglobin [16,800]) were all added to the column in the buffer described above. Elution was obtained with the same buffer as above.

(c) SDS-PAGE. SDS-PAGE was conducted as described by Laemmli (18). β-Galactosidase (116,000), phosphorylase B (97,400), BSA (66,500), ovalbumin (44,500), and carbonic anhydrase (29,000) were used as references.

(d) Nondenaturing gel electrophoresis. The mol wt was estimated by electrophoresis in a nondenaturing gel carried out in 12 × 14 cm slab gels as described by Hedrick and Smith (15). The acrylamide concentration was varied from 4 to 12% (w/v). BSA, ovalbumin, and carbonic anhydrase were standard proteins.

Purification Procedure. All steps were performed at 4°C. The soluble protein extract from spinach chloroplasts was loaded to a column of DEAE-Sepharose CL-6B (2.5 × 14.7 cm) equilibrated in buffer I. The column was washed with buffer I containing 30 mM KCl until the *A* (at 280 nm) of the eluate reached a value of about 0.05. The column was then eluted with a linear gradient of 30 to 300 mM KCl in buffer I with a total volume of 400 ml. The fractions containing SORase/DHQase activity were pooled and then concentrated by a Sartorius ultrafiltration unit equipped with a SM 145 39 membrane (exclusion weight 10 kD), and applied to a Sephacryl S 300 column (2.6 × 75 cm).

The enzyme was eluted with buffer I. Active fractions were combined and added directly to a Blue Sepharose column (1 × 67 cm) that had been equilibrated before with buffer I. After washing the column (same buffer) until the *A* at 280 nm was negligible, the enzyme was eluted with 135 ml of buffer I containing 320 mM KCl. Fractions with SORase/DHQase activity were combined and absorbed on a hydroxylapatite column (1 × 5 cm), which had been previously equilibrated with 30 mM K-phosphate (pH 7.0), 1 mM EDTA, 1.2 mM PMSF, 0.4 mM DTT (buffer II). Following an extensive wash with the same buffer, this column was developed with a linear gradient (50 ml) prepared from buffer II and 200 mM K-phosphate (pH 7.0) with the same additions as in buffer II. The 110 to 160 mM eluate containing the purified SORase/DHQase was dialyzed against 1 L of buffer I containing 50% (w/v) sucrose and stored at 0 to 4°C.

RESULTS

Chromatographic Procedures for Separation of Potential Isozymes of SORase/DHQase. The bifunctional protein (SORase/DHQase) was always found in the same fractions of a DEAE-Sepharose chromatography, regardless whether applying stroma or total leaf extract (Fig. 2).

Eluting the DEAE-Sepharose column described in Figure 2 with salt gradients up to 1 M KCl yielded identical elution profiles. No additional peaks of SORase or DHQase activity deriving from cytoplasmic isozymes were detected using gelfiltration on Sephacryl S 300 or hydroxylapatite chromatography (data not shown).

All assays were repeated with different buffers and several pH-values (sodium citrate [pH 6.8], Tris/HCl [pH 7.0], Tris/HCl [pH 8.0], glycine/NaOH [pH 9.5]) to exclude the possibility that the presence of isozymes were missed only because of possible different pH-optima or salt tolerances.

PAGE of Stroma and Total Leaf Extracts. When soluble protein fractions obtained from purified chloroplasts or total leaf homogenate were subjected to nondenaturing PAGE and specifically stained on the gel for enzyme activity, no band of an extraplastidic SORase was detectable; however, both gels showed a characteristic staining pattern. Four bands with enzyme activity were recognized (Fig. 3). In parallel experiments gel slices were obtained from the same PAGE as used in SORase activity determination. They were cut out at the identical R_F values as for SORase activity and eluted in buffer I. The DHQase assay revealed that only gel slices known to contain SORase activity showed DHQase activity. This may be interpreted as a group of four distinct enzymes (all inside the chloroplast) with such a similarity that only PAGE is able to separate them.

Assay of SORase and DHQase in Chloroplast and Cytoplasmic Fractions Obtained from Protoplasts. Another approach was used to elucidate the problem of SORase/DHQase distribution within spinach parenchym cells. A fractionation technique which allows separation of plastidic and cytoplasmic fractions from protoplasts was now applied (Table I).

The marker enzyme distribution (Table I) reveals that the stroma is virtually free of cytoplasmic enzymes, though the cytoplasmic fraction is considerably contaminated by chloroplast enzymes. Comparing the distribution of marker enzymes verifies that the activities of SORase and DHQase are confined to the fraction of soluble chloroplast protein (stroma). No activity was detectible outside the plastids.

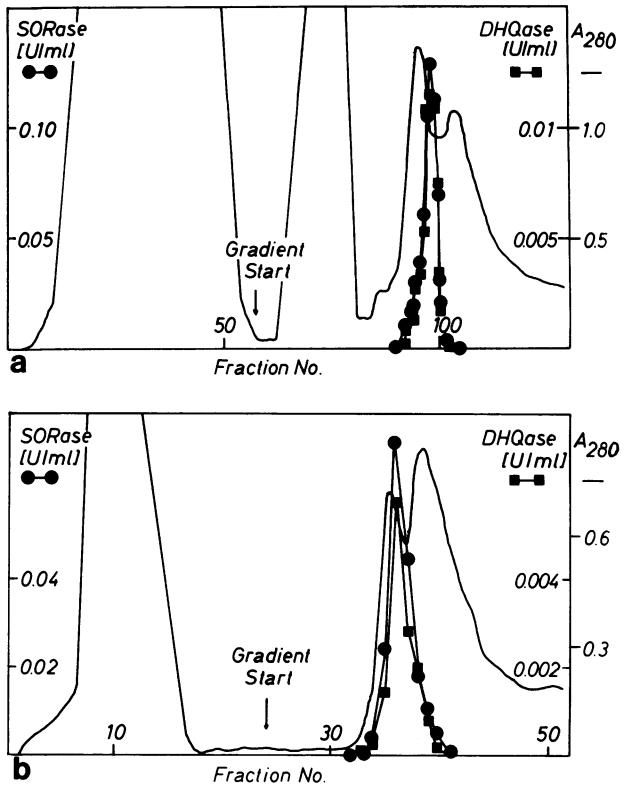

FIG. 2. Comparison of the results of DEAE-Sepharose chromatography of SORase/DHQase from total leaf extract (17.5 ml containing 2.67 units SORase and 0.21 units DHQase) (a), and chloroplast stroma (40 ml containing 2.91 units SORase and 0.23 units DHQase) (b). The column (2.5 × 21.5 cm) was washed with buffer I, 30 mM KCl, and then developed with a linear gradient up to 300 mM KCl in buffer I. The enzyme activities were eluted at 160 to 190 mM KCl. The recoveries of SORase/DHQase were 83% (a) and 90% (b), respectively.

Assay of SORase and DHQase in Chloroplast Subfractions. Activity of SORase and DHQase could be detected only in the chloroplast stroma fraction but not in the chloroplast membrane fractions (thylakoids and envelopes). These results show that within the plastids the sole site of SORase/DHQase is the soluble protein fraction.

Enzyme Purification. Results for the purification of SORase/DHQase from spinach chloroplast stroma are summarized in Table II.

The SORase/DHQase could be purified to apparent homogeneity (Fig. 7). The two enzymic activities are strictly associated and their ratio (SORase activity/DHQase activity; 9/1) remained fairly constant throughout the purification procedure (compare Table II). The enzymes were characterized after purification. At low protein concentrations SORase/DHQase gradually lost its activity within 2 d. Long-term storage is under investigation; glycerol or ampholines were not successful in stabilizing the purified enzyme.

The isolation of the bifunctional protein took 3 d and the enzyme appeared to be completely stable throughout the procedure provided that EDTA, DTT, and PMSF were present. Two steps, well established in protein purification, protamine sulfate precipitation and $(NH_4)_2SO_4$ fractionation had to be omitted because of strong SORase and DHQase inhibition. Tests with

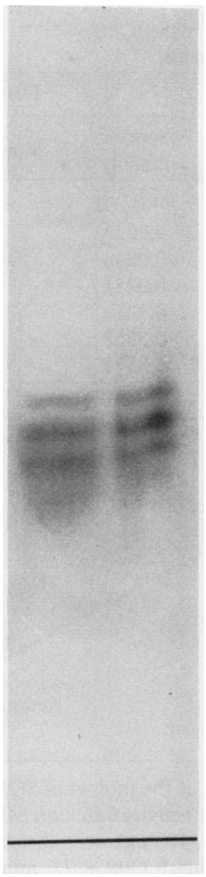

FIG. 3. Nondenaturing gel electrophoresis of SORase/DHQase from different sources, total leaf extract (0.01 unit SORase) in the left, chloroplast stroma (0.01 unit SORase) in the right lane were run and stained for SORase activity. Both multibanded patterns are essentially the same. Unfortunately, it is difficult to show the fourth band because the other more intensive bands are not quite dissoluble after exhaustive staining.

different salts ($[NH_4]_2SO_4$, $MgSO_4$, NH_4Cl and KCl) showed that NH_4^+ ion is a potent inhibitor of SORase/DHQase. At concentrations of 0.8 M NH_4^+ (this concentration corresponds to 20% [w/v] saturation of $(NH_4)_2SO_4$) the activity of SORase decreased to 12%, compared with the control. All the other ions had less effects, for instance 0.8 M $MgSO_4$ decreased the activity only to 67%. Very large losses of enzyme activity (down to 20% of the original activity) occurred after protamine sulfate precipitation (2% [v/v] of 1.5% [w/v] protamine sulfate solution pH 7.5 was added dropwise to the chloroplast stroma solution).

Hydroxylapatite chromatography proved to be a crucial step during the isolation. Extended contact of the enzyme with this column caused partial inactivation. To minimize this the column was run at a flow rate of 16 ml/h. This final purification step turned out as a very good method in concentrating SORase/DHQase. Dialysis was omitted if possible in favor of rapidity; nevertheless, it was necessary to dialyze the active fractions after purification.

The elution profile of hydroxylapatite chromatography is depicted in Figure 4. The two enzymic activities are evidently associated.

Enzyme Characterization. PAGE was carried out in two different ways to check the purity of a preparation. Nondenaturing gel electrophoresis showed a pattern of four bands when stained for protein which corresponded precisely with the position of the SORase when stained for enzyme activity. To examine the nature of this heterogeneity the purified enzyme was denatured by boiling in SDS and then subjected to SDS gel electrophoresis. This procedure produced only one distinct mobility form as detected by Coomassie blue staining (compare Fig. 7).

These results suggest that the native forms of these enzymes may represent charge isomers with such similarity that only gel electrophoresis is able to separate them. The method of Hedrick and Smith (15) was used to clarify whether or not the observed differences between the protein bands are caused by charge differences. Samples of SORase/DHQase were run on nondenaturing polyacrylamide gels at a series of different acrylamide concentrations. The log values of the relative mobility of the proteins (SORase/DHQase as well as marker proteins) are plotted against the gel concentration as per cent (Fig. 5). The lines for SORase/DHQase are parallel. This indicates that the distinct forms of the enzyme are the result of charge differences and not of mol wt differences.

The Hedrick and Smith (15) method was also used to determine the mol wt of the enzyme. Plots of the negative slopes from the graph (Fig. 5) against the known mol wt of the standards on two-cycle log paper allowed calculation of the protein mol wt (9). The value obtained by the linear plot produced as described above was 59 kD. This mol wt was the same for the four different SORase/DHQase forms.

Analytical gel filtration was also used to study the nature of this heterogeneity and to estimate the mol wt. The procedure, described under "Materials and Methods", resulted in a value of 69 kD for the enzyme, provided it has a globular shape. As expected there was no separation between the individual enzymes. This method did not rule out the possibility that separation could not be achieved because of resolution deficiency. We made further attempts to examine the specific differences of the

Table I. *Distribution of Shikimate Pathway Enzymes and Marker Enzymes in Protoplast Subfractions*

The shikimate pathway enzymes SORase and DHQase as well as marker-enzymes for the chloroplast stroma and the cytoplasm were assayed as described in "Materials and Methods". The protein concentrations were 14.0 mg/ml stroma (fraction volume 0.6 ml) and 4.0 mg/ml resulting supernatant of protoplast extract (fraction volume 2.04 ml).

Fraction	Enzyme							
	DHQase Units/ Fraction	%	SORase Units/ Fraction	%	Rev. GAPDH Units/ Fraction	%	Nonrev. GAPDH Units/ Fraction	%
Total protoplast extract	0.0180	100	0.232	100	2.12	100	0.367	100
Soluble chloroplast extract (stroma)	0.0156	87	0.205	88	1.89	89	0.0037	1
Resulting supernatant of protoplast extract	0.0024	13	0.027	12	0.23	11	0.363	99

Table II. *Purification of SORase/DHQase from Spinach Chloroplast Stroma*

The starting material was 8 kg deveined leaves. The purified enzyme was concentrated to 0.023 mg/ml.

Fraction	Volume	Total Protein	SORase			DHQase		
			Specific activity	Purification	Yield	Specific activity	Purification	Yield
	ml	*mg*	*units/mg*	*-fold*	*%*	*units/mg*	*-fold*	*%*
Stroma	24.5	229.8	0.023	1	100	0.0024	1	100
DEAE-Sepharose	50.0	19.95	0.228	10	86	0.0248	10	89
Ultrafiltration	6.3	17.27	0.229	10	75	0.0251	10	78
Sephacryl S 300	42.0	3.02	1.222	53	70	0.1250	52	68
Blue Sepharose	55.5	0.83	3.808	166	61	0.3863	160	59
Hydroxylapatite	12.0	0.06	36.28	1577	41	3.8571	1594	42

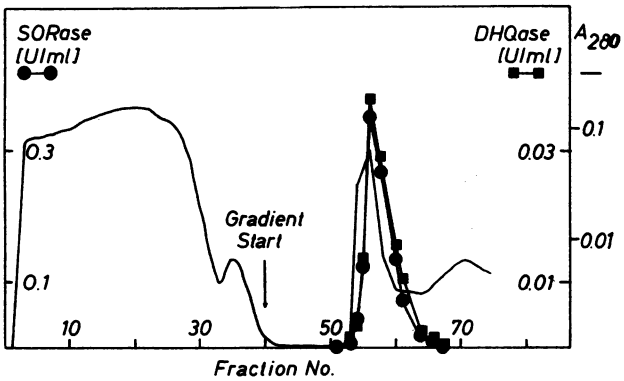

FIG. 4. Elution profile of SORase/DHQase and protein from the final purification step on a hydroxylapatite column. The pooled enzyme fractions from the Blue Sepharose chromatography were applied to the column. It was washed with buffer II until the absorbance of the eluate was zero. Elution was obtained after the application of a 50-ml linear gradient prepared from buffer II and 200 mM k-phosphate (pH 7.0) with the same additions as in buffer II.

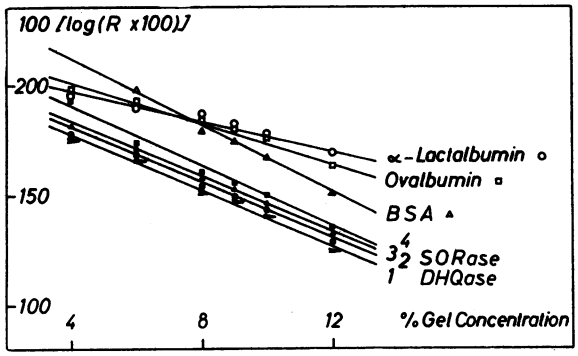

FIG. 5. Examination of multiple forms of SORase/DHQase by nondenaturing PAGE. SORase/DHQase (enzyme activity 0.004 unit SORase per lane) as well as marker proteins (Sigma, nondenatured protein marker kit, 5 μg each of them) were electrophoresed on a set of gels of various polyacrylamide concentrations according to the Hedrick and Smith method (15). Gels were stained for SORase activity or protein (as described in "Materials and Methods").

multiple forms of SORase/DHQase.

A HPLC unit (Bio-Rad) equipped with a gel filtration column produced a single peak of enzyme activity. Experiments with mol wt standards demonstrated the high resolution of this technique. The mol wt determined by HPLC was 65 kD (Fig. 6).

Our last approach to determine the mol wt of the enzymes was SDS PAGE. It gave essentially one band corresponding to a mol wt of 59 kD (Figs. 7 and 8).

It should be emphasized that the four distinct SORase/

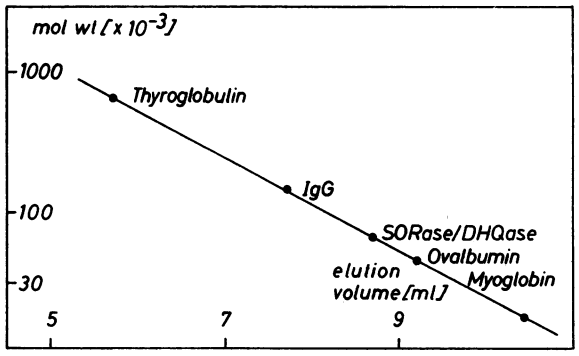

FIG. 6. Determination of the mol wt of SORase/DHQase by HPLC. SORase/DHQase (enzyme activity 0.02 unit SORase) as well as standard protein mixture (Bio-Rad, 100 μg) were chromatographed on a Bio-Sil TSK-250 gel filtration column (300 × 7.5 mm). The flow rate was 0.3 ml/min. Fractions of 0.1 ml were collected and assayed for both A_{254} and enzyme activity. The figure shows the primary data, mol wt *versus* elution volume.

DHQase forms were only separable under nondenaturing electrophoretic conditions. They were not resolved by use of denaturing PAGE or chromatographic procedures based on separation depending on differences in mol wt.

The multibanded pattern was analyzed to verify that the four forms of the bifunctional protein SORase/DHQase obtained with nondenaturing PAGE were not caused by the extraction, purification, or PAGE.

(a) A series of extracts were prepared differing the additions to a 50-mM Tris/HCl buffer (pH 7.5), 0.4 mM DTT (this buffer turned out to be the most suitable one in preserving enzyme activity).

The buffer included: 0.05% (w/v) PVP; 2 mM EDTA; 2 mM DTT; buffer without DTT; 2 mM 2-mercaptoethanol; 2 mM benzamidine; 2 mM PMSF; 1 μM pepstatin A; 1 μM tosyl-L-phenylalanine chloromethylketon (TPCK); or 0.42 mg/ml trypsin inhibitor. Buffer I was tested as well. The extracts were assayed for enzyme activity and subjected to PAGE (0.02 U SORase/lane). The resulting patterns were essentially the same except that without DTT here there was no detectable enzyme activity. The enzyme was very susceptible without any thiol reagents. The use of various protective agents should also conclusively exclude the possibility that a cytoplasmic isozyme, even if very unstable, was deactivated and therefore not detected in this test series.

(b) Fractions containing enzyme activity were electrophoresed by the nondenaturing method, during purification. Regardless at which stage, eluate from ion-exchange chromatography, gel filtration, etc. they all showed the same staining pattern.

(c) Two-dimensional PAGE ruled out the possibility that these forms were due to an artifact during electrophoresis. The multiple forms of SORase/DHQase were separated on a rod gel (7.5%

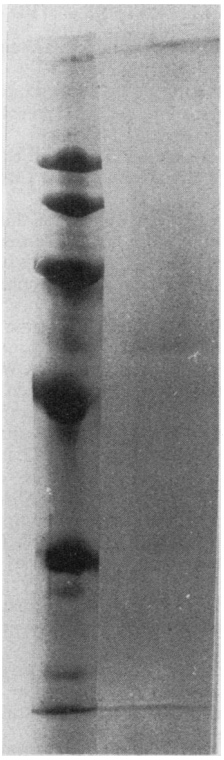

FIG. 7. Determination of the mol wt of SORase/DHQase by SDS-PAGE. The gel was loaded with 5 μg of each marker protein (Sigma, SDS marker kit) with 3 μg purified SORase/DHQase in lane 1 and 3 μg purified SORase/DHQase in lane 2. Electrophoresis was conducted in a 10% polyacrylamide gel according to the procedure of Laemmli (18).

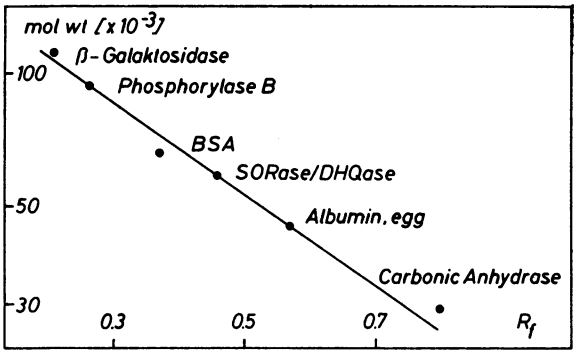

FIG. 8. Mol wt determination for the four purified SORase/DHQase forms. Purified enzymes and the indicated mol wt marker proteins (3 μg each) were electrophoresed in SDS polyacrylamide gels as described. The gels were stained for protein and the mobilities relative to the front (R_F) were calculated.

polyacrylamide) and then subjected to further electrophoresis in the second dimension on a slab gel (same polyacrylamide concentration with 0.005 units SORase/gel). Gels were specifically stained for SORase activity. An additional sample was run only in the second dimension showing no change between the distinct forms. There was no convertibility between the individual bands proving that the isozymic configurations were not affected by electrophoresis.

Kinetic Properties. Some kinetic properties of SORase/DHQase were studied. The experiments were performed at 20 ± 2°C in 0.1 M glycine/NaOH buffer (pH 9.5) (in the case of SORase) and 0.1 M Tris/HCl (pH 8.0) (in the case of DHQase), respectively, which were determined beforehand to be the pH optima. Both enzymes rapidly lost activity when pH was lowered. Activity was not detected at or below pH 6.4. The enzyme has an isoelectric point at pH 6.4 calculated from isoelectric focusing with simultaneous run of marker proteins (Radola, 21).

SORase showed a strong specifity to NADP(H) which could not be replaced by NAD(H). The apparent Michaelis constants for NADP was between 8 and 26 μM and that of shikimic acid was 0.2 mM.

We reported earlier higher K_m values for NADP. The superior purification method, higher insusceptibility, and that the tests carried out in this study were with each of the four different bands separated by PAGE and eluted from the sliced gel are the probable reasons for these differences.

The kinetic properties of the four SORase/DHQase forms investigated so far, are very similar. They exhibit the same pH optima, nearly the same K_m values, and the ratio of SORase to DHQase activity remained constant (9:1).

Metabolites from shikimate pathway as well as from related biosynthesises that might affect the activity of SORase/DHQase were tested for inhibition. Phenylalanine (1 mM), tyrosine (1 mM), tryptophan (1 mM), chorismate (1 mM), prephenate (1 mM), 4-hydroxyphenylpyruvate (5 mM), cinnamic acid (5 mM), coumaric acid (5 mM), and caffeic acid (5 mM) did not inhibit (the concentrations of the effectors are given in parentheses).

3,4-Dihydroxybenzoic acid (0.63 mM), quinic acid (0.32 mM), 3,5-dihydroxybenzoic acid (0.24 mM), and anthranilic acid (0.08 mM) all inhibited (the apparent K_i values obtained by a linear Dixon plot of 1/v against the concentration of inhibitors (I) are given in parentheses). The herbicide glyphosate (*N*-phosphonomethylglycine), a potent inhibitor of enolpyruvylshikimate-3-phosphate synthase (27), had no significant effect on the SORase/DHQase activity. It was applied in a range of 1 μM to 10 mM. SORase was inactivated at higher temperatures. When heated for 5 min at 50°C the remaining activity was 20%. The optimal temperature for the assay was also 50°C.

DISCUSSION

Localization of the Shikimate Pathway in Spinach Chloroplasts. The data presented in this paper demonstrate the localization of SORase and DHQase in the soluble chloroplast extract. The specific distribution of other enzymes of the shikimate pathway between the chloroplast and the surrounding cytoplasm in spinach leaf cells is not clear as yet. At the present stage of our research results here show that DAHP synthase (assayed by the incorporation of [^{14}C]phosphoenolpyruvate into DAHP, data not shown) exists in duplicate forms, one located inside the chloroplast, the other in the cytoplasm perhaps with another function than biosynthesis of aromatic amino acids. Dehydroquinate synthase has been found in the chloroplast stroma fraction (data not shown).

The next two reactions catalyzed by SORase/DHQase are confined to the chloroplast stroma. The same appears to be the case for 5-enolpyruvylshikimate-3-phosphate synthase. O. Joop and N. Amrhein (Bochum) reported in a personal communication that there were no signs for extraplastidic isozymes of 5-enolpyruvylshikimate-3-phosphate synthase in spinach leaves. These data clearly align the shikimate pathway with the chloroplast.

The soluble shikimate pathway enzymes in chloroplasts synthesize the aromatic percursors of α-tocopherol and plastoquinone. All steps in prenylquinone synthesis, starting with prenylation of homogentisate by prenyldiphosphate (29) (see Fig. 1), were firmly bound to the inner membrane of the chloroplast envelope (25, 26). The step connecting the shikimate pathway with prenylquinone synthesis, the formation of homogentisate from 4-hydroxyphenylpyruvate, was shown to be executed by a partly envelope-associated enzyme (11). The activity of the envelope-bound enzyme was high enough to fully support pren-

ylquinone synthesis. All these data support a continuous flow of aromatic precursors of the prenylquinones from the soluble protein fraction to the chloroplast envelope membrane (10).

Purification and Characterization. The purification and characterization of the bifunctional protein yielded 60 μg of SORase/DHQase from 8 kg deveined spinach leaves. The specific activity was enriched 1600-fold with a 40% yield in respect to soluble chloroplast protein. It is evident that a higher purification factor would be required to purify the enzyme to apparent homogeneity when starting with total leaf extract instead of chloroplast stroma. There are further advantages to isolate enzymically active proteins from chloroplasts provided they are localized exclusively in these organelles as it is shown to be the case for SORase/DHQase. First, the plastidic enzymes do not come in contact with plant phenolics released from the vacuole during maceration of leaves. Second, because the low amounts of nucleic acids in chloroplasts, protamine sulfate precipitation is not required as a step in purification. Protamine sulfate proved to be a deactivating agent of SORase/DHQase. The two enzymic activities (SORase and DHQase) purify on a single polypeptide. Characterization of the final preparation for homogeneity revealed four closely spaced bands on polyacrylamide gels identified on the basis of staining for protein and enzymic activity. These bands reduced to a single band when the enzyme was subjected to SDS PAGE; no subunits were detectable. The Hedrick and Smith (15) method provided a proof of our previous explanation that the multibanded pattern corresponds to four forms of the bifunctional protein, differing only in their charges. To clarify that these charge isomers are not due to an artifact caused by *in vitro* procedures, several examinations were made permitting a number of conclusions about these forms and their relationship in understanding of *in vivo* regulation. No significant differences were noted in their catalytic properties when each enzymic activity was compared. Further studies are underway concerning the kinetic behavior required to elucidate this issue.

A relevant question is, where do the multiple forms of SORase/DHQase originate from? It seems possible that the observed heterogeneity could be the result of different glycoid structures bound to the protein. Alternatively, phosphorylation could be responsible for the structural distinction between the different forms. The variability may reflect hybrid enzyme formation (polymorphism).

In any case, all our data reveal that the multiple forms of SORase/DHQase exist in spinach chloroplasts as different molecular forms of a single bifunctional polypeptide.

Concluding Remarks. Chloroplasts play a central role in the biosynthesis of nutritionally essential amino acids. In addition to the aspartate-derived amino acids (30) and the branched-chain amino acids (23), the aromatic amino acids are formed in the chloroplasts.

Although our experiments do not exclude the existence of the shikimate pathway outside the chloroplast in other plants or even in different tissues, it seems logical that if one enzyme is located only in the spinach chloroplast, that the shikimate pathway is to be found exclusively in the chloroplast. Large amounts of ATP, NADPH, as well as other photosynthetic metabolites that are necessary for energy-consuming pathways such as biosynthesis of aromatic amino acids are readily available. Finally, an intensive transport of intermediates of the shikimate pathway across the chloroplast envelope would result in difficulty.

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